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A Critical Role Of Cxcr2 Pdz Motif-Mediated Interactions In Endothelial Progenitor Cell Homing And Angiogenesis

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A CRITICAL ROLE OF CXCR2 PDZ MOTIF-MEDIATED INTERACTIONS IN ENDOTHELIAL PROGENITOR CELL HOMING AND ANGIOGENESIS

by

YUNING HOU

DISSERTATION

Submitted to the Graduate School of Wayne State University Detroit, Michigan in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

2016

MAJOR: BIOCHEMISTRY AND MOLECULAR BIOLOGY

Approved By:

_____________________________  _______________________
Advisor                          Date
DEDICATION

I would like to dedicate this dissertation to my family: My wife, Xiaonan Sun, for her unselfish support and encouragement during my research and life when I came across the difficulties during my research. I would thank my parents for their unconditional love during my whole life. I especially thank my parents for their understanding of my motivation in science and their support. I miss them so much.
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I wish I could fully express my thanks and appreciation to my advisor, Dr. Chunying Li. He has mentored me through my experiments, and in doing so, he has led me to enjoy the science and research. I am appreciative for the freedom that he has bestowed upon me to let me to develop my independence. His philosophy of mentoring is superb in encouraging me learn from outside by attending academic conferences and workshops to obtain novel ideas. I would also like to thank all my lab mates: Shukkur Muhammed. Farooq, Yanning Wu, Shuo Wang, Xiaoqing Guan, Marcello P. Castelvetere, Rhonda Prince, and Mostafa Omeis.

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I also would like to express my thankfulness to everyone in the department with whom I had the most meaningful years at Wayne.
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LIST OF ABBREVIATIONS

ABI  Ankle-brachial Index
AC   Adenylyl Cyclase
AP   Alkaline Phosphatase
CABG Coronary Artery Bypass Volume
cAMP Cyclic Adenosine Monophosphate
CB   Cord Blood
CCR2 Chemokine (C-C motif) Receptor 2
Cdc42 Cell Division Control Protein 42 homolog
CTD The last 45 amino acids located at the carboxyl terminus of CXCR2 were deleted
CXCL8 Chemokine (C-X-C Motif) Ligand 8
CXCL5 Chemokine (C-X-C Motif) Ligand 5
CXCL6 Chemokine (C-X-C Motif) Ligand 1
CXCL1 Chemokine (C-X-C Motif) Ligand 6
CXCL7 Chemokine (C-X-C Motif) Ligand 7
CXCR2 Chemokine (C-X-C Motif) Receptor 2
CXCR4 Chemokine (C-X-C Motif) Receptor 4
DAG  Diacylglycerol
Dil-Ac-LDL Acetylated Low Density Lipoprotein, Labeled with 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate
Dlg1 Drosophila Disc Large Tumor Suppressor
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>EBM</td>
<td>Endothelium Basal Medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EF</td>
<td>Ejection Fraction</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>EGM</td>
<td>Endothelial Cell Growth Medium</td>
</tr>
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<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
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<tr>
<td>ENA-78</td>
<td>Epithelial Neutrophil Activating Protein-78, also known as CXCL5</td>
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<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cells</td>
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<td>ERK</td>
<td>Extracellular Signal-Regulating Kinase</td>
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<td>ESV</td>
<td>End Systolic Volume</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FL</td>
<td>Full Length CXCR2</td>
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<tr>
<td>Flk1/KDR</td>
<td>Kinase Insert Domain Receptor</td>
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<td>GAP</td>
<td>GTPase-Activating Protein</td>
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<td>GCP-2</td>
<td>Granulocyte Chemotactic Peptide-2/also known as CXCL6</td>
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<tr>
<td>GDI</td>
<td>Guanosine Nucleotide Dissociation Inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Nucleotide Exchange Factor</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GROα</td>
<td>Growth-Regulated Protein α</td>
</tr>
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<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Hydrolase Enzymes that bind and hydrolyze Guanosine Triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8, also known as CXCL8</td>
</tr>
<tr>
<td>HDMEC</td>
<td>Human Dermal Microvascular Endothelial Cell</td>
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<tr>
<td>HPF</td>
<td>High Power Field</td>
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<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
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<td>IP3</td>
<td>Inositol-1, 4, 5-trisphosphate</td>
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<td>JNK</td>
<td>c-Jun N-Terminal Kinase</td>
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<tr>
<td>KC</td>
<td>Keratinocyte Chemoattractant</td>
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<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
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<td>LIX</td>
<td>LPS-induced CXC Chemokine</td>
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<td>LV</td>
<td>Left Ventricle</td>
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<td>MAGUK</td>
<td>Membrane-Associated Guanylate Kinase</td>
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<td>MAPK</td>
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<td>Monocyte Chemotactic Protein 1</td>
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<td>MI</td>
<td>Myocardial Infarction</td>
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<td>MIF</td>
<td>Macrophage Migration Inhibitory Factor</td>
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<td>MIP-2</td>
<td>Macrophage inflammatory protein 2</td>
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<td>MMP-9</td>
<td>Matrix Metallopeptidase 9</td>
</tr>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>MNC</td>
<td>Mononuclear cell</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
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<tr>
<td>NHERF1</td>
<td>Na⁺/H⁺ Exchange Regulatory Factor 1</td>
</tr>
<tr>
<td>NHERF2</td>
<td>Na⁺/H⁺ Exchange Regulatory Factor 2</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-Methyl-D-Aspartate Receptor</td>
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<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
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<td>Polyacrylamide Gel Electrophoresis</td>
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<td>PDZ</td>
<td>Post synaptic Density Protein (PSD95), Drosophila Disc Large Tumor Suppressor (Dlg1), and Zonula Occludens-1 Protein (Zo-1); PSD-95/Dlg1/ZO-1</td>
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<td>PDZK1</td>
<td>Na⁺/H⁺ Exchange Regulatory Factor 3</td>
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<td>Platelet Factor</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
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<td>PIP2</td>
<td>Phosphatidylinositol 4, 5-biphosphate</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLC-β</td>
<td>Phospholipase C, β Isozyme</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post Synaptic Density Protein 95</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>PICK1</td>
<td>Protein Interacting with C-kinase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>ROCK</td>
<td>Rho-Associated Kinase</td>
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<td>SCGM</td>
<td>Stem Cell Growth Medium</td>
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<td>SDF-1</td>
<td>Stromal Cell-Derived Factor 1</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SV</td>
<td>Stroke Volume</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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CHAPTER 1 INTRODUCTION

1.1. Endothelial Progenitor Cell Discovery

The process of postnatal formation of blood vessels was traditionally believed to result from proliferation of pre-existing mature endothelial cells, which is termed angiogenesis[1]. Thanks to Asahara and coworkers’ isolation of endothelial progenitor cells (EPCs) from human peripheral blood, our understanding to the mechanism responsible for new blood vessel formation was extended [2]. Their discovery of EPCs has introduced the concept of vasculogenesis [2]. After that, postnatal vasculogenesis was defined as the process that progenitor differentiate into mature endothelial cells and promote postnatal new blood vessel formation [2, 3].

Since the discovery of EPCs by Asahara’s group, a substantial amount of investigations showed that bone marrow-derived EPCs contribute to new blood vessel formation by promoting the function of endothelium, incorporating into sites of active neovascularization such as ischemic hind limbs, myocardium infarction, and progressing tumors [4-7]. Recently, EPCs has been reported to be an independent risk factor of cardiovascular diseases [8]. It is also reported that EPCs are decreased in artery disease [9-11] but elevated in several types of cancer [12]. These studies have highlighted the potential importance of EPCs in neovascularization or angiogenesis related diseases [13]. Understanding of EPC biology provides us a better chance to manipulate EPCs and treat blood vessel formation related diseases. EPCs can be easily obtained from bone marrow,
peripheral blood, and spleen, and they can be expanded or manipulated in vitro before transplantation.

1.2. Classification of Endothelial Progenitor Cells.

In the bone marrow, EPCs are derived from mesodermal cells which ultimately differentiate into endothelial cells [14, 15]. Since the discovery of EPCs in 1997, different groups have tried to define and characterize this population. However, despite those efforts and the increasing number of publications in EPC biology, no exclusive definition of EPCs has been identified. So the EPC is considered to be a group of cells that are derived from bone marrow, are able to proliferate, and are able to differentiate from progenitor phenotype to mature endothelial phenotype.

Different cell surface molecules have been mainly utilized to characterize and define EPCs. Initially, three markers were used to characterize the early EPC: CD133, CD34, and vascular endothelial growth factor receptor-2 (VEGFR2) which is also known as Flk-1 or kinase insert domain (KDR) [16, 17]. EPCs from peripheral blood express CD34 and VEGFR2 but no longer express CD133. Endothelial cells are also negative for CD133 [18]. So loss of CD133 expression is accompanied with EPC maturation.

During almost two decades of study, a variety of approaches to isolate EPCs were developed, such as flow cytometry to analyze EPC markers [2, 19] and several type of colony forming assays [8, 20-23]. These studies have indicated that EPC is a type of heterogeneous cell population rather than a single type of cell.
population. Figure 1.1 showed the putative cascade of bone-marrow derived EPC differentiation.

Figure 1.1 Putative origin and differentiation cascade of EPCs. Schematic diagram shows the potential differentiation cascade of EPC from hemangioblast cells. Figure is from [24]. Dash arrow line: Some isolated EPCs possess endothelial-like cell surface marker but function as monocytes, indicating the putative origin of that portion of EPCs is monocyte but lack of direct evidence.
Although different nomenclatures have been used in attempting to distinguish EPCs from other progenitor cells with angiogenic activities according to their morphology, expression of cell surface markers, and cellular phenotype, there is still no precise description to define them. One of the general classifications of EPCs is based on the time they have been cultured in vitro, according to which EPCs are divided into early and late EPC [25]. Generally, bone marrow or peripheral derived mononuclear cells differentiated EPCs start to emerge in fibronectin coated plated containing endothelial growth media within a week, which is considered to be early EPCs. These cells have low proliferation potential, display spindle shaped morphology and express VEGFR2 and VE-cadherin, CD14 and CD45 [7, 26, 27]. These early EPCs can be cultured for 4 weeks, while continued culturing resulted in morphological and phenotypical changes in the cells, such as loss of hematopoietic marker and gain of endothelial marker [26]. Late EPCs started to emerge after 2-3 weeks in cell culture. Different from early EPCs, late EPCs are more proliferative, and they incorporate into endothelial monolayer more rapidly [25].

A variety of studies have reported the functional significance of early and late EPCs. Early EPCs are reported to homing and incorporate into ischemic loci to promote new blood vessel formation mainly by secreting angiogenic factors [7, 18, 28-31]. Late EPCs is reported to promote revascularization in a synergistic manner with early EPCs [32]. Similar to early EPCs, in vivo studies have demonstrated that administration of late EPCs into ischemic mice also resulted in enhanced angiogenesis [7, 15, 33, 34]. Moreover, detailed studies indicated that
late EPCs participate in the process of blood vessel formation by directly incorporating into endothelial network and provide building blocks for neovascularization [4, 35, 36].

1.3. **EPC mobilization and homing**

EPCs reside in the stem cell niche of bone marrow which also contains fibroblasts and osteoblasts. Certain cytokines secreted from local microenvironments interrupt the interaction between EPCs and bone marrow stromal cells and improve the EPC transendothelial migration to promote the EPC entering the circulation system. A variety of chemokines and growth factors have been reported to contribute to EPC mobilization. Stromal derived factor 1 (SDF-1) has been documented to play critical roles in EPC mobilization, especially in the situation of ischemia. It is reported that disruption of SDF-1 mediated interaction between EPC and bone marrow stromal cells and elevation of circulating SDF-1 are important for EPC mobilization. Moreover, it is believed that SDF-1 mobilize EPCs in a matrix metalloproteinase 9 (MMP-9) dependent manner [37]. The SDF-1/CXCR4 signaling pathway has been reported to play important roles in EPC biology [38, 39].

Vascular endothelial growth factor (VEGF) has also been reported to contribute to EPC mobilization through promoting EPC proliferation and increasing the expression of MMP-9 [40]. Erythropoietin (EPO), colony stimulating factor (G-CSF) and granulocyte monocyte colony stimulating factor (GM-CSF) are also reported to be able to increase circulating EPC amount by stimulating EPC proliferation [41-44]. Moreover, in vitro and in vivo studies have demonstrated that
CXCL5 and CXCL8, which have been considered as pro-inflammatory chemokines at the beginning, also play important roles in EPC mobilization [45].

Mediated mainly by chemokine/chemokine receptor axis, EPCs will home to the sites of ischemia or vascular injury to participate in blood vessel formation either directly or indirectly. Accumulating evidence have shown that several chemokine/chemokine receptor pairs play major roles in regulating EPC homing. Monocyte chemoattractant protein-1 (MCP-1), which is one of the CCR2 ligands, is present in the sites of endothelial denudation [46]. It is also reported that MCP-1/CCR2 axis is essential for directing EPCs into tumor neovessels [47]. Moreover, it is well documented that SDF-1/CXCR4 axis acts as a key element for EPC recruitment towards hypoxic gradient and arterial injury sites [48, 49].

Recently, the in vivo studies have revealed the importance of CXCR2, a CXC chemokine receptor which was initially identified in leukocyte recruitment [50], in EPC recruitment [51]. In the study, CXCL1 and CXCL7, which are both CXCR2 ligands, were able to arrest flowing EPCs, and blockade of CXCR2 attenuated the recruitment of EPCs [51, 52]. Furthermore, it has also been reported that CXCL12 and CXCL7 mediated the homing of EPC at early stage after arterial injury and later stage of which was mediated by CXCL1 [53].

1.4. Paracrine action of EPC

During the process of investigating EPC biology, some groups have noticed that the observed beneficial effects were not proportional to the amount of incorporated EPCs, indicating certain unidentified roles that EPCs have played during those processes. Growing evidence indicates that EPCs participate in
maintaining the integrity of vascular structure by improving blood vessel formation through both secreting proangiogenic factors and incorporating into endothelial network to differentiate into mature endothelial cells. Reports from multiple groups have supported this notion that EPC is an important source of chemokines and growth factors. It is believed that EPCs release factors that promote the functions of resident mature endothelial cells [18, 54]. Recently, it is reported that under hypoxic condition EPCs secret several kinds of chemokines and growth factors including CXCL1, SDF-1, VEGF, and macrophage migration inhibitory factor (MIF), which promoted the EPC adhesion, migration, and transmigration through endothelial layers [55]. In agreement with this finding, another group has also detected several chemokines, such as growth-regulated protein-α (GROα), CXCL-8, EGF, and MCP-1, in EPC conditioned medium. Furthermore, the authors also found that the EPC-conditioned medium promoted endothelial cell viability, cell migration, and angiogenesis through phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase-kinase/extracellular signal-regulating kinase (MEK/ERK) pathways [56]. Moreover, the paracrine factors secreted by EPCs were also able to protect endothelial cells from apoptosis and promote their angiogenic potency [57]. These secreted factors not only exert their activities on endothelial cells but also promote the EPCs’ function, such as incorporating into growing capillaries [25].

Based on the previous findings, it is clear that EPCs need to undergo multiple steps in order to contribute to the regeneration of blood vessel as shown
in Figure 1.2. Dysregulation of any step during this process will prevent EPCs performing normal functions, which can be observed in diseases.

Figure 1.2 EPCs contribute to blood vessel regeneration. EPCs need to undergo multiple steps including mobilization, chemotaxis, invasion, migration, differentiation, as well as pro-angiogenic factor secretion to contribute to the blood vessel formation. (Figure adopted from [15])
1.5. EPCs and diseases

The first report describing the contribution of EPC to tumor related vasculogenesis was in 2001 [58]. Lyden et al. demonstrated that bone marrow from wild-type mice, rather than that from angiogenesis-defective Id-mutant mice, restored tumor angiogenesis and growth [58]. The contribution of EPCs to tumor blood vessel formation varies among different studies. Meanwhile, clinical study has also been carried out in patients suffered from cancer after bone marrow transplantation. The contribution of bone marrow derived EPCs to tumor angiogenesis varies from 1% to 12.1% [59]. On the other hand, EPCs also contribute to the tumor vascularization indirectly through their paracrine activity. It has been reported that blocking EPC mobilization has significantly reduced the tumor angiogenesis and progression [60]. Other groups also demonstrate that EPCs promote angiogenesis by releasing pro-angiogenic factors, such as VEGF and SDF-1 [61, 62]. Because of their contribution to tumor related vascularization, EPCs have been considered to be a potential biomarker in various clinical studies as shown in Table 1.1 (Reviewed in [63]). In summary, accumulating evidence indicates that EPCs contribute to tumor related blood vessel formation directly and indirectly, and consequently, they have been considered as a potential tumor biomarker.
Table 1.1 Studies evaluating circulating EPCs levels compared to healthy controls in cancer patients. Modified from [63]

<table>
<thead>
<tr>
<th>Author/Year</th>
<th>Cancer types</th>
<th>EPC definition</th>
<th>Circulating EPC levels</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ho/2006</td>
<td>Hepatocellular Carcinoma</td>
<td>CD34+ CD133+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dome/2006</td>
<td>Non-Small Cell Lung Cancer</td>
<td>CD34+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Zheng/2007</td>
<td>Glioma</td>
<td>CD34+ CD133+ VEGFR2+</td>
<td>increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yu/2007</td>
<td>Hepatocellular Carcinoma</td>
<td>CD34+ CD133+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Richter-Ehrenstein/2007</td>
<td>Breast Cancer</td>
<td>CD34+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Brunner/2008</td>
<td>Head and Neck Cancer</td>
<td>CD133+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bogos/2009</td>
<td>Small Cell Lung Cancer</td>
<td>CD34+ VEGFR3+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sieghart/2009</td>
<td>Hepatocellular Carcinoma</td>
<td>CD34+ CD133+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Goon/2009</td>
<td>Breast Cancer</td>
<td>CD34+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rafat/2010</td>
<td>Glioma</td>
<td>CD34+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Nowak/2010</td>
<td>Lung Cancer</td>
<td>CD34+ CD133+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Su/2010</td>
<td>Ovarian Cancer</td>
<td>CD34+ VEGFR2+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Table 1.1 Continued

<table>
<thead>
<tr>
<th>Author/Year</th>
<th>Cancer types</th>
<th>EPC definition</th>
<th>Circulating EPC levels</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhatt/2011</td>
<td>Renal Cell Carcinoma</td>
<td>CD34+ CD133+ CD146+ CD45-</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bhaskar/2012</td>
<td>Multiple Myeloma</td>
<td>CD31+ CD34+ CD133+ CD45-</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yang/2012</td>
<td>Renal Cell Carcinoma</td>
<td>CD34+ VEGFR2+ CD45-</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Qiu/2013</td>
<td>Ovarian Cancer</td>
<td>CD34+ VEGFR3+</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ha/2013</td>
<td>Gastric Cancer</td>
<td>CD34+ CD133+</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ramcharan/2013</td>
<td>Colorectal Cancer</td>
<td>CD34+ VEGFR2+ CD45-</td>
<td>Increased</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kim/2014</td>
<td>Ovarian Cancer</td>
<td>CD31+ CD133+ VEGFR2+ CD45-</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Kim/2014</td>
<td>Cervical Cancer</td>
<td>CD31+ CD133+ VEGFR2+ CD45-</td>
<td>Increased</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Moreover, increasing body of evidence has demonstrated the correlation between the amount and function of EPCs and the cardiovascular disease risk factors which includes diabetes, hypertension, aging, smoking, and hypercholesterolemia.

Diabetes is a critical cardiovascular risk factor and it is negatively associated with circulating EPC number and function. EPC dysfunction is a major problem in diabetes related cardiovascular symptoms and vascular complications [10, 64-66]. Under the diabetic conditions, the number of circulating EPCs are decreased. In both type 1 and type 2 diabetic patients, the amount of circulating EPCs are decreased compared to the healthy control subjects [10, 66-68]. Along with the decrease in the EPC counts, findings from clinical studies indicate that the EPC functions, such as proliferation, adhesion, and incorporation, are also impaired under the conditions of diabetes [10, 64, 69-71]. These impairment in EPC functions may be due to their decreased response to stimuli [72, 73] and dysfunction in paracrine abilities [10].

The EPC counts and functions are also negatively related to aging, which is an important cardiovascular disease risk factor. It has been reported that EPCs from younger mice restored the impaired blood vessel formation in older mice [74]. In clinical studies, decreased circulating EPC number with aging in patients with coronary artery disease have also been described by several groups [9, 75]. It has been proposed that the aging related EPC dysfunction may result from reduced levels of pro-angiogenic factors, enhanced senescence, and accelerated apoptosis [8, 76-78].
Lipid metabolism such as hypercholesterolemia has also been related to circulating EPC number and function. It has been reported that EPCs from patients with hypercholesterolemia display impaired proliferation, migration and adhesion activities [79, 80]. Meanwhile, increasing evidence has demonstrated the association between EPC number and function with other cardiovascular disease risk factors such as smoking and hypertension [9].

It is considered that mobilized EPCs migrate to the injured vascular, such as ischemic tissue or infarcted cardiomyocyte, to promote vascularization and tissue repair [7, 81-83]. Many pre-clinical investigations have confirmed the therapeutic potential of EPCs by using animal models with myocardial infarction and hind limb ischemia [54, 84]. In the clinical studies, EPCs also displayed their therapeutic capacities. A large number of pre-clinical studies have shown that patients with cardiovascular disease and severe leg ischemia benefited from EPC administration as shown in table 1.2. (Reviewed in [69])
Table 1.2 Therapeutic effect of EPC therapy

<table>
<thead>
<tr>
<th>Author</th>
<th>Disease</th>
<th>n</th>
<th>Method of delivery</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perin</td>
<td>Severe ischemic heart failure</td>
<td>21</td>
<td>Intramyocardial injection</td>
<td>Myocardial perfusion↑; EF↑; ESV↓</td>
</tr>
<tr>
<td>Strauer</td>
<td>Acute MI</td>
<td>10</td>
<td>Intracoronary infusion</td>
<td>Infarct area↓, ESV↓; Myocardial viability↑; SV↑</td>
</tr>
<tr>
<td>Assmus</td>
<td>Acute MI</td>
<td>40</td>
<td>Intracoronary infusion</td>
<td>EF↑; Local contractility↑; Myocardial viability↑; Coronary flow reserve↑; ESV↓</td>
</tr>
<tr>
<td>Wollert</td>
<td>Acute MI</td>
<td>30</td>
<td>Intracoronary infusion</td>
<td>EF↑</td>
</tr>
<tr>
<td>Fernandez-Aviles</td>
<td>Acute MI</td>
<td>20</td>
<td>Intracoronary infusion</td>
<td>EF↑; Local contractility↑; ESV↓</td>
</tr>
<tr>
<td>Hamano</td>
<td>CABG</td>
<td>5</td>
<td>Injection in myocardial CABG</td>
<td>Myocardial perfusion↑</td>
</tr>
<tr>
<td>Tateishi-Yuyama</td>
<td>Severe leg ischemia</td>
<td>25</td>
<td>Injection into the gastrocnemius muscle</td>
<td>ABI↑; physical tolerance↑; tissue perfusion↑; Rrest pain↓</td>
</tr>
</tbody>
</table>

Therapeutic of injection based EPC therapy in different diseases. CABG, coronary artery bypass graft; MI, myocardial infarction; EF, ejection fraction; ESV, end systolic volume; SV, stroke volume; LV, left ventricle; ABI, ankle-brachial index. (Table is adopted from Shantsila et al [69])
1.6. GPCR overview

G protein-coupled receptor, which is also known as seven transmembrane receptor, is a superfamily of integral membrane proteins containing one extracellular N-terminus, three intracellular loops, three extracellular loops, and one intracellular C-terminus [85]. GPCRs contain one of the largest and most investigated population of proteins in mammalian genomes [86]. GPCRs can be detected in all types of tissues and they participate in a variety of cellular activities. GPCRs transduce extracellular signals into the cells and regulate cellular activities through their bound G proteins [85, 87, 88]. G proteins, also known as guanine nucleotide-binding proteins, are composed of three non-identical subunits which are α, β, and γ subunits [89]. Each subunit contains several isoforms, which makes a variety of combinations of G proteins, resulting in the complex network of signaling transduction [89, 90]. G protein is reported to be associated with the third intracellular loop of the GPCRs, and binding of ligands to GPCRs results in their conformational change and, consequently, release of the heterotrimeric G proteins which further activate downstream signaling molecules, such as phospholipase C beta (PLC-β) and adenylyl cyclase (AC) [89, 91-93] (as shown in Table 1.3). Unregulated GPCRs have been reported in a variety of pathological conditions including cancer development and progression [94-96]. Accordingly, GPCR are also considered as a potential therapeutic target by pharmaceutical companies, and nearly 40% of modern drugs target GPCRs [97]. Moreover, those drugs have only targeted a small portion of identified GPCRs. On the other hand, there are still large amount of GPCRs, whose functions and ligands are still not clear, have been
termed as orphan receptors [98-100]. Further studying GPCRs will improve our understanding of this group of proteins and eventually improve the development of disease therapies.

Table 1.3. G-protein subunits and their functions. (modified from [89])

<table>
<thead>
<tr>
<th>G-protein</th>
<th>Effector</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ga1, Ga2, Ga13, Ga6AB, Ga2</td>
<td>Adenylyl cyclase</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Ga1, Ga2, Ga5iast</td>
<td>Phosphodiesterase</td>
<td>Activation</td>
</tr>
<tr>
<td>Ga5, Ga5XL, Ga5i2l</td>
<td>Adenylyl cyclase</td>
<td>Stimulate</td>
</tr>
<tr>
<td>Gaq, Ga11, Ga14-16</td>
<td>Phospholipase Cβ</td>
<td>Stimulate</td>
</tr>
<tr>
<td>Ga12, Ga13</td>
<td>Rho guanine nucleotide-exchange factors</td>
<td>Stimulate</td>
</tr>
<tr>
<td>Gβγ-subunit</td>
<td>Phospholipase A, Phospholipase Cβ1-3, Adenylyl cyclase (type II, IV),</td>
<td>Activation</td>
</tr>
<tr>
<td></td>
<td>N-type calcium channel, P/Q-type calcium channel, Adenylyl cyclase (type I, III, V, VI, VII), PI3K, p21-activated protein kinase</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

1.7. GPCR signaling

G proteins are composed of three non-identical subunits [89]. G proteins usually exist in the heterotrimeric form containing α, β, and γ subunit [101, 102]. It is reported that α subunit contains 17 isoforms, the β subunit contains 5 isoforms, and γ subunit has 12 isoforms [89, 90]. When bound to GDP, the α subunit forms the complex with βγ heterodimer and associate with GPCRs, and when the α subunit binds GTP, it functionally dissociates from the βγ complex and regulates the downstream signaling effectors [91]. Generally, G proteins are termed
according to α subunit, which give rise to the $G_s$ (contains $G_{αs}$); $G_q$ (includes $G_{αq}$); $G_i$ (includes $G_{αi}$); and so on. $G_s$ couples to a large number of GPCRs and activates AC, which is responsible for the synthesis of the second messenger, cyclic AMP (cAMP). On the other hand, $G_i$ inhibits the cAMP synthesis by inhibiting the activity of adenylyl cyclase. $G_q$ is reported to stimulate PLC-β, and $G_{α12}$ activates the activities of Rho-Guanine Nucleotide Exchange Factors (GEFs) [89, 103]. Meanwhile, β and γ subunits function in the form of heterodimer are also capable of transducing signaling toward its own downstream effectors, such as PLC-β, AC, and $K^+$ channels [104-108] (as shown in table 1.3). Moreover, G proteins have also been reported to activate p38 mitogen-activated protein kinase (p38MAPK), extracellular signaling-related protein kinase (ERK1/2), and c-Jun N-terminal kinase (JNK) pathways [109-112].

1.8. Chemokine and CXCR2

Chemokines are a subtype of chemotactic cytokines, which were identified as cytokines that activated neutrophils [113]. They exert their activities to regulate cellular functions through GPCRs [114-116]. Based on the spacing of the first two cysteines at the N-terminus, chemokines have been divided into four subgroups: C, CC, CXC, and CX3C chemokines, in which X represents any non-cysteine amino acids [117-119] as shown in Figure 1.3. Within the chemokine sub-families, CXC chemokines are further classified into two categories based on the presence of a specific short peptide sequence: glutamic acid-leucine-arginine (ELR) at the N-terminus. Accordingly, CXC chemokine are subclassified into ELR$^+$ and ELR$^-$ CXC chemokines [120]. ELR$^+$ CXC chemokines include: interleukin-8 (IL-
8/CXCL8), epithelial neutrophil activating protein-78 (ENA-78/CXCL5), granulocyte chemotactic peptide-2 (GCP-2/CXCL6), neutrophilic activating protein (NAP-2/CXCL7), melanoma growth stimulatory activities (GROα,β,γ/CXCL1-3) [117]. It has been well accepted that ELR+ CXC chemokines are actively involved in angiogenesis [121, 122], recruitment of stromal cells to tumor microenvironment, and cancer progression [96]. By contrast, ELR- CXC chemokines, platelet factor-4 (PF-4) has been shown to have angiostatic properties [120]. Since Murphy et al identified the CXCR1 and CXCR2 in neutrophils in 1991, more and more chemokine receptors have been identified [123]. These chemokine receptors share several common properties. All of them are integral membrane proteins containing one extracellular N-terminus, three intracellular loops, three extracellular loops, and one intracellular C-terminus [85]. These chemokine receptors can be modified through glycosylation at their N-terminus, and their activities can be regulated by serine and threonine phosphorylation at their intracellular C-terminus. Moreover, the cysteines located at the first and second extracellular loops usually form a disulfide [124]. Generally, many chemokines have more than one receptors and many chemokine receptors have multiple ligands [117]. For example, ELR+ chemokine IL-8 stimulates both CXCR1 and CXCR2, which are highly homologous [123, 125]. CXCR1 binds to IL-8 or CXCL6, however, CXCR2 is able to interact with CXCL1, 2, 3, 5, 6, 7 and 8 [116].

CXCR2 is widely expressed in a variety of cell types including neutrophils [50], epithelial cells [96], smooth muscle cells, monocytes, and endothelial progenitor cells [45]. It has been reported to be involved in multiple cellular
activities such as cell proliferation, migration, angiogenesis, as well as survival [50, 96]. Activated by its ligands, G protein dissociates from CXCR2 and activate different downstream effectors such as cAMP/PKA, PKC, PLC, PI3K/AKT, Ras/Raf/MAPK, and even NF-κB pathways to regulate different cell functions [126-129].

Figure 1.3. Classification of chemokine. Based on the spacing of the first two cysteines at the N-terminus, chemokines have been divided into four subgroups: C, CC, CXC, and CX3C chemokines, in which X represents any non-cysteine amino acids. CX3C chemokines possess additional mucin-like domain and cytoplasmic domain. (Figure from [130])
1.9. **PDZ domain, PDZ motif and PDZ mediated protein-protein interaction**

PDZ domain, the name of which comes from the first three proteins observed containing this domain: post synaptic density protein 95 (PSD-95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1), mediates protein-protein interactions by recognizing and physically interacting with short binding motifs which usually is located at the carboxyl termini of proteins [131, 132]. Previous studies from our lab and others’ have demonstrated that PDZ domain mediated protein-protein complexes play important roles in facilitating signaling transduction in different types of cells [50, 96, 133]. Composed of one β strand and one α helices, the binding groove of PDZ domain specifically recognize and bind the short binding motif, which is usually 4 ~ 5 residues in length, referred as PDZ motif [134, 135]. The nomenclature for the PDZ motif is defined as follows: the last amino acid at carboxyl terminal amino acid is defined as P₀ residue, and subsequently, the rest of the residues towards the amino terminus are named as P₋₁, P₋₂, P₋₃, etc. [136]. Oriented peptide library approach was used to study the preference of amino acids within this PDZ motif [136]. Comprehensive previous studies indicated that the P₀ and P₋₂ residues are the most critical residues for recognition and physical interaction between PDZ motif and PDZ domain [136]. Specific hydrophobic amino acids, Valine (V) and Leucine (L), are preferred at P₀ position for PDZ domain recognition and binding [134, 137]. Meanwhile, the amino acid preference at P₋₂ position varies, the amino acid there could be hydroxylated, charged or hydrophobic [138]. Based on the bound PDZ motif, PDZ domains are classified into several subgroups [138, 139].
Generally, they are divided into three classes: class I PDZ domains specifically recognize S/T-X-φ-COOH [89, 90] (φ represents hydrophobic amino acid residue and X represents any amino acid residue); class II PDZ domains specifically recognize the φ-X-φ-COOH [85, 91]; and class III PDZ domains recognize the motif X-X-C-COOH [140, 141]. Moreover, there are a few other PDZ domains that do not fit into this classification [142-144]. Proteins containing PDZ domains, such as Na+/H+ exchange regulatory factor 1 (NHERF1) and Na+/H+ exchange regulatory factor 2 (NHERF2), provide scaffold for organization of both membrane receptors or ion channels and downstream effectors to proximity location, leading to facilitated signaling transduction. Based on the status of possessed PDZ domains, PDZ domain containing proteins (PDZ proteins) can be divided into two groups: ones that only contain PDZ domain, NHERF1 and NHERF1; and proteins that contain PDZ domain along with other domains, such as membrane-associated guanylate kinase (MAGUK) proteins [82]. Within the groups of proteins that contain PDZ domain(s) only, NHERF1 which is also called ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50), as well as its isoform NHERF2 are expressed in a variety types of cells. Possessing two highly homologous PDZ domains, PDZ1 and PDZ2, NHERF1 and NHERF2 provide scaffold for a variety of signaling proteins including GPCRs [133]. Also, both NHERF1 and NHERF2 regulate a wide range of cell signaling and cellular events [145]. They usually function as scaffold proteins to associate with membrane receptors and their downstream effectors. CXCR2 possesses a PDZ motif (-S-T-T-L) at tis carboxyl termini, meanwhile, its downstream effector, PLC-β isoforms, also contain consensus PDZ motif (-X-S/T-
X-L/V). Our previous studies have demonstrated that class I PDZ domain mediated multiprotein complex, including CXCR2, NHERF1, PLC-β, play important roles in facilitating specific and efficient signaling transduction in different cell lines [50, 96] (Figure 1.4).

![Figure 1.4](image-url)

Figure 1.4. Structure of NHERF1 PDZ2 in complex with CXCR2 C-terminus containing PDZ motif and NHERF1 PDZ1 in complex with PLC-β3 C-terminus with PDZ motif.
(A). Ribbon diagram of NHERF1 PDZ2 recognizes CXCR2 PDZ motif. PDZ2 is shown in orange and CXCR2 C-terminus peptide is shown in blue. (B). Ribbon diagram of NHERF1 PDZ1 recognizes PLC-β3 C-terminus peptide. PDZ1 is shown in green and PLC-β3 is shown in magenta. For both PDZ1 and PDZ2, the α-helices and β-strands are labeled according to their position in the sequence. (Diagrams are modified from [146, 147])
1.10. CXCR2 in angiogenesis

Angiogenesis plays important roles in multiple biological processes, such as wound healing and tumor progression [148, 149]. Ligands of CXCR2 such as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 have been reported to be critical in promoting angiogenesis, and these findings have highlighted the significance of CXCR2 in angiogenesis [150-154]. Moreover, through corneal micropocket assays, the angiogenic activity induced by CXCR2 ligands was significantly attenuated in the presence of CXCR2 neutralizing antibody or under the condition of CXCR2 depletion [155]. Meanwhile, CXCR2 has also been reported to promote endothelial cell tube formation, proliferation, and inhibit endothelial cell from apoptosis [156].

Cellular functions of endothelial cells such as migration and proliferation also play important roles during the process of angiogenesis. CXCL1 was reported to be able to enhance human umbilical vein endothelial cell proliferation [157]. It has also been demonstrated that CXCL1 promotes endothelial cell migration and proliferation through ERK1/2 and p38 MAPK signaling pathways [157, 158].

1.11. CXCR2 in EPC

EPCs are defined according to expressing markers of progenitor, possessing the capacity of colony-forming, and being able to differentiate into endothelial cells. According to these characteristics, EPCs play important roles in blood vessel formation directly and indirectly, which contain multiple steps. The CXCR2 has also been detected in EPCs in previous studies and it has been shown that CXCR2 regulates several EPC activities such as homing, transmigration, and reendothelialization [51, 159, 160]. Findings from previous studies showed that
multiple CXCR2 ligands are positively involved in EPCs activities. It has been demonstrated that CXCL1 and CXCL7, function through their receptor CXCR2, play critical role in EPC recruitment, adhesion, and incorporation during the multiple steps after arterial damage [53]. More specifically, during the process of homing to the arterial injury sites, CXCL1 and CXCL7, promote the adhesion of EPCs to fibronectin or integrin ligands. Accordingly, CXCR2 is termed as an arrest receptor in EPCs. Compared to CXCR4, which plays critical roles in EPC mobilization, CXCR2 is more important during EPC recruitment [51, 159]. Another CXCR2 ligands, CXCL8, is also involved in CXCR2 mediated EPC transwell migration and capillary tube formation. Previous studies have shown that under hypoxic condition, upregulated expression of CXCL8 from ischemic tissue mediates EPC migration toward chemotaxis gradient [161, 162]. Furthermore, CXCL8 axis also regulates the angiogenic, proliferative, and migratory activities in mature endothelial cells in both paracrine and autocrine manner [156, 163]. Under certain pathological conditions environmental circumstances also influence the behaviors of EPCs, sometimes through affecting chemokines and chemokine receptors. In blood vessel formation related disorders, ischemia is one of the major environments. Hypoxic condition has been reported to upregulate the expression of CXCR2 and its cognate ligand, CXCL1, in EPCs. In the presence of CXCL1/CXCR2, the transmigration, adhesion, and angiogenic potencies of EPCs have been stimulated. Moreover, CXCL1/CXCR2 axis is believed to play crucial roles in initiating of neovascularization by recruiting EPCs [55]. Similarly, IL-8 secreted by ischemic cardiac endothelium is also reported to function as a
mediator for EPC myocardial homing by generating a chemoattractant gradient [162].

1.12. Rho GTPase and RhoA/ROCK signaling

Small GTPases are composed of a large population of hydrolase enzymes that function independently as molecular switches to regulate a variety of cellular activities. Activated by guanine nucleotide exchange factors (GEF) and inactivated by GTPase-activating proteins (GAP), small GTPases cycle between GTP bound active conformation and GDP bound inactive conformation. When they are activated, GTP bound GTPase will transduce signaling toward their downstream effectors until the GTP is hydrolyzed as shown in Figure 1.5. Among hundreds of GTPases, Rho, Rac, and Cdc42 belong to Rho GTPase subfamily, are the three most extensively studied members. Their contributions to the regulation of cytoskeleton assembly and regulation have been well demonstrated in a variety of cell lines. It has been shown that, Cdc42 and Rac are involved in mediating membrane protrusion while RhoA regulates the membrane retraction in fibroblasts. RhoA has been reported to mediate the wound closure [164, 165]. Moreover, these members of Rho GTPases are also associated with regulating cellular activities other than cell motility. They were reported to regulate cell polarity, G1 cell cycle progression, proliferation, and survival [166].

Among these well studied GTPase, RhoA and its principle effector Rho-associated kinase (ROCK), which is a serine/threonine protein kinase, have been the study subjects of a variety of groups [167]. Involved in cytoskeleton organization, the RhoA/ROCK signaling axis has been shown to contribute to the
regulation of several cellular events, such as cell focal adhesion and stress fiber assembly [168], activation of integrin [169], migration, contraction, proliferation, cell cycle progression, survival, and apoptosis [170]. A growing body of evidence from basic research and clinical study suggest that RhoA/ROCK signaling axis play important roles the development of cardiac structure and conduction system during embryonic [171, 172]. Accordingly, RhoA/ROCK signaling pathway have been demonstrated to be associated with several cardiovascular diseases, including hypertension, coronary artery spasm, ischemic stroke, pulmonary hypertension, and heart failure [173-177]. Moreover, recruitment of bone marrow derived cells has also been attenuated under the condition of ROCK deficiency [178].
Figure 1.5. Regulation of Rho GTPase activity.
Rho GTPase cycles between GDP-bound (inactive) and GTP-bound (active) states. GEFs stimulates the release of GDP from Rho GTPase, which allows it to binding GTP. GAP hydrolysis the GTPase bound GTP to inactivate it. GDI binds to GDP-bound Rho GTPase to prevent it from activation and keep it away from the plasma membrane, which is their place for action.
As RhoA exerts its activities mainly through ROCK proteins to regulate a variety of cellular activities, ROCK proteins have also been studied. The ROCK family has two members, ROCK1 and ROCK2, which share 92% homology in their catalytic domains. Selective inhibitors, such as Y27632 [179] and fasudil [180], targeting ROCKs at their ATP-dependent kinase domain have been used to study the role of RhoA/ROCK signaling axis in different disorders. RhoA/ROCK and their downstream targets are responsible for cell migration and motility, and Y27632 or siRNA targeting ROCKs attenuated the RhoA/ROCK mediated cell migration and proliferation in different cell lines [181-187]. Moreover, the roles of RhoA/ROCK signaling axis have been extensively explored in cancer and cancer related cells, such as cancer associated endothelial cells [160, 188-193].

Under the circumstance of malignancy, decreased cell proliferation, migration, and invasion, as well as increased apoptosis, were observed in different types of cell lines when RhoA/ROCK signaling pathways were inhibited [194-200]. Meanwhile, different mechanisms such as reduced stress fiber and focal adhesion formation, decreased expression of proteolytic enzymes, and cytoskeleton rearrangement disorder [194, 195, 201] have been proposed to explain those observed results. These findings highlighted the importance of RhoA/ROCK signaling pathway in the cell motility, survival, proliferation, as well as gene expression.

Moreover, RhoA/ROCK signaling pathway is also reported to play important roles in the process of blood vessel formation [202, 203]. Expressed in endothelial cells, RhoA/ROCK system is involved in the VEGF mediated angiogenesis, and
inhibiting this signaling results in impaired precapillary cords formation and endothelial migration [204-208]. In addition, human umbilical vein endothelial cells (HUVECs) overexpressing RhoA display increased migratory and angiogenic potency, which indicates the importance of RhoA signaling in endothelial cell activities [209]. Previous findings indicated that RhoA affects the endothelial cell functions mainly through regulating actin cytoskeleton organization [210]. In addition, RhoA/ROCK pathway has been suggested to be an upstream regulator of MAPK signaling molecules including ERK1/2, p38 MAPK, and JNK [211-213]. The MAPK pathways are closely associated with a variety of cellular activities [212, 214]. To be specific, the ERK1/2 pathway, which has been well studied, is involved in cell growth, proliferation, differentiation, and migration [215]. Inhibition of ROCK decreased the activation of ERK1/2 and consequently attenuated the cell migration and proliferation in glioblastoma cells [214]. Recently, Zoledronate, which is used to treat hypercalcemia in clinic, has been shown to attenuate the angiogenesis in EPCs through inhibiting the activation of RhoA and MAPK signaling [216]. These findings suggest the association of MAPK signaling and RhoA/ROCK pathway in EPCs.

On the other hand, it has been demonstrated that interfering of CXCR2 mediated signaling transduction, either through CXCR2 neutralizing or CXCR2 knockout, has significant effect in endothelial cell migration and angiogenesis [217, 218]. Moreover, recruitment of EPCs from bone marrow to the sites of neovascularization was also reported to be mediated by CXCR2 in mice [219]. In addition, substantial evidence has accumulated that CXCR2 signaling activates
the MAPK signaling molecules, including ERK1/2, p38 MAPK, and JNK, in a variety of cell lines to regulate multiple cellular activities [220-223]. Interestingly, RhoA also plays important roles in all these blood vessel formation related cellular activities, such as migration, angiogenesis, and neovascularization. Taken together, it is possible that CXCR2 mediated angiogenesis and neovascularization are mediated through RhoA in endothelial cells and EPCs.
CHAPTER 2 MATERIAL AND METHODS

2.1. Antibodies and reagents

Anti-human and mouse CXCR2 and PLC-β3 antibodies as well as mouse anti-NHERF1 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-NHERF1 polyclonal antibody was bought from Sigma-Aldrich. Recombinant chemokines, IL-8/CXCL8, growth-regulated protein alpha (CXCL1/GROα/KC), and macrophage inflammatory protein 2 (MIP-2/murine CXCL2) as well as growth factor VEGF (both mouse and human) were obtained from ProSpec (East Brunswick, NJ). Growth factor reduced Matrigel matrix, glutathione agarose beads and Transwell inserts were purchased from BD Bioscience. The human and murine CXCR2 C-terminus peptides (biotin-conjugate at N-terminus): WT (biotin-FVGSSSGHTSTTL for human CXCR2 C-tail; and Biotin-FVSSSANTSTTL for mouse CXCR2 C-tail) and PDZ motif deletion (∆TTL) were synthesized by Genemed Synthesis, Inc. (San Antonio, TX). CXCR2 inhibitor SB225002 was purchased from Tocris Bioscience. Chariot™ peptide/protein delivery reagent was purchased from Active Motif (Carlsbad, CA). Lipofectamine 2000, Hanks’ buffered salt solution (HBSS), Fura-2 were obtained from Invitrogen (Carlsbad, CA). GST PDZ fusion proteins (GST-NHERF1, NHERF2, or PDZK1) were purified using glutathione agarose beads (BD Bioscience), eluted with 50 mM glutathione, and used in pull-down. Fibronectin for EPCs attachment in culture plate was purchased from BD biosciences (Franklin, NJ).

2.2. Plasmid, cloning, and mutagenesis
C-terminal fragments of murine CXCR2 (last 45 amino acids; a.a. 315–359 for murine CXCR2) were generated by PCR cloning into pEGFP-C3 vector (Invitrogen). CXCR2 C-terminus PDZ motif deletion mutation (ΔTTL) was generated using the QuikChange™ Site-directed Mutagenesis kit (Agilent Technologies), and also cloned into pEGFP-C3 vector for plasmid construction.

2.3. **EPC isolation and culture**

Mouse bone marrow (BM) cells were obtained by flushing the tibias and femurs from wildtype C57BL/6J mice with PBS. The collected BM mononuclear cells (MNCs) were isolated from collected bone marrow cells by density centrifugation over Histopaque1083 (Sigma). The MNCs were then collected and counted. Eighty million MNCs were seeded into each fibronectin (10 μg/ml) coated 60-mm petri dish with endothelial cell basal medium-2 (EBM-2) (Lonza) supplemented with endothelial cell growth medium-2 (EGM-2) MV SingleQuots (Lonza) containing FBS (5%), vascular endothelial growth factor (VEGF), fibroblast growth factor-B, epidermal growth factor, insulin-like growth factor 1, hydrocortisone, ascorbic acid and antibiotics. Cells were cultured at 37°C with 5% CO₂. After 72 hours in culture, non-adherent cells were removed, and culture medium was changed every other day. EPCs were characterized by incorporation of Dil labeled acetylated low density lipoprotein (Dil-Ac-LDL) and staining of BS-Lectin I (Vector laboratories, Burlingame, CA), and flow cytometric analysis of specific EPC surface markers (CD34 and VEGFR2) on day 7. During cultivation, the morphological changes of EPCs were recorded using inverted microscopy. Mouse BM-derived EPCs were also obtained from Celprogen Inc. (Torrance, CA).
The EPCs have been characterized by the Celprogen Company and are stained positive for CD34, CD45, Sca-1, AP, and demonstrate incorporation of acetylated LDL. The cells were maintained in EBM-2 supplemented with EGM-2 MV SingleQuots, and sub-cultured every 2 days on murine fibronectin-coated culture plates. The cells between passages 2–8 were used for the experiments.

Cell freezing and thawing were used to maintain the early passage batches of cells for long term storage. Exponentially growing cells (approximately 80% confluency) were treated with trypsin in a 37°C incubator. Then, 3 ml growth medium were added to abrogate trypsin activity. The mixture was then transferred to a 15-ml sterile tube and centrifuged at 100g for 7 min to harvest cell pellet. The cell pellet was re-suspended in 5 ml freezing medium [growth medium containing 20% FBS and 10% (v/v) dimethyl sulfoxide (DMSO)]. Then aliquots of 1ml containing 2 x 10^6 cells were transferred in cryogenic foam boxes at -80°C freezer overnight and then immersed in liquid nitrogen. Cells were recovered from liquid nitrogen by rapid thawing in a 37°C water bath, and then immediately re-suspended in the appropriate pre-warmed culture medium and transferred to a 100 mm^2 Petri dish, and medium was changed after 24 hours.

Human cord blood (CB)-derived EPCs (AC133^+ progenitor cells) were isolated from the cord blood obtained from volunteers under an Institutional Review Board-approved protocol by the Henry Ford Health System. The cord blood MNC population was generated by Ficoll gradient centrifugation and was enriched for AC133^+ cells by immune-magnetic positive selection using the MidiMACS system (Miltenyi Biotec, Auburn CA) according to the manufacturer's protocol. Upon
isolation AC133 + cells were suspended in CellGro® Stem Cell Growth Medium (SCGM) (CellGenix) supplemented with 40 ng/ml of stem cell factor, 40 ng/ml of FMS-like tyrosine kinase 3 and 10 ng/ml of thrombopoietin (all from CellGenix), as described before [224]. Cells were maintained under these conditions at 37°C with 5% CO₂ as a suspension culture to expand for up to 30 days with the cell concentration kept between $5 \times 10^5$–$1 \times 10^6$ cells/ml. For cellular functional studies (such as adhesion and migration), primary AC133+ CB-EPC cells were first differentiated in EGM-2 medium supplemented with 2% FBS and 2 ng/ml of VEGF and plated in 6-well plate coated with human fibronectin at a concentration of $2 \times 10^5$ cells/ml. Cells were allowed to differentiate for 2 weeks. The medium was changed every 2–3 days, and the cells were monitored by inverted phase contrast microscopy to evaluate the morphological changes during differentiation, as described before [224].

2.4. Cell adhesion assay

Chariot™ peptide delivery reagent was used to deliver CXCR2 C-terminal peptides (WT and ΔTTL) into the cells as reported before for adhesion assay. Briefly, EPCs were cultured in fibronectin coated dishes until they reached 50% confluency. CXCR2 C-tail peptides (WT and ΔTTL) diluted in sterilized water were then mixed with Chariot™ reagent diluted in PBS at room temperature for 30 min to form chariot-peptide complex. The complex was then incubated with EPCs at 37°C for 1 hour, and in the control group, EPCs were incubated with Chariot™ reagent only under the same condition. The cells were then detached from culture dish with trypsin. Ten thousand EPCs were seeded into each well of fibronectin
coated 96-well plate at 37°C for 1 hour in starve medium (EBM-2 medium containing 0.5% FBS) supplemented with 100ng/ml MIP-2. After 1 hour, non-adherent EPCs were removed by PBS washing and attached cells were quantified by counting under 3 randomly selected high power field (HPF) in each well using an inverted microscopy. Experiment was repeated three times and each condition was triplicated.

2.5. Chemotaxic migration assay

Chemotaxic migration assay was performed using 24-well cell culture insert (BD bioscience) with 6.5-mm diameter polycarbonate filters (8-μm pore size) with EPCs. Filter membranes were pre-coated with 0.1% gelatin diluted in migration medium (0.5% FBS in EBM-2) for 2 h at 37 °C and air-dried afterward. EPCs in culture dish were delivered with CXCR2 C-terminal peptides (WT or ΔTTL) or Chariot™ reagent only, which was used as negative control, as described above. EPCs were then detached and seeded in to each culture insert. EPCs (1 x 10^5) in 100μl of migration medium (0.5% FBS in EBM-2) were loaded in the upper chamber of gelatin-coated Transwell, and 500μl migration medium containing MIP-2 (100 ng/ml) was added in the lower chamber. The Transwell was then returned to the 37 °C incubator for 3 h to allow the EPCs to migrate toward chemoattractant gradient. After incubation, the remaining cells in the upper chamber were removed by cotton swap. The migrated cells were fixed and stained with Diff-Quick stain kit (IMEB, San Marcos, CA) according to the manufacturer’s protocol. Experiment was repeated three times and each group was triplicated. Stained adherent cells
were counted in 3 randomly selected fields per well using HPF under an inverted microscopy.

2.6. In vitro incorporation assay

EPCs were delivered with CXCR2 C-terminal peptides (WT or ΔTTL) or Chariot™ reagent alone, which was used as negative control, as described above. After that, EPCs were incubated with Dil-Ac-LDL (red fluorescence; Vector Laboratories) in growth medium or, alternatively, incubated with Calcein Am (green fluorescence, Invitrogen) in serum free medium according to the manufacturer’s protocol respectively. Fluorophore-labeled EPCs (2 x 10^3), delivered with different CXCR2 C-terminal peptides or delivery reagent, were co-cultured with human dermal microvascular endothelial cells (HDMECs; 1 x 10^4) in growth factor reduced matrigel (BD biosciences) coated 96-well plates (round bottomed) in EBM-2 medium containing 100ng/ml MIP-2. The cell mixture were kept in the 37°C incubator for 3 hours to form tubular structure. The incorporated and non-incorporated EPCs were quantified using ImageJ software. The percentage of incorporated EPCs was calculated and plotted. The proportion of EPCs in tubule structures was determined in 10 random fields under a fluorescence microscopy.

2.7. Generating stable transfected EPC cell line

EPCs were allowed to grow in culture plates till they reached 90% confluency which is optimum for transfection. Then the cells were transfected with pEGFP-C3 vector expressing different murine CXCR2 C-termini, WT and PDZ motif deleted, (pEGFP-CXCR2 C-tail WT and pEGFP-CXCR2 C-tail ΔTTL) or pEGFP-C3 vector alone into EPCs using lipofectamine 2000 reagent (Invitrogen)
according to the manufacturer’s instruction. Cells were allowed to grow for another 48 hours and then were further diluted (1:1,000 dilution) and transferred into new culture dish. Another plate with same amount of un-transfected cells was incubated under the same condition as negative control. Stable expression of the constructs in murine EPCs were obtained by using G418 sulfate selection. The expression of GFP was confirmed by fluorescent microscopy and western blotting.

2.8. **In vivo matrigel plug angiogenesis assay**

EPCs stably transfected with pEGFP-CXCR2 C-tail WT and pEGFP-CXCR2 C-tail ΔTTL or the vector only were used in matrigel plug angiogenesis assay. Briefly, $5 \times 10^5$ EPCs stably expressing CXCR2 C-tail constructs or vector alone in 100μl of PBS were mixed with 500μl of growth factor reduced Matrigel containing 40 units/ml heparin and 200ng/ml MIP-2. The Matrigel containing EPC mixtures were implanted by a single subcutaneous injection into the mid-dorsal region of wild-type C57BL/6/J mice. Ten days after the injection matrigel plugs containing EPCs expressing different constructs were collected. The obtained matrigel plugs were prepared for histological study (H&E staining and anti-CD31 immunostaining). Microvessel density (CD31$^+$ cells) was quantified and compared in multiple mice between different groups where EPCs expressed different constructs. Image analyses of the areas of CD31$^+$ blood vessels, vessel number, and length of vessel perimeter were carried out as reported [225]. All the animal studies were accomplished under the protocol approved by Wayne State University Institutional Animal Care and Use Committee.
2.9. **Intracellular calcium mobilization assay**

EPCs (2 × 10^6/ml) were delivered with CXCR2 C-terminal peptides (WT or ΔTTL) or Chariot™ reagent alone as described above. The peptide-delivered EPCs were then labeled with 5μM Fura-2 AM in HBSS buffer (Ca^{2+} and Mg^{2+} free) at 37 °C for 45 min. The cells were then re-suspended in regular HBSS buffer at the concentration of 10^6 EPCs/ml. Two milliliter re-suspended EPCs were loaded into a continuously stirred cuvette at 37°C in a PTI fluorescence spectrophotometer (Photon Technology International). The fluorescence of cell suspensions was continuously monitored in a PTI fluorescence spectrophotometer at excitation wavelengths of 340 nm and 380 nm, and emission wavelength of 510 nm. The intracellular calcium concentration ([Ca^{2+}]_i) was expressed as the fluorescence ratio at 340nm and 380nm (R). EPCs were stimulated with 100ng/ml MIP-2 once the baseline was stabilized. At the end of each test, ionomycin (5μM, a calcium-specific ionophore) were added to trigger maximal fluorescence ratio (R_{max}). After that, 10mM EGTA (pH > 8) were added to chelate the Ca^{2+} to obtain minimum fluorescence ratio (R_{min}). The intracellular calcium level (in nM) was proportional to the ratio of fluorescence at 340/380. The equation describing this relationship is as follows:

\[
[\text{Ca}^{2+}]_i (\text{nM}) = K_d \times \left[ \frac{(R - R_{min})}{R_{max} - R} \right] \times Sfb
\]

In this equation, \( K_d = 225 \text{ nM} \) (under the condition that Ca2+ binds to Fura-2 AM at 37°C), Sfb = ratio of fluorescence at 380nm under ionomycin and EGTA treated conditions.
2.10. Pull down assay

EPCs endogenously express CXCR2 were lysed in binding buffer (PBS + 0.2% Triton X-100) supplemented with a mixture of protease inhibitors (containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and phosphatase inhibitor mixture (Sigma). Cell lysates were mixed with the lysis buffer at 4°C for 15 min followed by centrifugation at 17,000g for 15 min at 4°C. The concentration of total proteins was measured with Bradford method. Then the cleared supernatant was equally mixed with GST or different GST fused PDZ domain containing proteins (GST-NHERF1, GST-NHERF2, and GST-PDZK1) at 4°C for 2 h for direct binding. After 2 hours incubation, same amount (20ul) of glutathione agarose beads (BD biosciences, San Jose, CA) were equally added and mixed overnight at 4°C. Thereafter, the protein bound glutathione agarose beads were pelleted by low speed centrifugation (800 x g, 1 min) followed by three times of wash using lysis buffer to get rid of loosely bound proteins. After that, the mixture of glutathione agarose beads and proteins were eluted in Laemmli sample buffer containing β-mercaptoethanol. Eluted proteins were subjected to western blotting to detect CXCR2.

2.11. Co-immunoprecipitation

EPCs were solubilized in binding buffer (PBS containing 0.2% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). After centrifugation (17,000 x g, 15 min at 4°C), cleared supernatant were collected and total protein concentration was measured. Then the cleared lysates were processed for co-
immunoprecipitation using a kit for co-immunoprecipitation (Thermo Scientific). Resin from the kit was used to immobilize the normal IgG control and anti-CXCR2 IgG according to the manufacturer’s instruction. Co-precipitated proteins were eluted in Laemmli sample buffer containing β-mercaptoethanol. Eluted proteins were separated by SDS-PAGE and probed for PLC-β3 and NHERF1.

2.12. Western blot

Denatured proteins, either from total cell lysate or from binding assay elution, were loaded onto Mini-PROTEAN TGX Gels (Bio-Rad) for separation. Then the separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using Bio-Rad electrophoresis system. The transfer blot filter paper (Bio-Rad) and the PVDF membrane (Bio-Rad) were pre-soaked in transfer buffer. Transfer was carried out at a constant intensity of 60V for 90 min. Following transfer, the membrane was then blocked in 5% non-fat dry milk solution in 1X TBST (Tris-Buffered Saline and Tween 20) at room temperature for 1 hour and incubated in primary antibody 1:1,000 diluted with 1X TBST. Incubation was carried out with gentle shaking at 4ºC overnight. The membrane was then washed six times by gentle shaking in 1X TBST at room temperature. It was then incubated with a suitable secondary antibody conjugated with the horseradish peroxidase (HRP) 1:20,000 diluted in TBST-5% milk for 1 hour at room temperature with gentle shaking. The membrane was then washed in TBST. The membrane was then incubated with a mixture of the SuperSignal West Pico Chemiluminescent Substrate (Pierce) or SuperSignal West Femto maximum sensitivity substrate (Pierce) for 5 min at room temperature. The membrane was then exposed in UVP
Biospectrum 500 imaging system (UVP). The signals were obtained for 5 seconds to 30 minutes depending on the intensity of the signals and the substrate that was used. The pictures were processed using NIH ImageJ software.

2.13. EPC isolation and culture

Mouse BM-derived EPCs were obtained from Celprogen Inc. (Torrance, CA). The EPCs have been characterized by the Celprogen Company and are stained positive for CD34, CD45, Sca-1, alkaline phosphatase (AP), and demonstrate incorporation of Dil-Ac-LDL. The cells were maintained in EBM-2 supplemented with EGM-2 MV SingleQuots, and sub-cultured every 2 days on murine fibronectin-coated culture plates. The cells between passages 2–8 were used for the experiments.

Cell freezing and thawing were used to maintain the early passage batches of cells for long term storage. Exponentially growing cells (approximately 80% confluency) were treated with trypsin in a 37°C incubator. Then, 3 ml growth medium were added to abrogate trypsin activity. The mixture was then transferred to a 15ml sterile tube and centrifuged at 100g for 7 min to harvest cell pellet. The cell pellet was re-suspended in 5 ml freezing medium [growth medium containing 20% FBS and 10% (v/v) dimethyl sulfoxide (DMSO)]. Then aliquots of 1ml containing 2 x 10^6 cells were transferred in cryogenic foam boxes at -80°C freezer overnight and then immersed in liquid nitrogen. Cells were recovered from liquid nitrogen by rapid thawing in a 37°C water bath, and then immediately re-
suspended in the appropriate pre-warmed culture medium and transferred to a 100 mm² Petri dish, and medium was changed after 24 hours.

2.14. Immunofluorescence staining

EPCs were seeded on glass coverslips pre-coated with fibronectin (10μg/ml) until they reached 40% confluency and were starved in serum free medium before stimulation. EPCs were then delivered with WT CXCR2 C-terminus peptide or Chariot™ reagent alone before MIP-2 stimulation. At the same time, another group of EPCs were pre-incubated with ROCK inhibitor (Y27632) for 1 hour as negative control. After that the cells were then treated with 100ng/ml MIP-2 in serum free medium for different time spans (0, 6, 12min) followed by washing with 37°C PBS to remove stimulants. The cells were then fixed by 4% paraformaldehyde in PBS (pH = 7.0) at room temperature for 10 min and washed with PBS at room temperature for 30 min. The paraformaldehyde fixed cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 mins at room temperature followed by washing with PBS for 30 min at room temperature to get rid of remaining detergent. The coverslips were then transferred to a humid chamber and were incubated with 100nM Acti-stain™ 555 phalloidin (cytoskeleton Inc.) prepared in PBS at room temperature in the dark for 30min to stain F-actin. Thereafter, the nuclei were stained with 1μg/ml DAPI (Invitrogen) for 1 min. Then, the coverslips were washed with PBS and inverted on a drop of mounting media on glass slides. Pictures were taken under a fluorescence microscopy.

2.15. RhoA activation assay
The activation status of RhoA was performed using RhoA pull-down activation assay kit (Cytoskeleton, Inc.) according to the manufacturer’s instruction. Briefly, EPCs were seeded into fibronectin coated plates till 40% confluency. Then EPCs were starved in serum free medium followed by incubation with 100ng/ml MIP-2 in serum free medium for 0, 1, 3, 6min. After chemokine stimulation, medium was aspirated and cells were washed with PBS followed by aspiration of PBS. Ice-cold lysis buffer provided by the kit was used to lyse the cells. After that, cell lysate from each group were collected by cell scraper and clarified by centrifugation (10,000 x g, 1 min at 4°C). After measurement of protein concentration, 25ug cell lysate from each group was obtained and used as input and 400ug total cell lysate from each group was used for pull-down assay. Same amount (50ug) rhotekin-RBD beads, which specifically binds to activated RhoA, were used for pull-down in each group. Binding was carried out at 4°C for 1 hour then the beads were pelleted. When the binding was completed, the rhotekin-RBD beads were pelleted. The proteins bound to the beads were eluted in Laemmli sample buffer containing β-mercaptoethanol and boiled for 2 min. The eluted and denatured proteins were then subjected to western blot to detect the bound activated RhoA as previous mentioned except for the equilibration step. The equilibration was performed after electrophoresis. During equilibration, the gel was immersed in western blot buffer (containing 25mM Tris pH 8.3, 192mM glycine, and 15% methanol dissolved in water).

2.16. MAPK and Akt signaling activation assay
EPCs were cultured in fibronectin coated 6-well plates to reach 80% confluency and were starved in serum free medium before stimulation. After starvation, EPCs were delivered with WT CXCR2 C-terminus peptide or Chariot™ reagent alone. Meanwhile, another group of EPCs were pre-incubated with 10μM ROCK inhibitor Y27632 for 1 hour. Then cells from each group were stimulated with 100ng/ml MIP-2 for different time spans (0, 1, 3, 6, and 12 min). Cells were then washed with PBS to remove any remaining chemokines and were solubilized in RIPA buffer (150 mM NaCl, pH 8.0, 50 mM Tris-base, 1% (v/v) Nonidet P-40, 0.5% (v/v) Na-deoxycholate, 0.1% (v/v) SDS) supplemented with protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin) and a mixture of phosphatase inhibitors (40mg/ml sodium fluoride, 20mg/ml sodium orthovanadate, and 65mg/ml sodium pyrophosphate). Cells were lysed in 4°C for 15 min and were centrifuged to pellet nuclei and cell debris. Cleared supernatant were collected and total protein concentration was measured in each sample with Bradford method. After concentration measurement, 20μg of each sample were mixed with Laemmli sample buffer containing β-mercaptoethanol. Denatured proteins were subjected to western blot to detect phosphorylation status of signaling molecules belonging to MAPK pathway, such as Akt, JNK, p38MAPK, and ERK1/2.

2.17. Statistical analysis

All experiments are repeated at least three times. Analyzed data is presented as mean ± SE. Statistical significance of differences was evaluated with student’s t-test. When p < 0.05, the data was considered as statistically significant.
CHAPTER 3 RESULTS

3.1. CXCR2 PDZ-mediated macromolecular complex in EPC homing and angiogenesis

3.1.1. Rationale

Angiogenesis, which is composed of endothelial cells migrate from pre-existing blood vessels and proliferate to form blood vessels, was believed to be the only mechanism of new blood vessel formation until Asahara and his colleagues isolated EPCs twenty years ago [26, 226, 227]. Despite the debate of EPC characterization and definition, our knowledge of EPCs biology has extensively improved during the past two decades. Based on the morphological and functional features, EPCs can be generally divided into two groups: early EPCs and late EPCs. Early EPCs, which exist at the early stage in culture, possess spindle shape and contribute to angiogenesis by producing angiogenic cytokines such as IL-8 and VEGF. Late EPCs display cobblestone shape and express different cell surface markers. Moreover, late EPCs with the potential of long term proliferation, are able to incorporate into endothelial network and differentiate into mature endothelial cells to facilitate new blood vessel formation [2, 20, 228-230]. Findings from preclinical studies have demonstrated the contribution of early and late EPCs to the blood vessel formation under the condition of ischemia [25, 32]. It has been shown that these two groups of cells synergistically promote the neovascularization, mainly through IL-8 secretion, when infused together into animal model [32]. Due to the roles that EPCs play during angiogenesis and neovascularization in a variety of disorders [12, 53, 231-234], they have been considered to be a potential therapeutic target. However, administration of EPCs
in clinical studies seems not fully encouraging [235]. Accordingly, deep understanding of EPC biology, especially the underlying molecular mechanism of the angiogenic and vasculogenic functions in EPCs, will significantly improve the translational study.

It has been reported that both CXCR2 neutralizing antibody and CXCR2 depletion dramatically decreased endothelial cell migratory and angiogenic capacities [217, 218]. Moreover, recruitment of EPCs from bone marrow to the sites of neovascularization was also reported to be mediated by CXCR2 in mice [219]. These studies have revealed the importance of CXCR2 in regulating cellular functions during angiogenesis and neovascularization. Importantly, our recent studies have further demonstrated that the PDZ motif located at the C-terminus of CXCR2 is involved in regulating intracellular signaling transduction and cellular functions in neutrophil and pancreatic cancer cells [50, 96]. In our studies, we showed that NHERF1, a scaffolding protein containing two PDZ domains, clustered CXCR2 and its downstream signaling molecule PLC-β to form macromolecular complex through PDZ mediated protein-protein interactions in neutrophil and pancreatic cancer cells. These multiprotein complexes were involved in a variety of cellular activities, such as cell migration, proliferation, and calcium mobilization. Therefore, it is possible that the CXCR2, through its PDZ motif, can also interact with PDZ domain containing scaffolding proteins, which further interact with other downstream proteins to form multiprotein complex in EPCs. However, the existence of this PDZ mediated CXCR2 involved
macromolecular complex as well as its influence in EPCs have not been evaluated so far.

3.1.2. Results

3.1.2.1. EPC isolation and characterization

In addition to using commercially available mouse EPCs (Celprogen Inc.), EPCs were isolated from the tibias and femurs of C57BL/6J according to the protocols previously reported [13, 29]. Isolated EPCs were cultured in EPC specific medium for in vitro expansion. After 4 days of cultivation, the isolated EPCs gradually adhered to the fibronectin coated growing surface and displayed spindle-shaped morphology as shown in Figure 3.1.1A, which is similar to the morphology reported by Asahara as well as others [2, 236]. Those attached cells were characterized by Dil-Ac-LDL up-taking and FITC-conjugated BS-Lectin staining as shown in Figure 3.1.1B. AC133+ EPCs isolated from human cord blood were also cultured according to previously reported protocol [224] as shown in Figure 3.1.1C.
Figure 3.1.1. EPC isolation and characterization.

(A). Morphology of EPCs isolated from mice bone marrow (BM-EPCs) in culture at day 1, 7, and 14 (phase contrast images, 4x or 10x magnificent). (B). Images for phase contrast, Dil-Ac-LDL (red) up-taking and FITC conjugated BS-Lectin I (green), and nuclei staining (DAPI, blue) of bone marrow derived EPCs (10x magnificent). (C). Morphology of EPCs isolated from human cord blood in cell culture at day 1, 14, and 28 in cell culture (10 x magnificent).
3.1.2.2. **CXCR2 signaling plays important roles in EPC migration and angiogenesis in mice.**

EPCs need to migrate toward and subsequently adhere to the angiogenic active site to contribute to the formation of new blood vessels and CXCR2 is also reported to be important in regulating angiogenesis. The role of CXCR2 was evaluated in these processes in EPCs, including EPC migration, adhesion, invasion, and incorporation into endothelial network. The commercially available murine EPCs (Celprogen Inc.) were used in these experiments. The data shows that compared with controls, EPCs, which were cultured in basal medium (EBM-2 containing 0.5% FBS), VEGF (used as positive control), MIP-2, and CXCL8 treatment significantly improved EPC migration (Figure 3.1.2), adhesion (Figure 3.1.3), invasion (Figure 3.1.4), and incorporation into endothelium tubular structure (Figure 3.1.5) as shown in Figures respectively. On the other hand, MIP-2 and CXCL8 mediated EPC migration, adhesion, incorporation into endothelium network, and tubular structure formation were significantly attenuated by inhibiting CXCR2 mediated signaling transduction through CXCR2 neutralizing antibody [237] or CXCR2 antagonist SB225002 [238]. However, the promotive activities generated by VEGF were not affected by CXCR2 signaling interfering, which is consistent with previous findings [51, 219].
Figure 3.1.2 CXCR2 promotes murine EPCs migration. Pictures (A) and Bar graphs (B) showed MIP-2, CXCL8, and VEGF (all 100ng/ml) promoted the migration of EPCs (pre-treated in goat serum); CXCR2 antiserum (EPCs pre-treated with neutralizing antibody in goat serum) inhibited the MIP-2 and CXCL8 mediated EPCs migration but VEGF mediated EPC migration was not affected (n=4, p < 0.05). (C) EPC migration was improved by MIP-2, CXCL8, and VEGF (all 100ng/ml) and the promotive effect of MIP-2 and CXCL8 could be inhibited by pre-incubation with CXCR2 antagonist SB225002. While VEGF mediated increased migration was not affected by the CXCR2 antagonist pre-incubation (n=4, p < 0.05). (NT: no cytokine treatment, HPF: high power field).
Figure 3.1.3. CXCR2 promotes murine EPC adhere to fibronectin. Pictures (A) and bar graph (B) showed that MIP-2, CXCL8, and VEGF promoted EPC adhere to fibronectin coated surface compared with EPCs without stimulation (NT: no cytokine treatment). Pre-incubation of CXCR2 antiserum (goat serum containing CXCR2 neutralizing antibody) significantly inhibited MIP-2 and CXCL8 promoted EPC adhesion but had not effect on VEGF promoted ECP adhesion to fibronectin. (n=3, p< 0.05)
Figure 3.1.4. CXCR2 promotes EPC invasion activity in vitro. MIP-2, CXCL8, and VEGF (all 100ng/ml) facilitate the invasion of EPCs through matrigel. The administration of CXCR2 antagonist SB225002 significantly attenuated the promotive effect generated by MIP-2 and CXCL8 while had no effect on VEGF promoted EPC invasion. (n=4, p < 0.05).
Figure 3.1.5. CXCR2 promotes the incorporation of EPCs into endothelial network in vitro.

DAPI labeled EPCs (blue), pre-incubated in goat serum, incorporation into endothelium (HDMEC shown as transparent cells) tubular network in matrigel was significantly improved by CXCR2 ligands, MIP-2 and CXCL8, and VEGF as shown in fluorescent images (A) and bar graph (B). Pre-incubation of EPCs in CXCR2 antiserum significantly reduced the EPCs incorporation into endothelium tubule structures in response to CXCR2 ligands, but had no apparent effect on VEGF promoted increased incorporation. (n=4, p < 0.05).
Human cord blood derived EPCs were also used to confirm the findings in murine EPCs. Accordingly, the role of CXCR2 in human EPC migration and incorporation into endothelial tubular structures were evaluated respectively. Human cord blood EPCs were obtained as reported [224, 239-241]. The results suggested that the migratory potential and the ability of incorporating into endothelial tubular structure of EPC were increased in response to the CXCL8 and VEGF compared to that of the cells without any treatment. Moreover, when EPCs were pre-incubated with CXCR2 antagonist SB225002, the promotive effect of CXCL8 on EPCs were significantly reduced, while promotive effect of VEGF on EPCs were not apparently affected (Figure 3.1.6). These findings were consistent with those from murine EPCs.
Figure 3.1.6. CXCR2 promotes human EPC migratory and incorporative capacities in vitro.

(A). Migration of human cord blood derived EPCs was promoted by CXCL8 and VEGF (all 100ng/ml). The increased migration mediated by CXCL8 treatment was significantly interfered by pre-incubation of EPCs with CXCR2 antagonist SB225002 but that mediated by VEGF was not affected (n=4, p < 0.05). (B) EPCs in vitro incorporation into HDMVEC tubule structure was facilitated by CXCL8 and VEGF treatment (all 100ng/ml). The promoted incorporation mediated by CXCL8 was attenuated by pre-incubation with CXCR2 antagonist SB225002 but mediated by VEGF was not affected significantly (n =3, p < 0.05) (NT: not cytokine treated, SB: SB225002).
3.1.2.3. Disrupting CXCR2 PDZ motif-mediated interactions inhibits EPC in vitro adhesion.

Our previous studies have demonstrated the importance of CXCR2 PDZ motif (TTL) in facilitating CXCR2 mediated intracellular signaling transduction in neutrophil and pancreatic cancer cells [50, 96]. It has been demonstrated that CXCR2 is actively involved in EPC homing and angiogenesis [51, 219]. So it is possible that the PDZ motif of CXCR2 is also involved in the CXCR2 mediated EPC activities including migration, adhesion, invasion, and incorporation into endothelial cell network. For this purpose, synthesized biotin labeled CXCR2 C-terminus containing PDZ motif (Biotin-FVSSSSANTSTTL; WT) or without PDZ motif (Biotin-FVSSSSANTS; ΔTTL) were used for EPC functional assays in vitro. Murine EPCs were firstly delivered with different CXCR2 C-terminal peptides (WT or ΔTTL) or the delivery reagent alone (Chariot™) and then were seeded onto fibronectin coated culture dishes in starve medium (EBM-2 containing 0.5% FBS) supplemented with or with MIP-2 (100ng/ml). After 1 hour, adherent cells were counted. The result showed that the adhesion of WT CXCR2 C-terminus peptide delivered EPCs to fibronectin in response to MIP-2 was significantly attenuated compared to that of EPCs delivered with Chariot™ or ΔTTL CXCR2 C-terminus peptide (Figure 3.1.7A). The same concept was also evaluated in human cord blood derived EPCs. Human cord blood derived EPCs were delivered with human CXCR2 C-terminus peptides with or without PDZ motif (WT or ΔTTL) or Chariot™ alone. Then those cells were used for adhesion assay mediated by CXCL8. The data showed that WT peptide delivery significantly inhibited the human cord blood derived EPCs adhesion to fibronectin under the stimulation of CXCL8 (Figure
3.1.7B). These findings indicated that PDZ motif of CXCR2 plays an important role in EPC adhesion.
Figure 3.1.7. WT CXCR2 C-terminus peptides delivery inhibited EPCs adhesion to fibronectin mediated by CXCR2 signaling. (A). Picture and bar graph of murine EPCs delivered with WT or ΔTTL CXCR2 C-terminus peptide, or Chariot™ reagent alone for adhesion assay under the stimulation of MIP-2. WT peptide delivery significantly decreased the EPC adhesion to fibronectin. (n = 4, p < 0.05) (B). Human cord blood derived EPCs were also delivered with human WT or ΔTTL peptide or delivery reagent alone for adhesion assay under the stimulation of CXCL8. WT peptide significantly attenuated the EPC adhesion to fibronectin compared to those deliveries with ΔTTL peptide or Chariot™ reagent alone. (n = 4, p < 0.05)
3.1.2.4. Disrupting CXCR2 PDZ motif-mediated interactions inhibits EPC in vitro migration

Transwell migration assay were performed using both murine EPCs and human cord blood derived EPCs. Similarly, EPCs were subject to delivery of CXCR2 C-terminus peptides (WT or ΔTTL) or Chariot™ alone followed by transwell migration assay. The results from transwell migration assay showed that EPCs delivered with WT peptides displayed significantly reduced migratory potential under the stimulation of MIP-2 or CXCL8, which was not observed in EPCs delivered with ΔTTL peptides (Figure 3.1.8). These data indicated that CXCR2 PDZ motif is important in EPC migratory activity in both human and mice.
Figure 3.1.8. WT CXCR2 C-terminus peptides delivery inhibited EPCs migration. (A). Picture and bar graph of murine EPCs delivered with WT or ΔTTL CXCR2 C-terminus peptide, or Chariot™ reagent alone for migration assay under the stimulation of MIP-2. WT peptide delivery significantly decreased the MIP-2 induced EPC migration. (n = 4, p < 0.05) (B). Human cord blood derived EPCs were also delivered with human WT or ΔTTL peptide or delivery reagent alone for migration assay under the stimulation of CXCL8. WT peptide significantly attenuated the EPC migration compared to that delivery with ΔTTL peptide or Chariot™ reagent alone. (n = 4, p < 0.05)
3.1.2.5. **Disrupting CXCR2 PDZ motif-mediated interactions blocks EPC in vitro incorporation into endothelial tubule structure.**

It has been suggested that EPCs need to act in concert with pre-existing endothelial cells to form blood vessels, but not to form new vessels de novo [64]. In addition, it has also been documented that CXCR2 neutralizing antibody dramatically attenuated the incorporation of human EPCs into the arterial injury site in the carotid arteries of athymic nude mice [51], indicating that CXCR2 play an important role in the process of EPC incorporation. In order to evaluate the role of CXCR2 PDZ motif-mediated interactions in the process of EPC incorporation into endothelial tubular structure, CXCR2 peptides (WT or ΔTTL) or Chariot™ were delivered into EPCs. After that, the cells were labeled with Dil-Ac-LDL and were cultured with HDMECs in growth factor reduced matrigel respectively in the presence of MIP-2. The result showed that WT peptide delivery significantly interfered the incorporation of EPCs into endothelial tubular structure while ΔTTL peptide delivery did not significantly affect the EPC incorporation induced by MIP-2. (Figure 3.1.9)
Figure 3.1.9. Disrupting PDZ-mediated interaction attenuated the incorporation of EPCs to the endothelial tubular network. WT CXCR2 C-terminus peptides delivery significantly reduced the incorporation of EPCs into the endothelial tubular network. While ΔTTL peptide delivery did not achieve any significant inhibition on EPC incorporation compared to control group which delivered with Chariot™ reagent alone. (n=3, p< 0.05)
Disrupting CXCR2 PDZ motif-mediated interactions decreases EPC in vivo angiogenesis in mice.

EPCs have been reported to contribute to neovascularization through incorporating into the vessel formation site and differentiating into mature endothelial cells [242, 243]. In order to investigate the role that PDZ motif of CXCR2 plays in EPC neovascularization in vivo, matrigel plug assay was performed using murine EPCs. For this purpose, EPCs that stably express CXCR2 C-terminus peptide were established. Constructs expressing CXCR2 C-terminus peptides that contains the last 45 amino acids of CXCR2 were cloned into pEGFP-C3 vector (pEGFP-CXCR2 C-terminus WT). The PDZ motif deleted mutation (pEGFP-CXCR2 C-terminus ΔTTL) was also generated with commercial available mutagenesis kit. The generated constructs and empty vector (pEGFP-C3) were then delivered into EPCs with lipofectamine 2000 separately. Stable cell lines consistently expressing transfected constructs were obtained by G418 selection. The expression of the constructs was evaluated by fluorescent microscopy and western blot as shown in Figure 3.1.10.
Figure 3.1.10. Stable cell lines expressing CXCR2 C-terminus peptides or GFP only.
(A). Pictures of EPCs expressing pEGFP-C3 CXCR2 C-terminus WT or ΔTTL under fluorescent microscopy. (B). Western blot detecting the expression of GFP-CXCR2 C-terminus WT or ΔTTL peptides or GFP alone in EPC cell lysates (IB: GFP).
When the stable cell lines were established, the cells were used for matrigel plug assay. EPCs expressing different CXCR2 C-terminus constructs were mixed with growth factor reduced matrigel containing MIP-2. The matrigel containing EPCs were subcutaneously injected into wildtype C57BL/6J mice. Ten days after administration, the matrigel plugs were obtained and prepared for histological investigations. Visually, less blood vessels were observed from the matrigel plug containing EPCs expressing WT CXCR2 C-terminus compared to those containing EPCs expressing vector alone or ΔTTL CXCR2 C-terminus. The matrigel plugs were then subjected to H&E staining and immunohistochemistry (IHC) staining. Data from H&E staining showed that the tubular structure formation was significantly decreased in matrigel plugs containing pEGFP-C3 CXCR2 C-terminus WT transfected EPCs compared with those containing EPCs expressing ΔTTL CXCR2 C-terminus (Figure 3.1.11A). The expression of endothelial marker, CD31, in the matrigel was also evaluated. The data from IHC demonstrated that the expression of CD31 was significantly lower in WT group compared with ΔTTL group (Figure 3.1.11B). The microvessel density represented by CD31+ cells were quantified and compared among groups. The result showed that the microvessel number in the matrigel from WT group was significantly lower than that from ΔTTL and vector control groups (Figure 3.1.11C). The plugs from WT and ΔTTL groups were homogenized and the homogenates were used for western blot to confirm the expression of GFP fused CXCR2 C-terminus peptide (Figure 3.1.11D).
Figure 3.1.11. Disrupting CXCR2 PDZ motif-mediated interactions reduces EPC in vivo angiogenesis in mice
(A). H&E staining of newly formed blood vessels (arrows) in matrigels containing EPCs transfected with pEGFP CXCR2 WT (left) or ΔTTL (right) C-termini. (B). IHC staining of CD31 in matrigels containing EPCs expressing pEGFP CXCR2 WT (left) or ΔTTL (right) C-termini. (C). Quantification of CD31 positive blood vessels in matrigel plugs from matrigels with EPCs expressing pEGFP CXCR2 WT (left) or ΔTTL (right) C-termini. (n= 5 mice in each group, p< 0.01) (D). Western blot using anti GFP antibody to detect the expression of GFP fused CXCR2 WT (left) and ΔTTL (right) C-terminus.
3.1.2.7. Disrupting CXCR2 PDZ motif-mediated interactions reduces CXCR2-mediated EPC intracellular calcium mobilization

CXCR2 is able to couple to the pertussis toxin-sensitive G<sub>i</sub> proteins to stimulate phospholipase C (PLC) activities [50]. GPCR-induced PLC activation catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2), resulting in two second messengers: diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) which can trigger the intracellular calcium mobilization [244]. It is well accepted that calcium signaling plays important roles in cytoskeletal reorganization and cell migration [245, 246]. Recently, it has been reported that calcium signaling is involved in the EPC migration toward the sites of neovascularization and vascular remodeling [247]. In order to study the role of CXCR2 PDZ-mediated interaction in calcium signaling in the cells, intracellular calcium mobilization assay was performed in vitro. CXCR2 C-terminus peptides (WT or ΔTTL) or Chariot<sup>TM</sup> reagent alone were delivered into murine EPCs separately. Then calcium mobilization under the stimulation of MIP-2 (100ng/ml) was performed and the results were calculated as described in the method. The result demonstrated that the MIP-2 induced calcium mobilization was significantly attenuated in EPCs delivered with WT CXCR2 C-terminus peptide, but not in those delivered with ΔTTL CXCR2 C-terminus peptide or Chariot<sup>TM</sup> reagent alone (Figure 3.1.12). These data suggested that PDZ-mediated interaction plays an important role in calcium mobilization in EPCs.
Figure 3.1.12. Disrupting CXCR2 PDZ-mediated interactions attenuated MIP-2 induced calcium mobilization in EPCs. EPCs were delivered with different CXCR2 C-terminus peptide (WT or ΔTTL) or Chariot™ respectively and were treated with MIP-2 (100ng/ml) to induce calcium mobilization. The cells were incubated with Fura-2 (5μM) and the fluorescence was monitored in a PTI fluorescence spectrophotometer under different wavelength. The changes of intracellular calcium level (nM) in different groups were obtained and calculated as previously described (Wu et al., 2012). (n=4, p < 0.01).
CXCR2 interacts with relevant signaling proteins via PDZ-based interactions.

CXCR2 processes a consensus PDZ motif (-STTL-COOH) at its carboxyl terminus, which could potentially bind to certain proteins containing PDZ motif. Based on PDZ array (Panomics) and previous studies [50, 96] several PDZ scaffold proteins which interact with CXCR2 through its C-terminal PDZ motif have been identified. GST pull-down assay was performed to investigate whether endogenous CXCR2 in EPCs interacts with PDZ scaffold proteins as previously described [50]. EPCs were lysed and cleared cell lysate were incubated with GST fused PDZ proteins (GST-NHERF1, GST-NHERF2, and GST-PDZK1) or GST for binding. After that, the GST-fusion proteins with their bound proteins were pull-down by glutathione agarose beads for western blot to detect CXCR2. The result demonstrated that CXCR2 was pulled down by NHERF1 rather than NHERF2 or PDZK1 (Figure 3.1.13A), indicating that endogenous CXCR2 prefers to interact NHERF1 in EPCs. In order to investigate if the binding between NHERF1 and CXCR2 happens endogenously, co-immunoprecipitation assay was performed. EPCs were lysed and cleared cell lysate were used for co-immunoprecipitation according to the instruction of manufacturer. The result showed that endogenous NHERF1 could be co-immunoprecipitated with CXCR2 in EPCs (Figure 3.1.13B).

As introduced above, CXCR2 couples to Gi, which is sensitive to pertussis toxin, to active PLC-β isoforms. In addition, we have demonstrated that PLC-β2 forms multiprotein complex with CXCR2 and NHERF1 through PDZ-based interaction in neutrophils [50]. We have also reported a NHERF1 interact with PLC-β43 and CXCR2 to form macromolecular complex in a PDZ dependent manner in
pancreatic cancer cells [96]. More importantly, it was reported that PLC-β2 deficiency in EPC results in interfered migration and intracellular calcium mobilization [247]. As demonstrated above, disrupting PDZ-mediated interactions attenuated CXCR2 mediated calcium mobilization in EPCs, which suggests that PLCβ isoform may form macromolecular complex with CXCR2 through PDZ based interaction. For this purpose, co-immunoprecipitation assay was performed using CXCR2 antibody. The data demonstrated that PLC-β3 was co-immunoprecipitated with CXCR2 (Figure 3.1.13C). Like CXCR2, PLC-β3 also contains a PDZ motif at its C-terminus [96], so it is highly possible that NHERF1, possessing two PDZ domains, functions as a scaffold protein to cluster CXCR2 and PLC-β3 to proximity to form a macromolecular complex through PDZ-mediated interactions to facilitate specific and efficient signaling transduction in EPCs.
Figure 3.1.13. CXCR2 interact with NHERF1 and PLC-β3 through PDZ-mediated interactions.
(A). endogenous CXCR2 was pulled down by GST NHERF1 protein. (B). Endogenous NHERF1 was co-immunoprecipitated with endogenous CXCR2 in EPC. (C). Endogenous PLC-β3 was co-immunoprecipitated with endogenous CXCR2 in EPC.
3.1.3. Discussion

A large body of evidence has accumulated to indicate that EPCs, which is considered to be a group of cells that are derived from bone marrow, have the capacity to migrate, proliferate, and differentiate from progenitor phenotype into mature endothelial cells in a variety of systems. EPCs have been demonstrated to be important in adult neovascularization and contribute to vascular repair as well as tumor growth [2, 248]. The homing of EPCs toward the new blood vessel formation sites is a complex process which includes chemotactic migration, invasion, adhesion, incorporation, and finally differentiation [243]. These processes are regulated by a variety of biologically active factors such as cytokines, chemokines, and growth factors. During two decades of investigation of EPC biology, CXCR2 and CXCR4 and their ligands have been reported to play critical roles in EPC homing through regulating EPC mobilization, recruitment, and firm adhesion injury [51, 162, 219, 249-251]. Although EPCs have been studied for two decades in different areas, the underlying mechanisms of how EPCs contribute to postnatal neovascularization still needs further investigation.

CXCR2 possesses a PDZ motif at its C-terminus, and the PDZ motif has been reported to modulate CXCR2 mediated cellular chemotaxis [252]. We have recently demonstrated the role of PDZ mediated CXCR2 involved macromolecular complex in neutrophil and pancreatic cancer cells. In neutrophils, disrupting PDZ-mediated interaction by exogenous peptide (short peptide containing CXCR2 PDZ motif) significantly interrupted CXCR2-mediated calcium mobilization and migration [50]. Similarly, in pancreatic cancer cells, introducing exogenous peptide
containing CXCR2 PDZ motif has significantly attenuated proliferation and invasion in vitro and inhibited tumor growth in vivo [96].

In this study, the importance of CXCR2 PDZ-mediated interaction in EPC motility and angiogenic potency has been demonstrated through functional assays and biochemical studies. Through introducing exogenous CXCR2 C-terminus peptide (WT or PDZ motif deleted) or stably transfecting DNA constructs expressing CXCR2 C-terminal fragments into EPCs, the CXCR2 PDZ-mediated interactions have been interrupted. Consequently, the abilities of EPCs incorporating into endothelium network (Figure 3.1.9) and postnatal neovascularization (Figure 3.1.11) was significantly interfered. Moreover, the intracellular calcium mobilization was also affected due to the interrupted CXCR2 PDZ-mediated interaction (Figure 3.1.12). Based on our previous findings, these results indicated that a PDZ-mediated CXCR2 involved multiprotein complex may be formed in EPCs to regulate those cellular activities. A growing body of evidence indicate that proteins containing PDZ domains function as scaffolding proteins to cluster multiple proteins to form macromolecular complexes which promote specific and efficient signaling transduction [145, 253-255].

It has been reported that antiserum against the PDZ motif of shCD146, which is a cell adhesion molecule, inhibited the adhesion of EPCs to activated endothelial cells. This study indicated that PDZ motif plays important roles in EPC biology [256]. Recently, we have also demonstrated that NHERF1 clustered CXCR2 and PLC-β to proximity in neutrophil and pancreatic cancer cells to regulate CXCR2 mediated signaling transduction [50, 96]. We further reported the
crystal structures that demonstrated the interaction between NHERF1 and CXCR2 as well as NHERF1 and PLC-β3 [146, 147, 257]. PLC-β isoforms have been documented to be involved in GPCR-mediated cellular activities. Among the 4 members of PLC-β family, PLC-β3 is widely expressed in different tissues [258] and can be activated by Gβγ subunits [259]. PLC-β3 has been reported to interact with PDZ domain containing proteins including NHERF1 and NHERF2 through the PDZ motif located at its C-terminus in a variety of cell lines [260-262]. Based on these findings, it is reasonable to speculate that the PDZ-mediated CXCR2 involved multiprotein complex may also exist in EPCs to regulate signaling transduction. In the present study, the biochemical assay demonstrated that in EPCs NHERF1 clustered CXCR2 and PLC-β3 to form macromolecular complex. Accumulating evidence suggested that the assembly of the macromolecular complex beneath cellular membrane facilitated the transduction of signals from environment into the cells, which subsequently affected the cellular activities specifically and efficiently [50, 263-266]. Furthermore, functional assays suggested the importance of this multiprotein complex in EPC recruitment and incorporation into endothelial tubule structure.

These findings suggested a therapeutic implication of PDZ-mediated CXCR2 involved multiprotein complex in diseases related to blood vessel formation. EPCs are involved in a variety of disorders including pathologic angiogenesis, tumor progression, and airway remodeling in asthma [219, 249, 267, 268]. Therefore, inhibiting the angiogenic potency of EPCs may provide an approach to control new blood vessel formation in these disorders. In vivo matrigel
plug assay has demonstrated that competing the PDZ-mediated interaction between CXCR2 and NHERF1 by introducing CXCR2 C-terminus peptides into EPCs has significantly hindered the EPC involved blood vessel formation. These findings have supported the notion that targeting PDZ-mediated interaction in EPC may have therapeutic implication in angiogenesis related diseases. In addition, Aarts et al. reported that disrupting the PDZ based interaction between N-methyl-D-aspartate receptor (NMDAR) and PSD-95 has inhibited the activation of neuronal nitric oxide synthase (nNOS), which subsequently reduced the stroke induced brain injury [269]. Moreover, the PDZ domain of adaptor protein PICK1 has recently been identified as a potential therapeutic target for neuropathic pain, excitotoxicity, and cocaine addiction [270, 271]. Therefore, targeting PDZ-mediated CXCR2 involved macromolecular complex in EPC may convey promising therapeutic implication in diseases associated with blood vessel formation such as tumor progression.
3.2. RhoA is involved in CXCR2 signaling in EPC

3.2.1. Rationale

Substantial proof has accumulated that CXCR2 signaling activates the MAPK signaling molecules, including ERK1/2, p38 MAPK, and JNK, in a variety of cell lines to regulate multiple cellular activities [220-223, 272]. It is well accepted that MAPK signaling pathway regulates cell migration in cancer cells [273], endothelial cell [274], and EPCs [216]. MAPK signaling is important in the process of angiogenesis due to its contribution to the migratory and proliferation in endothelial cells and EPCs, which are critical events in angiogenesis. On the other hand, the regulatory effects of CXCR2 on the motility and angiogenic activities in EPCs has been reported as well. Li et al. also reported that EPC mobilization and neovascularization were significantly hindered in CXCR2 knockout mice [45]. Furthermore, the promotive role of CXCR2 in angiogenesis have been well appreciated [217, 275]. These findings indicate the putative involvement of MAPK signaling in the CXCR2 regulated EPC functions.

Another important signaling effector downstream of CXCR2 is PI3K/Akt. It has been demonstrated that PI3K/Akt and MAPK are involved in CXCR2 mediated actin remodeling in neutrophil [276]. Moreover, PI3K/Akt signaling is also involved in CXCR2 mediated chemotaxis and the process of epithelial-mesenchymal transition (EMT) [277, 278]. More importantly, PI3K/Akt has been reported to contribute to EPCs involved angiogenesis [279]. It is reasonable to propose that CXCR2 may regulate the homing and angiogenesis in EPCs through PI3K/Akt and/or MAPK signaling pathways.
Interestingly, accumulating evidence has shown that RhoA is associated with the activities of both MAPK and PI3K/Akt signaling. It has been recently reported that RhoA is involved in the regulation of MAPK signaling activation in EPCs [216]. RhoA/ROCK pathway has been suggested be an upstream regulator of MAPK signaling molecules including ERK1/2, p38 MAPK, and JNK [211, 212, 280]. Inhibition of ROCK decreased the activation of ERK1/2 and consequently attenuated the cell migration and proliferation in glioblastoma cells [214]. Recently, Zoledronate, which is used to treat hypercalcemia in clinic, has been shown to attenuate the angiogenesis in EPCs through inhibiting the activation of RhoA and MAPK pathway [216]. Meanwhile, RhoA also regulates the activity of PI3K/Akt signaling in a variety of cell lines. For instance, activated by RhoA, PI3K/Akt/MAPK signaling leads to upregulated production of hepatic growth factor in macrophage [281]. In addition, C3 exoenzyme, an inhibitor of RhoA, inhibited the activation of Akt in endothelial cells [282], indicating that RhoA activates Akt in endothelial cells. These findings provide the possibility that RhoA/ROCK pathway may be also involved in regulating MAPK and PI3K/Akt signaling in EPCs.

RhoA activity is regulated by a variety of upstream signaling molecules. It has been reported that MIP-2, which is one of the murine CXCR2 ligands, activated RhoA in murine endothelial cells. Moreover, inhibition of RhoA signaling through ROCK inhibitor or siRNA targeting RhoA has significantly decreased endothelial cell migration during MIP-2/CXCR2 mediated angiogenesis [283]. Furthermore, CXCL8 has also been shown to be responsible for the actin cytoskeleton rearrangement for assembly of stress fibers and membrane ruffling through Rac1
and RhoA mediated signaling in HUVECs to promote the cell migration. In addition, dominant negative RhoA and constitutively active RhoA mutations were able to attenuated and facilitate CXCL8 mediated endothelial cell migration respectively, indicating the regulatory capacity of RhoA in this process [284]. It has been demonstrated that LIX, and MIP-2 and their cognate receptor CXCR2 are actively involved in ischemia induced angiogenesis, and blockade of CXCR2 diminished this process. Importantly, significant activation of RhoA has also been observed during this CXCR2 mediated angiogenesis [285]. All these findings have highlighted the importance of RhoA in CXCR2 signaling pathway.

Taken together, it is reasonable to hypothesize that CXCR2 regulates EPC migration and angiogenesis in a RhoA/ROCK, PI3K/Akt, and MAPK dependent manner.

3.2.2. Results

3.2.2.1. MIP-2 treatment results in activation of RhoA in EPCs

It is known that cell motility requires cells to experience alternating phases of protrusion and contraction. During migration, cells attach and adhere to the substrate such as ECM, and the adhesion provides the force for the cell body contraction and tail retraction. RhoA regulated stress fiber plays critical roles in these processes [286, 287], such as promoting cell adhesion and migration [288]. In my data, I have demonstrated that MIP-2 induced the migration of EPCs (Figure 3.1.8). Moreover, it has been reported that MIP-2 was able to activate RhoA in endothelial cells to contribute to neovascularization [283, 285]. However, whether RhoA is involved in MIP-2 mediated migration in EPCs has not been studied. I
hypothesize that MIP-2 may activate RhoA through CXCR2 in EPCs to modulate EPCs functions. RhoA activation assay was used to test this hypothesis. In this assay, EPCs were incubated with MIP-2 for 0, 1, 3, and 6 min and were lysed. Then, the same amount of cleared cell lysates (400μg) from each group was used for GST-Rhotekin pulldown assay, and 5% of total cell lysate in each group was used as input. The amount of active RhoA present in the cells was determined by western blotting. Figure 3.2.1 demonstrated that RhoA was activated in EPCs by MIP-2 (100ng/ml). The data showed that RhoA was activated 6 min after the MIP-2 was administrated. This data demonstrated that RhoA could be activated by MIP-2/CXCR2 signaling pathway in EPCs.

![Figure 3.2.1. MIP-2 activated RhoA in EPCs. Upper panel shows that RhoA was activated by MIP-2 (100ng/ml) after 6 min of stimulation in EPCs. Lower panel shows the input (5% of cell lysate used for pulldown assay), which was used as loading control.]

3.2.2.2. PDZ-mediated interaction is involved in the activation of RhoA

From the previous data, I have demonstrated that MIP-2/CXCR2 mediated EPCs migration, which could be attenuated by interrupting CXCR2 PDZ motif mediated interaction within the cells (Figure 3.1.8). Meanwhile, I have also shown
that RhoA, which provides forces for cell body contraction and tail retraction during cell migration [286, 287], was also activated by MIP-2 treatment (Figure 3.2.1). While whether the CXCR2 PDZ motif-mediated interaction is involved in MIP-2 induced RhoA activation has not been investigated. RhoA and its major effector, ROCK, regulate actin reorganization to promote the assembly of focal adhesions and formation of actin stress fibers [289, 290]. I hypothesize that CXCR2 PDZ motif mediated interaction may play a role during the MIP-2 induced activation of RhoA.

In order to test my hypothesis, I pre-incubated EPCs with ROCK inhibitor Y27632 for 1 hour or delivered CXCR2 C-terminus peptide or Chariot™ reagent alone into EPCs before MIP-2 stimulation. Then cells were stimulated with MIP-2 (100ng/ml) for 0, 6, 12 min respectively. After that, immunofluorescence staining was performed to detect the actin formed stress fibers in each group. Figure 3.2.2 showed that stress fibers could be observed in the control group (left column), in which EPCs were delivered with Chariot™ reagent alone, 6min after MIP-2 stimulation. No stress fiber was observed in the Y27632 pre-incubated group (right column). However, compared to the control group, the formation of stress fibers in EPCs was delayed (observed 12min after MIP-2 treatment) and attenuated when the CXCR2 C-terminus peptide was delivered into EPCs as shown in the figure (middle column), indicating that CXCR2 PDZ motif-mediated interaction in EPCs is involved in activation of RhoA.
Figure 3.2.2. Disrupting CXCR2 PDZ motif-mediated interaction attenuated the activation of RhoA.
RhoA activation in EPCs was reflected by stress fiber formation after MIP-2 stimulation. Left column: Stress fibers were formed 6min after MIP-2 (100ng/ml) treatment in EPCs delivered with Chariot™ reagent alone. Middle column: In EPCs delivered with CXCR2 C-terminus PDZ peptide, the stress fibers were formed 12min after MIP-2 (100ng/ml) treatment in EPCs, which was delayed compared to that in control group. In addition, the strength of the signal was weak compared to that in control group. Right column: The MIP-2 induced formation of stress fibers were barely observed in EPCs pre-incubated with ROCK inhibitor Y27632.
3.2.2.3. RhoA is involved in the MIP-2/CXCR2 signaling pathway

It has been reported that CXCR2 signaling activates the MAPK signaling and PI3K/Akt signaling to regulate a variety of EPC functions including, mobilization, migration, and proliferation [43, 280]. Meanwhile, RhoA/ROCK has also been reported to regulate PI3K/Akt and MAPK signaling in endothelial cells and EPCs [283, 215]. Moreover, a clinical used drug to treat hypercalcemia, Zoledronate, has been reported to attenuate the angiogenic potential of EPCs by inhibiting the activities of RhoA and MAPK signaling [215]. My data has shown that MIP-2/CXCR2 signaling activated RhoA to form stress fiber, which is important in EPC migration and angiogenesis, and this activation was attenuated by disrupting CXCR2 PDZ motif mediated interaction in EPCs. It is possible that MAPK signaling or PI3K/Akt signaling may be involved in the MIP-2 induced EPC migration and disrupting PDZ mediated interactions would affect the activation of these signaling. In order to test this hypothesis, EPCs were starved and pre-incubated with Y27632, or delivered with WT CXCR2 C-terminus peptide or Chariot™ reagent alone before MIP-2 stimulation. Then cells were treated with MIP-2 (100ng/ml) for 0, 1, 3, 6, and 12min respectively. After that the activation status of JNK, p38, ERK1/2, and Akt has been detected and compared among different groups as shown in Figure 3.3. The data shows that MIP-2 induced the phosphorylation of p38 (Figure 3.2.3.A), ERK1/2 (Figure 3.2.3.B), and Akt (Figure 3.2.3. C and D) in EPCs, but not JNK (data not shown), indicating that these molecules could be activated by CXCR2 signaling. In addition, introducing WT CXCR2 C-terminus peptide into the EPCs to compete the PDZ mediated interactions attenuated the phosphorylation of those
molecules, indicating that CXCR2 PDZ motif-mediated interactions plays important role in CXCR2 mediated MAPK and PI3K/Akt activation. Moreover, Y27632 pre-incubation has also inhibited the activation of those CXCR2 activated signaling molecules, which suggests that RhoA/ROCK is also involved in CXCR2 mediated MAPK and PI3K/Akt signaling transduction.

Figure 3.2.3. RhoA/ROCK and PDZ-mediated interaction is involved in CXCR2 induced activation of p38, ERK1/2, and Akt. EPCs were pre-incubated with Y27632, or delivered with WT CXCR2 C-terminus peptide or Chariot™ reagent alone before followed by starvation and MIP-2 stimulation for 0, 1, 3, 6, and 12 min. The data has shown that MIP-2 activated p38 (A, upper panel), ERK1/2 (B, upper panel), and Akt (C and D, upper panel) in EPCs after 3 min incubation (upper panel), while the activation of p38 was not observed in WT CXCR2 C-terminus peptide delivered EPCs (A-D, middle panel) and Y27632 pre-incubated EPCs (A-D, lower panel).
3.2.3. Discussion

The importance of EPC in postnatal vascularization indicates its therapeutic potential. EPCs are involved in a variety of blood formation related disorders. Their instructive function (secret pro-angiogenic factors) and constructive function (incorporation and differentiation) are dysfunctional in cardiovascular diseases and diabetes which require blood vessel formation [9, 10, 72, 77, 78]. On the other hand, in the malignancies, EPCs contribute to tumor growth and progression through promoting angiogenesis [59-61]. So it is necessary to understand the underlying mechanism that regulates EPCs functions in these disorders. This study has shown that interrupting PDZ mediated interaction in EPCs attenuated the cell adhesion (Figure 3.1.7), migration (Figure 3.1.8), and angiogenesis (Figure 3.1.11). These studies have suggested the potential therapeutic value of CXCR2 PDZ motif-mediated interaction within EPCs. However, the underlying mechanism of how this PDZ-mediated interaction affect the cellular function has not been fully studied. Results from this study showed that RhoA, which is responsible for the formation of stress fibers and focal adhesions that play important roles in cell migration, was activated through MIP-2/CXCR2 axis (Figure 3.2.1). I have further demonstrated that interrupting PDZ mediated interaction in EPCs attenuated the MIP-2 induced stress fiber formation (Figure 3.2.2). Moreover, disrupting PDZ mediated interactions in EPCs have also attenuated the MIP-2 induced phosphorylation of p38 MAPK, ERK1/2, and Akt. In addition, similar results were observed when RhoA/ROCK signaling was inhibited in the cells (Figure 3.2.3). These results suggested that RhoA/ROCK and MAPK as well as PI3K/Akt may
contribute to the MIP-2 mediated chemotaxis of EPCs and PDZ mediated interactions may play a role in regulating the signaling transduction. This study has provided a potential signaling transduction pathway in regulating EPCs function. However, the exact role of each signaling molecules remains mysterious, which directs future studies toward the investigation of their functions during this process. It is worthy to determine the signaling cascades or network that are involved in this regulation process which could be helpful in identifying specific and efficient therapeutic target.
CHAPTER 4 CONCLUSION AND FUTURE DIRECTION

4.1. Conclusion

Derived from bone marrow, EPCs contribute to the adult vascularization through secreting pro-angiogenic factors and incorporating into angiogenic sites to differentiate into mature endothelial cells [4, 18]. The functions of EPC reflect its importance in physiological and pathological conditions. Mobilized by a variety of factors, EPCs will home to the sites of ischemia or vascular injury to promote the blood vessel formation [49-51]. Thus, a comprehensive understanding of the biology of EPC and the underlying mechanisms that regulate its homing and angiogenic activities could be helpful to identify potentially disease specific molecular targets in the cells for developing improved therapies.

My research has demonstrated that the PDZ motif of CXCR2 plays important roles in EPC motility and angiogenic activity in vivo and in vitro. Disrupting the CXCR2 PDZ motif-mediated interactions in EPCs attenuated neovascularization, incorporation into endothelial network, and migration. Moreover, based on our previous finding of PDZ based CXCR2 involved macromolecular complex in neutrophil and pancreatic cancer cells [50, 96], the biochemical study of my research has suggested a CXCR2-NHERF1-PLCβ3 complex in EPCs. In addition, I have also studied the possible mechanism that CXCR2 PDZ-mediated interaction affects EPC functions. I have found that the chemotaxis stimulus of EPCs activated RhoA in the EPCs and disrupting the CXCR2 PDZ-mediated protein-protein interactions attenuated the activation of RhoA as well as downstream signaling molecules including p38 MAPK, ERK1/2,
and Akt. The findings suggest the involvement of these signaling molecules in the CXCR2 mediated EPC cellular activities and also indicates their activations can be regulated by CXCR2 PDZ mediated interaction. My study, for the first time, demonstrated the importance of CXCR2 PDZ motif-mediated interaction in EPC biology and provided the potential involved signaling pathways. These findings contribute to our understanding of EPC biology and may be valuable in identification of novel therapeutic targets. My dissertation study provides the possibility that targeting the CXCR2 PDZ motif-mediated macromolecular complex is an effective approach to regulate the EPC dependent angiogenesis in different diseases.

4.2. Future direction

4.2.1. The role of MAPK signaling and PI3K/Akt signaling pathways in cellular functions have been intensively investigated.

The MAPK signaling is composed of p38 MAPK pathway, MEK/ERK pathway, and JNK pathway [220-222]. It is well documented that p38 MAPK, ERK1/2, and Akt are involved in cell migration [216, 278] and angiogenesis [279, 291-293]. Although I have shown in my results that disrupting CXCR2 PDZ motif-mediated interaction attenuated the activation of p38 MAPK, ERK1/2, and Akt under the stimulation of MIP-2 in EPCs, it is still not known their exact role of them during MIP-2 induced EPC cellular activities. In order to have a more comprehensive understanding of the mechanisms that PDZ mediated interaction regulates EPC homing and angiogenesis, inhibitory studies will be performed.

I speculate that the contribution of three signaling pathways in EPC functions are not equal. To test my hypothesis, I will block p38 MAPK (using
SB203580), ERK1/2 (U0126), or Akt (MK2206) signaling pathways in EPCs separately and perform EPC migration assay, adhesion assay, and in vitro tube formation assay under the stimulation of MIP-2. EPCs without inhibitor treatment will be used as negative control. I will observe the effects of different inhibitors on EPCs adhesion, migration, and in vitro angiogenesis to study how the three signaling pathways are involved in each EPC function.

In parallel, I will also knockdown the expression of p38 MAPK, ERK1/2, or Akt by transiently transfect commercial available siRNAs targeting each signaling molecule respectively in EPCs. EPCs incubated with transfection reagent alone will be used as negative control. After the silencing each of the signaling molecules, I will stimulate the EPCs with MIP-2 and perform adhesion, migration, and in vitro tube formation assays. The effect of each pathway inhibition on each EPC function will be recorded and compared to study how the three signaling pathways are involved in each EPC function.

4.2.2. Further study the PDZ mediated interaction on RhoA activation.

As a member of small GTPase, the activity of RhoA is regulated by nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). My dissertation results showed that RhoA activation was affected by CXCR2 PDZ motif-mediated interactions (Figure 3.2.2). However, how did the PDZ-based interaction influence RhoA activation is still unclear. Our previous findings in neutrophil and pancreatic cancer cells [50, 96] have suggested the presence of PDZ-mediated CXCR2 involved complex in the cells to promote efficient and specific signaling transduction. Thus, I hypothesize that CXCR2 may form a protein
complex through its PDZ motif-mediated interaction with GEF to facilitate RhoA activation and disrupting this PDZ-mediated interaction would attenuate the activation of RhoA.

I will perform immunoprecipitation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect CXCR2 bound proteins mediated by PDZ-based interaction. To do this, I will clone full length CXCR2 or CXCR2 without PDZ motif with 3HA tagged at N-terminus into pcDNA3.1 vector (pcDNA3.1-3HA-CXCR2-FL; pcDNA3.1-3HA-CXCR2-dTTL). After that, I will transfect EPCs with pcDNA3.1-3HA-CXCR2-FL, pcDNA3.1-3HA-CXCR2-dTTL, or pcDNA3.1 vector alone respectively and stimulate cells with MIP-2. Then cells from each group will be lysed and 3HA tagged CXCR2-FL or CXCR2-dTTL and their bound proteins will be immunoprecipitated by anti HA agarose. The precipitated proteins will then be eluted and separated in a SDS-PAGE gel and digested to produce peptide for the tandem mass spectrometry to identify the bound proteins. After that, identified proteins from EPC expressing CXCR2-FL, CXCR2-dTTL, and pcDNA3.1 empty vector will be compared to obtain unique proteins including GEFs only found in CXCR2-FL group. It is possible that the list of unique proteins may contain the potential adaptor protein or GEFs containing PDZ domain.
REFERENCES


67. !!! INVALID CITATION !!! {}.


ABSTRACT

A CRITICAL ROLE OF CXCR2 PDZ MOTIF-MEDIATED INTERACTIONS IN ENDOTHELIAL PROGENITOR CELL HOMING AND ANGIOGENESIS

by

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Bone marrow-derived endothelial progenitor cells (EPCs) participate in postnatal vascularization in response to growth factors, cytokines, and chemokines. Chemokine receptor CXCR2 and its cognate ligands are reported to mediate EPC recruitment and angiogenesis. CXCR2 possesses a consensus PSD-95/DlgA/ZO-1 (PDZ) motif at its carboxyl terminus. The PDZ motif has been reported to regulate cellular signaling and functions. Here we investigated the potential role of the PDZ motif in CXCR2-mediated EPC motility and angiogenesis. We have found that introducing exogenous CXCR2 C-terminus significantly attenuated in vitro EPC migration and angiogenic activities in response to CXCR2 ligands, as well as in vivo EPC angiogenesis. Meanwhile, the activation of RhoA, p38, ERK1/2, and Akt has also been inhibited due to the exogenous CXCR2 C-terminus. On the other hand, delivering CXCR2 C-terminus without PDZ motif into EPCs did not significantly affect the functions of EPCs. Moreover, biochemistry study suggested that NHERF1, a PDZ domain containing scaffolding protein, preferred to interact with CXCR2 and its downstream effector, PLC-β3 in EPCs.
Taken together, the data has demonstrated the role of CXCR2 PDZ motif-mediated macromolecular complex in EPC homing and angiogenesis and also indicated potentially involved signaling pathways. The findings suggested that targeting CXCR2 PDZ motif-mediated interactions in EPCs may be a novel therapeutic approach in EPC dependent angiogenesis related diseases.
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4). Yuning Hou, Xiaoqing Guan, Shukkur M. Farooq, Xiaonan Sun, Peijun Wang, Zhe Yang, Chunying Li. Stem Cell Therapeutics for Cardiovascular Diseases. In: Saura C. Sahu (Eds.), Stem Cells in Toxicology and Medicine (in press). Publisher: Johns Wiley and Sons Ltd.


