

**Vascular Stem/Progenitor Cell
Migration Induced by Smooth Muscle
Cell-derived CCL2 and CXCL1**

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Abstract

Recent studies have shown that Sca-1⁺ (stem cell antigen-1) stem/progenitor cells within blood vessel walls may contribute to neointima formation, but the mechanism behind their recruitment has not been explored. In this work Sca-1⁺ progenitor cells were cultivated from mouse vein graft tissue and found to exhibit increased migration when co-cultured with smooth muscle cells (SMCs) or when treated with SMC-derived conditioned medium. This migration was associated with elevated levels of chemokines, CCL2 (chemokine (C-C motif) ligand 2) and CXCL1 (chemokine (C-X-C motif) ligand 1), and their corresponding receptors on Sca-1⁺ progenitors, CCR2 (chemokine (C-C motif) receptor 2) and CXCR2 (chemokine (C-X-C motif) receptor 2), which were also up-regulated following SMC conditioned medium treatment. Knockdown of either receptor in Sca-1⁺ progenitors significantly inhibited cell migration. The GTPases Cdc42 and Rac1 were activated by both CCL2 and CXCL1 stimulation and p38 phosphorylation was increased. However, only Rac1 inhibition significantly reduced migration and p38 phosphorylation. After Sca-1⁺ progenitors labelled with GFP were applied to the adventitial side of wire-injured mouse femoral arteries, a large proportion of GFP-Sca-1⁺-cells were observed in neointimal lesions, and a marked increase in neointimal lesion formation was seen 1 week post-operation. Interestingly, Sca-1⁺ progenitor migration from the adventitia to the neointima was abrogated and neointima formation diminished in a wire injury model using CCL2^{-/-} mice. These findings suggest vascular stem/progenitor cell migration from the adventitia to the neointima can be induced by SMC release of chemokines which act via CCR2/Rac1/p38 and CXCR2/Rac1/p38 signalling pathways.

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Declaration

I, Baoqi Yu, confirm that the research included within this thesis is my own work. I have been involved in the design, planning and conduct of all the experiments and the thesis writing. This includes cell functional experiments, molecular biology methods, mouse femoral artery injury and analysis of the results.

Expert assistance was provided in some aspects of the project by the following colleagues from the Cardiovascular Division of King's College London.

Dr Yanhua Hu isolated vascular progenitor cells, Dr Claire Potter performed Confocal Microscopy, Dr Mei Mei Wong performed FACs, Mr Zhongyi Zhang prepared tissue paraffin section and Dr Dereck Warren analysed data of single cell tracking.

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Abbreviations

ACE	Angiotensin-Converting Enzyme
α -SMA	alpha-Smooth Muscle Actin
bFGF	basic Fibroblast Growth Factor
CABG	Coronary Artery Bypass Grafting
CCL2	chemokine (C-C motif) Ligand 2
CCR2	chemokine (C-C motif) Receptor 2
CD	Clusters of Differentiation
Cdc42	Cell division control protein 42 homolog
cDNA	complementary DNA
c-kit	proto-oncogene c-kit
CM	conditioned medium
ctrl	control
CXCL1	chemokine (C-X-C motif) Ligand 1
CXCR2	chemokine (C-X-C motif) Receptor 2
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	double distilled water
DES	Drug-Eluting Stents
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
dNTPs	deoxyribonucleotide triphosphates
ECM	Extracellular Matrix
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic Acid

ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
EPCs	Endothelial Progenitor Cells
ERK1/2	Extracellular signal-Regulated Kinase 1/2
ESCs	Embryonic Stem Cells
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum
Flk-1	Fetal Liver Kinase-1
GEF	Guanine nucleotide Exchange Factor
GFP	green fluorescent protein
GPCR	G Protein-Coupled Receptors
GTP	Guanosine Triphosphate
HE stain	Haematoxylin and Eosin stain
hES-MCs	human ESC-derived Mesenchymal Cells
HLA-DR	Human Leukocyte Antigen - antigen D Related
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukins
iPS cells	induced Pluripotent Stem cells
JNK	c-Jun N-terminal kinase
LDL	Low Density Lipoprotein
LIF	Leukemia Inhibitory Factor
Mac-1	Macrophage-1 antigen
MEF	Mouse Embryonic Fibroblasts
MMPs	Matrix Metalloproteinases

mRNA	messenger RNA
MSCs	Mesenchymal Stem Cells
mTOR	mammalian Target Of Rapamycin
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NG2	Neuron-Glial antigen 2
NO	Nitric Oxide
OCT3	Organic Cation Transporter 3
PAI-1	Plasminogen Activator Inhibitor-1
PCI	Percutaneous Coronary Intervention
PDGF	Platelet-Derived Growth Factor
PDGF-BB	Platelet-Derived Growth Factor subunit B
PDGFR- β	beta-type Platelet-Derived Growth Factor Receptor
PLC β	Phospholipase C beta
PSGL-1	P-selectin Glycoprotein Ligand-1
PTCA	Percutaneous Transluminal Coronary Angioplasty
qPCR	quantitative real time Polymerase Chain Reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RT-PCR	Reverse transcription polymerase chain reaction
SAPK	Stress-Activated Protein Kinases
Sca-1	Stem cells antigen-1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Shh	Sonic hedgehog
shRNA	small hairpin RNA

siRNA	small interfering RNA
SMCs	Smooth Muscle Cells
SMMHC	Smooth Muscle Myosin Heavy Chain
SOX2	SRY (sex determining region Y)-box 2
SSEA-3	Stage-Specific Embryonic Antigen 3
TGF- β	Transforming Growth Factor beta
Th1 cells	Type 1 helper T cells
TNF- α	Tumour Necrosis Factor alpha
tPA	tissue Plasminogen Activator
μg	microgram
μl	microliter
μm	micrometre
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VPC	Vascular Progenitor Cell

Chapter 1

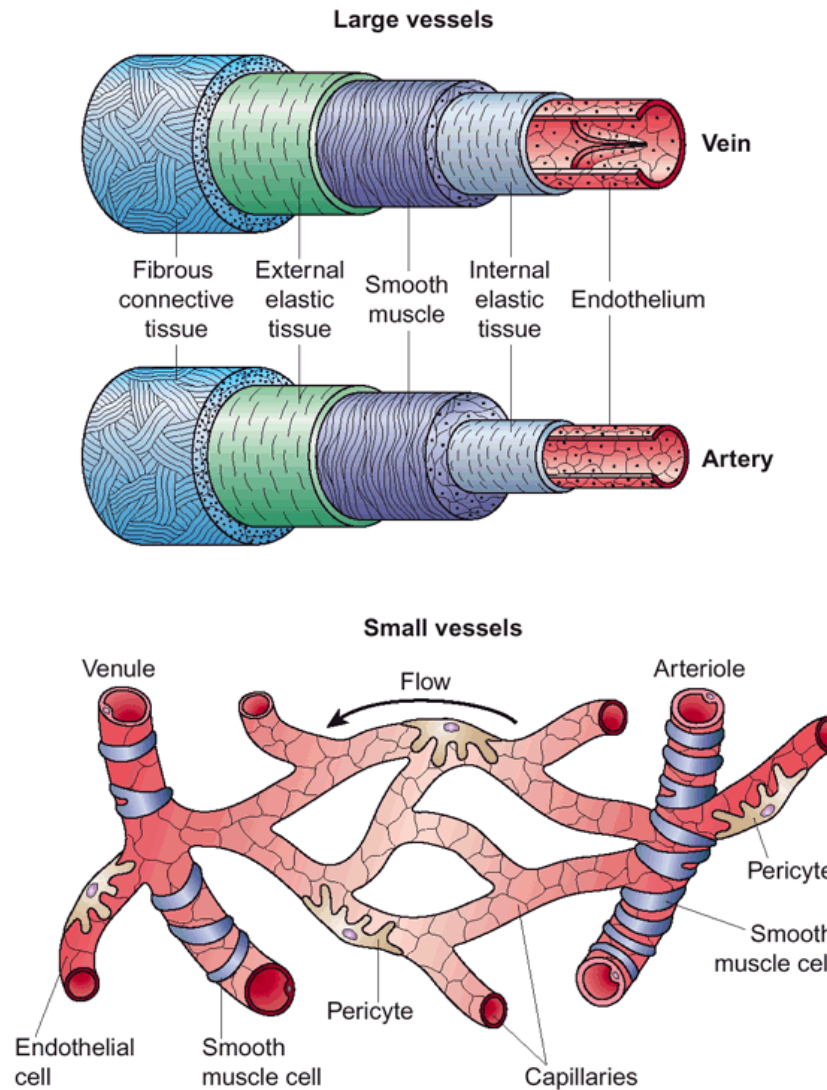
Introduction

1.1 The vascular system and vessel structure

1.1.1 The classification of vessels

The blood vessels, which transport blood throughout the whole body, are very important parts of the cardiovascular system. There are 5 main types of blood vessels: arteries, arterioles, capillaries, venules and veins. Each time the heart beats, blood is pumped into the aorta (large artery) which then flows through its branches into progressively smaller arterioles. These arterioles then carry blood into every part of the body. Consequently, they connect with capillaries that exchange oxygen, carbon dioxide, water and chemicals between blood and tissues. After this stage of its journey, blood returns to smaller venules from capillaries and gradually merges into bigger veins before finishing its return journey to the heart.

1.1.2 The structure of vessels



Ondine Cleaver & Douglas A Melton, *Nature Medicine*, 2003

Figure 1.1 The structure of blood vessel wall. The schema shows the structures of an artery, vein and capillary. The large vessels (artery and vein) contain multiple layers of cells and extracellular tissues. In contrast, the small vessels (arteriole, venule and capillary) are only composed of a layer of endothelial cells with a loose covering of smooth muscle cells and pericytes (Cleaver & Melton, 2003).

As shown in Figure 1.1, diameters of different vessels (from 25mm in the aorta to 8 μ m in capillaries) and their tolerances of circulating blood pressure are different, except in capillaries, in which the vessel wall is formed of a single layer of cells. The arteries and the veins are both

composed of 3 layers of tissues in the vessel walls. From the inside (lumen side) of the vessel wall outwards, they are the tunica intima, tunica media and tunica adventitia.

The tunica intima is the thinnest layer of the vessel wall, which consists of a single layer of endothelial cells (EC) linked by a polysaccharide intercellular matrix and surrounded by the internal elastic lamina. Though this layer can be seen clearly under the light microscope when viewing large arteries it appears to be thin or incomplete in arterioles, veins and venules. The tunica intima forms the only layer of cells in the vessel wall of capillaries indicating the necessity of the endothelium throughout the entire vascular system. As endothelium is an important barrier to separate circulating blood and other parts of the vessel wall, endothelial cells which express markers PECAM-1 (CD31) and VE-Cadherin (CD144) play a crucial role in maintaining vascular homeostasis and initiating various responses to vascular injury.

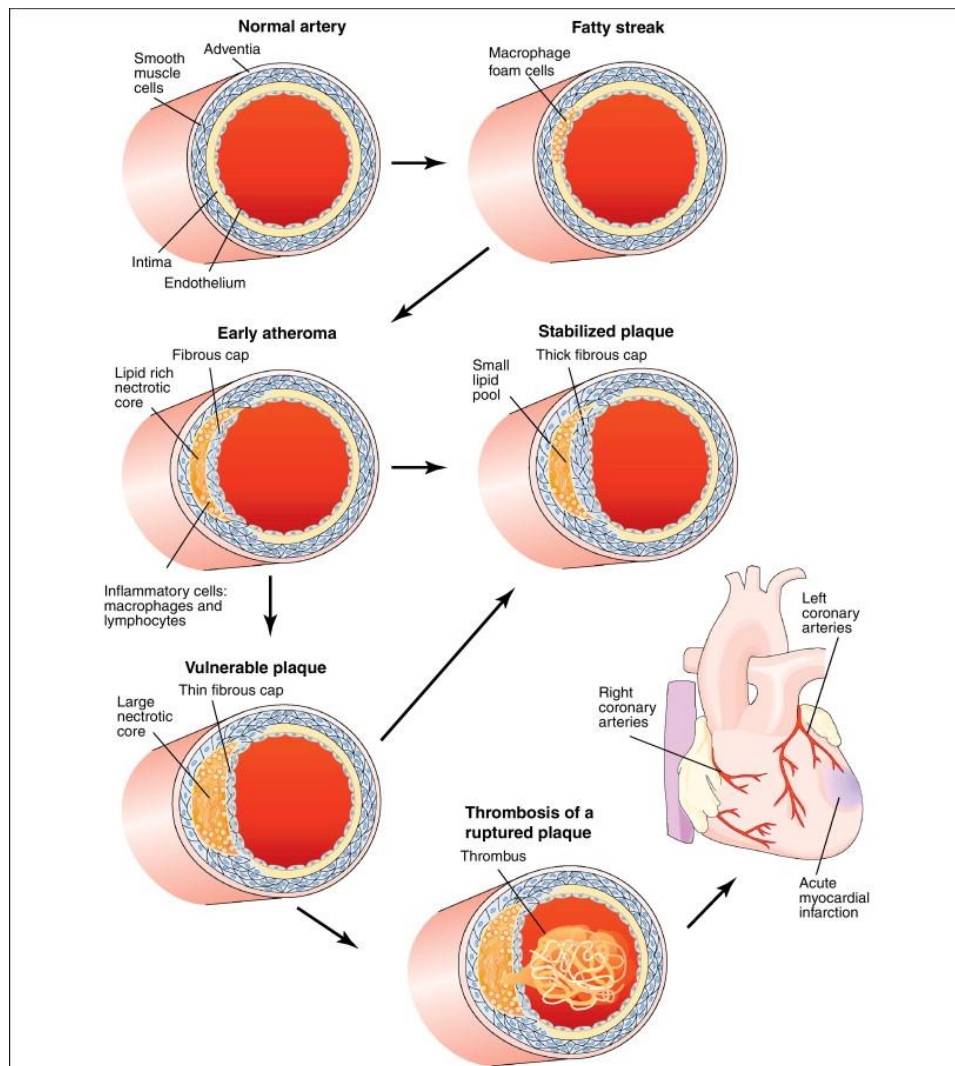
The outer layer in contact with the tunica intima is the tunica media. This layer is comprised of a large number of smooth muscle cells (SMC), and is much thicker in arteries than in veins, due to the constriction and dilation functions of arteries, which is necessary to control the blood volume supplied to different parts of the body and to maintain blood pressure throughout the circulation. The external elastic lamina forms the boundary between the tunica media and tunica adventitia and is also much thicker in arteries than in veins for similar reasons. SMCs are usually identified by alpha-smooth muscle actin (α -SMA), calponin, myocardin and smooth muscle myosin heavy chain (SMMHC) and are the major cell type in the tunica media. In physiological conditions, they control vascular tone to regulate blood volume and pressure in vessels, while in pathological conditions, they participate in repair of the injured vessel wall.

The tunica adventitia is the outermost layer of the vessel wall and is mainly composed of collagen and elastic fibres which make it the strongest layer of the vessel wall. It can enhance the stability of the vessel by anchoring the vessel to nearby tissues and protecting from overexpansion of the vessel wall. In large vessels, the vasa vasorum is present in the tunica adventitia to supply nutrients to the outer layer of the vessels, while the inner layer of the vessel wall can be nourished by blood from the lumen.

1.2 The pathophysiology of atherosclerosis

Atherosclerosis is one of the leading causes of mortality and morbidity in the world. It is a multifactorial vascular disease influenced by genetic, gendered and environmental factors. It is the most common kind of arteriosclerosis and usually gives rise to coronary artery disease, carotid artery disease, peripheral arterial diseases and cerebrovascular diseases (e.g. acute coronary syndrome (ACS), myocardial infarction or stroke). The main characteristics of atherosclerosis are lipid deposition and vessel stiffness, which result in thickening of the vessel wall and narrowing of the lumen. As shown in Figure 1.2 the underlying pathophysiology of atherosclerosis is initiated by endothelial dysfunction, caused by physical or chemical offence such as hypertension, shear stress of disturbed laminar flow in the branch of the vessel, reactive oxygen species in the circulation (e.g. smoking), hyperlipidaemia and hyperglycaemia. These factors activate endothelial cells to provoke a cascade of inflammatory responses.

In the initial stages, molecules and lipoproteins (e.g. low density lipoprotein (LDL)) from plasma leak into subendothelial areas through the impaired endothelium. At the injured intima sites, retained LDL is prone to modification (e.g. oxidation) into a cytotoxic form (e.g. ox-LDL) by factors such as reactive oxygen species, myeloperoxidase, lipoxygenases, and/or nitric oxide synthase which induces atherosclerosis (Glass & Witztum, 2001).



Aldons J .Lusis et al., Annu. Rev. Genomics Hum. Genet., 2004

Figure 1.2 The pathological development of atherosclerosis. Transverse views of alterations of the vessel wall and lumen area in atherosclerotic coronary arteries. Initially, the fatty streak is formed by the accumulation of macrophage-derived foam cells in the sub-endothelium area. In the early atheroma, a lipid-rich necrotic core consisting of necrotic foam cells and SMCs debris is surrounded by a fibrous cap and infiltrated with inflammatory cells. The thickness of the fibrous cap, which is composed of SMCs and extracellular matrix, determines the stabilization of lesions. In the late stage, thrombosis formation is related to vulnerable plaque rupture (Lusis, Mar, & Pajukanta, 2004).

Subsequently, chemokines that are derived either from platelets in the blood or released from ox-LDL activated ECs attract inflammatory cells such as monocytes and T lymphocytes to migrate into sub-endothelial sites. During this process, ox-LDL also triggers defective endothelial cells to express adhesion molecules (e.g. VCAM-1) on their surface, which leads to immune cells adhering to the endothelial cells before extravasation into the sub-endothelium area.

The fatty streak, the earliest lesion visible without focal magnification, is the first sign of atherosclerosis. It consists of a large number of foam cells (monocyte-derived-macrophages that have ingested ox-LDL) and some T lymphocytes. As the disease progresses, foam cells become more lipid-laden and undergo death (apoptosis and necrosis), which subsequently results in an accumulation of cell debris and necrotic core formation from lipids.

At the same time, inflammatory cells in the intima secrete chemokines and cytokines that stimulate proliferating SMCs to migrate from the media to the intima. At this stage, atherosclerosis is established and a fibrous cap is formed from proliferative smooth muscle cells and collagen. This cap makes the lesion relatively stable due to its restriction of the necrotic core inside.

Over time, a continuous accumulation of lipid and production of inflammatory molecules by infiltrated inflammatory cells causes the lesion to become unstable. Macrophages will then replace SMC in the fibrous cap to create an inflamed cap, which is more susceptible to rupture. Once the plaque ruptures, platelets and the clotting cascade response result in thrombotic occlusion of the vessels.

1.3 Treatment of atherosclerosis and complications

1.3.1 Drug treatments

Based on the proposed cause of atherosclerosis, different medical treatments are prescribed to patients. In cases of hypertension angiotensin-converting enzyme (ACE) inhibitors, β -blockers, calcium channel blockers and thiazide diuretics are used to lower the blood pressure by reducing the amount of water in the blood and/or dilating the arteries. For hyperlipidaemia, statins are commonly used medicines, which lower cholesterol levels in the circulation by inhibiting the effects of HMG-CoA reductase, to control the production of cholesterol in the liver. To prevent thrombosis, antiplatelet agents, such as aspirin, are used to stop platelets binding to each other and forming a clot on the damaged vessel wall. Alternatively, anticoagulants, such as warfarin, are applied to decrease blood coagulation through inhibition of vitamin K epoxide reductase. The level of blood glucose also needs to be controlled in cases of diabetes. As atherosclerosis is an inflammatory disease, anti-inflammatory drugs are also indispensable in atherosclerosis treatment, for example, statins which can block GTPase isoprenylation in ECs, macrophages, dendritic cells and T cells, and which also have an effect on both the innate and adaptive immunological reactions (Bu, Griffin, & Lichtman, 2011).

1.3.2 Coronary artery bypass grafting

In some cases, severe atherosclerosis results in a narrowed or even blocked vessel lumen, which will reduce or stop blood flow supplying organs such as the heart or brain, thereby leading to myocardial infarction or stroke. To prevent or rectify these risks, surgeries are required to widen the lesion area or bypass the site of the blockage. Coronary artery bypass grafting (CABG) is carried out by attaching a part of vessel derived from a great saphenous vein or internal mammary artery to the coronary artery to bypass the narrowed or blocked area. This new graft creates a channel to restore normal blood perfusion to the part of the myocardium previously supplied by the obstructed coronary artery.

1.3.3 Percutaneous transluminal coronary angioplasty

In the cardiovascular system, percutaneous coronary intervention (PCI) (Figure 1.3), also known as coronary angioplasty, can improve blood flow by widening the narrowed or blocked vessel using a balloon and holding the vessel open using a stent. Stents are made of a metal mesh and are usually coated with different drugs which can be released into lesion sites continuously to prevent the artery from re-narrowing.



Panoulas VF and Colombo A, *Nat Rev Cardiol.*, 2014

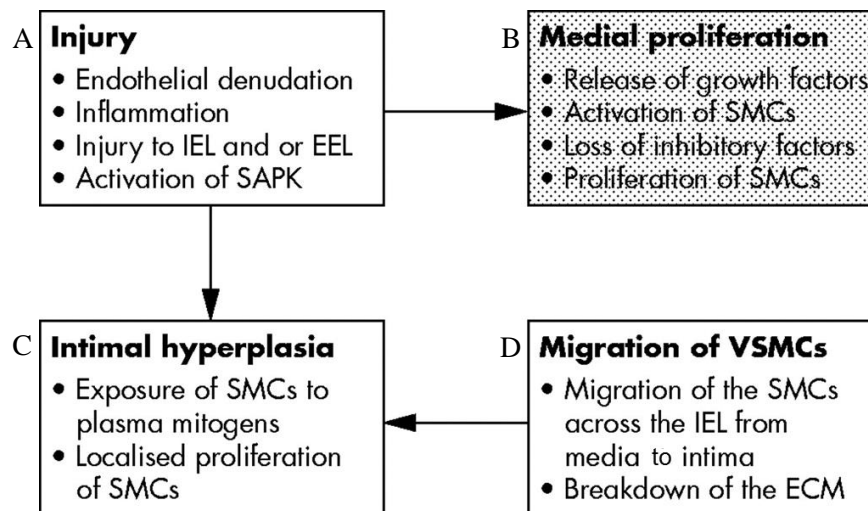
Figure 1.3 Schematic representation of a percutaneous coronary intervention. The catheter, containing a deflated balloon within a closed stent, is inserted into the narrow coronary artery. Once the stent reaches the plaque lesion site, the balloon is inflated and the stent is expanded to widen the narrowed artery. The catheter with the balloon is withdrawn, leaving the stent to support the lumen opening in the artery. The cross-section shows the compressed plaque and the stent-widened artery (Panoulas & Colombo, 2014).

1.3.4 Complications

1.3.4.1 Post-angioplasty restenosis

Due to the advantages of a less invasive procedure and a more rapid recovery rate than surgery, PCI is the first-line treatment for obstructive coronary artery disease, however, it may not always be successful long-term due to complex vascular remodelling processes such as post-angioplasty restenosis (Holmes et al., 1984; Nobuyoshi et al., 1988; Nobuyoshi et al., 1991). Restenosis leads to vessel re-occlusion and patients needing revascularization procedures, which

increases the risk of morbidity and mortality of the disease. The flow chart in Figure 1.4 indicates a cascade of mechanisms leading to neointimal hyperplasia.



Mitra et al., J Clin Pathol., 2006

Figure 1.4 A series of cellular and molecular responses in the vascular wall during post-angioplasty restenosis. A. **Injury**, damage to the endothelium and the elastic lamina activates the inflammatory response and the SAPK (stress-activated protein kinases) signalling pathway, which results in (B) **Medial proliferation**, activation of SMCs and release of growth factors, (C) **Intimal hyperplasia**, due to localised SMCs proliferation and interaction with plasma mitogens. Intimal hyperplasia is also caused by (D) **Migration of VSMCs**, medial SMCs migration to the intimal layer with disruption of the ECM.

At an early stage (days to weeks) after angioplasty, endothelial injury initiates thrombus formation (Virmani, Kolodgie, Farb, & Lafont, 2003), and fibrin and platelet accumulation activate an inflammatory response. Specifically, leukocytes infiltrate into the vessel wall across the stent site, particularly monocyte-derived macrophages (Gradus-Pizlo et al., 2003). In human studies, the staining for a specific macrophage marker (CD68) on sections of patients' coronary arteries showed that macrophage infiltration into the neointima is correlated with neointima expansion (Burke, Kolodgie, Farb, Weber, & Virmani, 2002). However, others argue that rather than macrophages it is neutrophils that infiltrate into the media and this was seen early after injury in rabbit vessels. Medial SMC proliferation was reduced because of the inhibition of this

process (Welt, Edelman, Simon, & Rogers, 2000). Deposited platelets, monocytes and local SMCs can recruit more leukocytes to migrate into the injured site through the release of cytokines, growth factors and chemokines. Growth factors such as transforming growth factor beta (TGF- β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) can induce EC and SMC proliferation.

During the late phase (weeks to months), the dominant change in the vessel wall is medial SMCs dedifferentiation and subsequent proliferation and migration towards the intima. SMCs also release intracellular hyaluronan, proteoglycans and collagens. These factors are major components of the neointima (Rollins, 1997) and interact to stabilize the Extracellular matrix (ECM). The ECM can then facilitate SMC and inflammatory cell adhesion and migration, and thus plays an important role in vessel repair and the remodelling process. The ECM is initially comprised from fibrin, fibrinogen, and fibronectin. Following the subsequent digestion of hyaluronan, inflammatory cells produce metalloproteinases (MMPs) that break down collagen type 1 to promote permanent wound healing (Wight & Potter-Perigo, 2011).

1.3.4.2 Late stent thrombosis after drug-eluting stent application

Although the bare metal stent has successfully treated many coronary atherosclerosis patients and has effectively prevented acute myocardium infarction, the high incidence of post-angioplasty restenosis is the main challenge in PCI (Serruys et al., 1994). In the decade since the development and application of drug-eluting stents (DES) in PCI, there has been a revolutionary improvement in treatment. These DES are coated with drugs that target SMC proliferation, and this local delivery of treatments has been widely applied in the clinic, to reduce the occurrence of post-angioplasty restenosis and vessel re-narrowing.

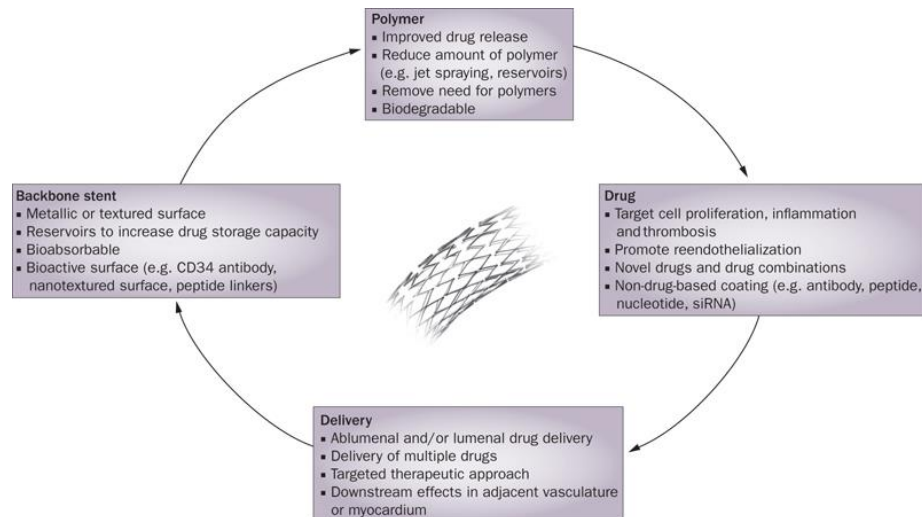
Sirolimus, as an inhibitor of mammalian target of rapamycin (mTOR), can suppress cell proliferation by inhibiting multiple regulators of the cell cycle (Marx, Jayaraman, Go, & Marks, 1995). Sirolimus has anti-proliferative and anti-migratory effects on vascular SMCs through inhibition of the G1 phase of the cell cycle (Wessely, Schomig, & Kastrati, 2006).

Compared with bare metal stents, DESs have remarkable advantages in preventing restenosis, but they still have some limitations, for example there is no significant difference in late stent thrombosis incidence between bare metal and drug eluting stents (Joner et al., 2006).

Late stent thrombosis usually occurs at least 1 month after stent implantation and is characterised by occlusion of the coronary artery, caused by thrombosis formation in the stent, which results in a high rate of myocardial infarction occurrence (Daemen et al., 2007). This problem is attributed to the delay in arterial healing after PCI due to fibrin deposition and decreased re-endothelialisation (Bavry & Bhatt, 2008; D. W. Park et al., 2006). The possible reasons for late thrombosis after stent implantation are variable; one of the reasons is the premature termination of anti-platelet therapy (McFadden et al., 2004). Furthermore, others have found that the parts of the stent-implanted vessel wall where incompletely healed had much higher risk of thrombosis. The morphology of the atherosclerotic lesion may alter the proportion of coverage of cells on the stent which depends on the local drug concentration. Researchers demonstrated that at vessel sections with reduced evidence of thrombosis there was less neointimal formation (Farb, Burke, Kolodgie, & Virmani, 2003; Finn et al., 2007).

1.3.5 Optimization and further studies

As described above, DESs have proved a beneficial treatment for coronary atherosclerosis but much could still be done to improve their success long-term. The main factors affecting DES performance are the stent backbone, the drug eluted, the polymer and the delivery system (Figure 1.5). Improvements may be focused on development of bio-absorbable stents, coating stents with drugs, antibodies, peptides, nucleotides or a combination which can induce re-endothelialisation and promote anti-thrombotic processes, and improve the way of drug delivery, for example through control of drug release direction to deliver the drugs to their specific targets (e.g. SMC or EC), which would suppress SMC proliferation as well as improve endothelium healing. For some coronary artery disease patients with other complications (e.g. diabetes), the lesions are more difficult to treat due to multiple risk factors, so a greater range of drugs could be beneficial.



Wessely et al., *Nature Reviews Cardiology*, 2010

Figure 1.5 The important components of drug-eluting stent performance. The schema shows the 4 main factors influencing drug-eluting stent performance. These include stent backbone, polymer, drug and way of delivery. Each box lists the specific function and modifications which are being developed for each element.

Though PCI is widely applied in the clinic, complications still cause a bad prognosis for the disease. In recent years a proposed alternative to surgical intervention put forward is the use of regenerative medicine to encourage vessel repair or to develop artificial vessels by application of stem/progenitor cells.

1.3.6 Endovascular intervention for peripheral artery disease

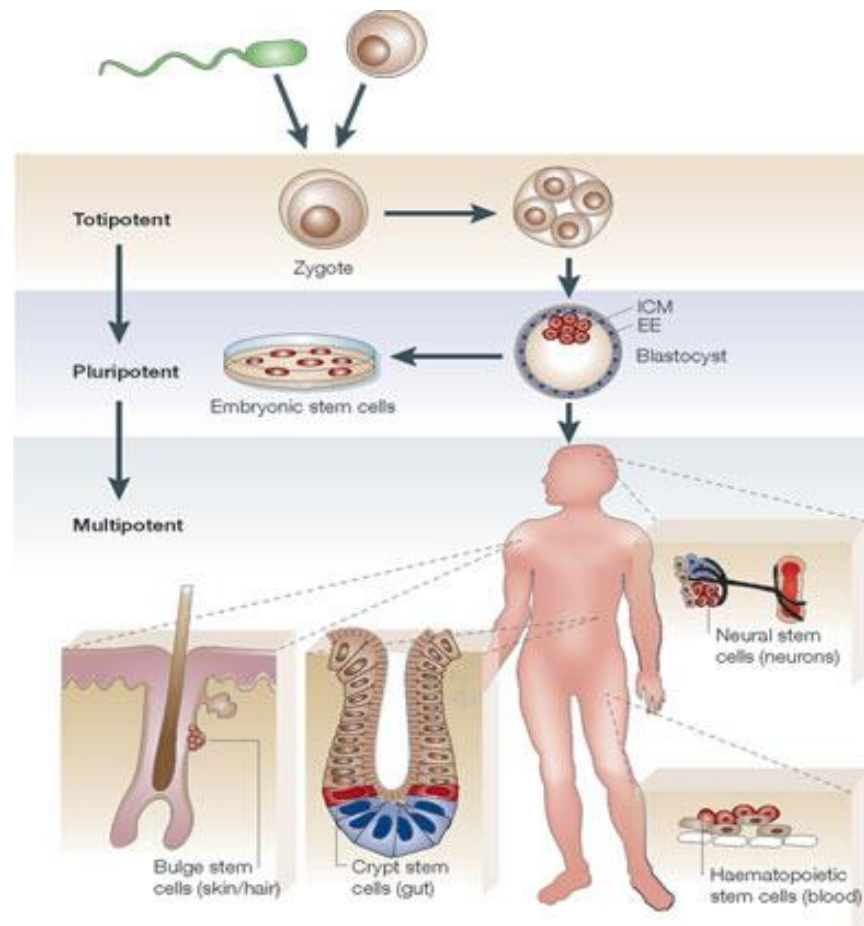
Besides coronary arteries, atherosclerosis is also formed in carotid arteries (Ku, Giddens, Zarins, & Glagov, 1985), cerebral arteries (Sacco, Kargman, Gu, & Zamanillo, 1995), renal arteries (Missouris, Buckenham, Cappuccio, & MacGregor, 1994) and peripheral arteries (Heinrich, Schulte, Schonfeld, Kohler, & Assmann, 1995) among others. In most cases, peripheral artery disease caused by lower extremity atherosclerosis leads to intermittent claudication and critical limb ischemia. Bajwa et al. reviewed several current imaging modalities which are being applied on patients to assess tissue perfusion of the lower limbs (Bajwa et al., 2014). The superficial femoral artery, due to its anatomical features of flexion and torsion, can easily

develop localized inflammatory injury and is a common site for peripheral artery disease occurrence and development (Wood et al., 2006). As the progressive lesion develops in the superficial femoral artery, chronic total occlusion will completely obstruct the blood supply to the lower limb which eventually results in severe ischemic ulcer formation or gangrene (Jude, Oyibo, Chalmers, & Boulton, 2001).

Similarly to percutaneous coronary intervention, peripheral vascular intervention can be used to treat patients with peripheral artery disease. In aortoiliac segments, peripheral vascular intervention achieves successful long-term patency, while in femoropopliteal and infrapopliteal segments, a high incidence of restenosis is still the major challenge of this technique. Peripheral restenosis was found to occur in 5%-70% of patients, depending on lesion localization, complexity and procedural factors (Deloose et al., 2014; Schmidt et al., 2010). To improve the long-term outcome of patients after self-expanding stent implantation, drug-eluting stents are widely applied as clinical treatments. As the first-generation DES, compared with bare metal stents in clinical trials, sirolimus-coated stents did not show significant improvement in target lesion revascularization and restenosis rates after follow-up at 24 months, which may be due to an inflammatory response to the polymer matrix (Duda et al., 2006). Furthermore, the results of other trials also reported a high incidence rate of stent fracture in approximately 30% of patients (Duda et al., 2005; Duda et al., 2002). Recently, the second-generation of DES was developed by coating paclitaxel directly on the stents to abolish the inflammatory effects of the polymer matrix. As an alternative, a drug-coated balloon is also applied in the clinic, since it can provide anti-proliferative drugs without having to deploy a stent as a permanent inflammatory stimulus for neointima formation (Byrne et al., 2013). Ongoing trials should focus on the comparison of long-term outcomes of patients with different treatment technologies. The further basic investigation is required to thoroughly understand the pathophysiology of post-angioplasty restenosis in order to explore possible therapeutic targets.

1.4 Stem cells

In the past 30 years, research into stem cells has been identified as a promising area for regenerative medicine. Stem cells have the notable potential to self-renew and differentiate into many cell types. These abilities are crucial not only during embryo development but also in self-repair throughout the lifespan of the organism.



Craig E. Eckfeldt et al., *Nature Reviews Molecular Cell Biology*, 2005

Figure 1.6 Development of human embryonic and adult stem cells. Human embryonic stem cells are derived from the inner cells of a blastocyst which is the divided product of a zygote, a fusion of the egg and sperm. These embryonic stem cells are either self-renewing or pluripotent. Human adult stem cells reside in different adult tissues and they are also capable of self-renewal and multipotent differentiation. Whether differentiated cells are lineage restricted to the original organs of their origin or have potential to differentiate into other cell types remains uncertain (Eckfeldt, Mendenhall, & Verfaillie, 2005).

There are two broad categories of stem cells, embryonic stem cells (ESC) and adult stem cells (somatic stem cells) (Figure 1.6). ESCs are derived from the inner cell mass of blastocysts and are pluripotent and able to generate every kind of cell in the body. Adult stem cells reside in specific tissues and are multipotent, only differentiating into specific lineages of cells but thought to act as major sources of cells to replenish injured adult tissues.

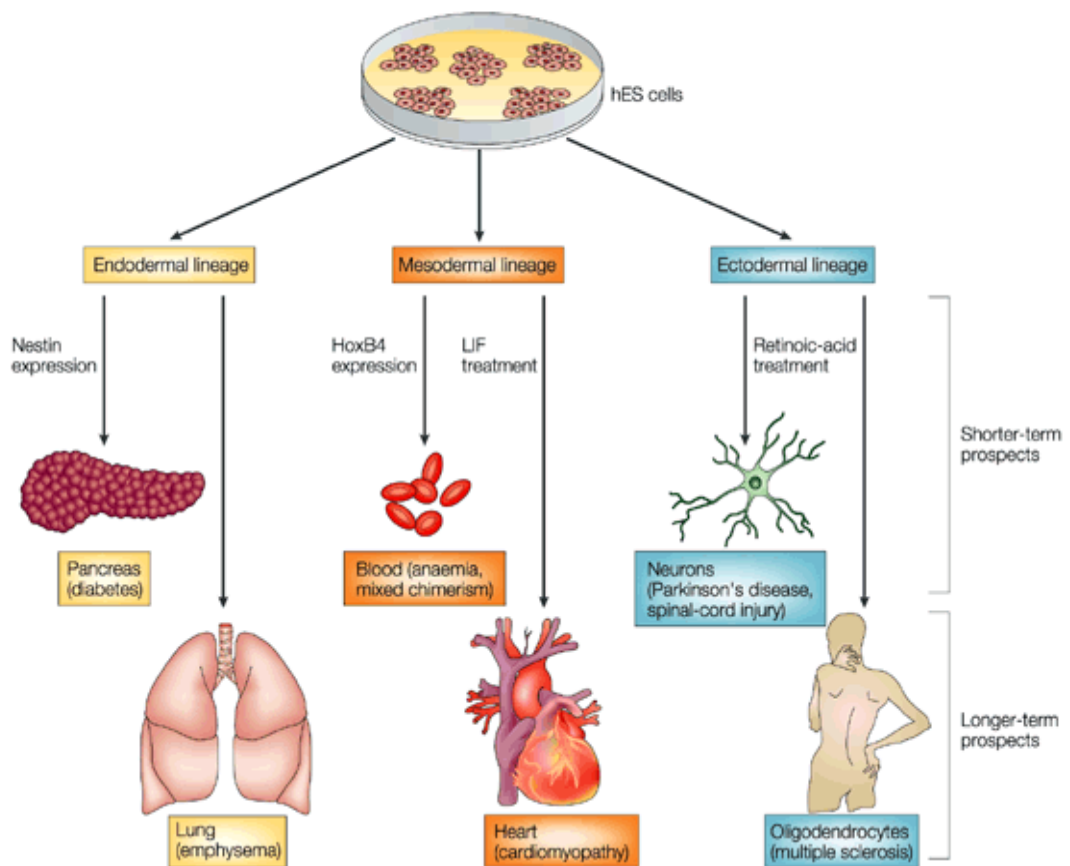
Recent research into stem cells is not only limited to drug screening and embryo development studies, but also focused on patient-derived cell-based personal therapies and regenerative medicines. For example, atherosclerosis is one of the major causes of morbidity and mortality, to treat this disease stem cells can potentially be used to repair damaged vessels, to prepare graft tissue and to develop cell culture models to increase our understanding of the disease process.

1.4.1 Embryonic stem cells

1.4.1.1 Overview of ESCs

ESCs derived from the inner cell mass of blastocyst stage embryos are pluripotent stem cells with self-renewal ability. They have a high proliferative potential and an unlimited capacity to differentiate into all cell types (Reubinoff, Pera, Fong, Trounson, & Bongso, 2000; Wong et al., 2014) (Figure 1.7). In 1981, mouse ESCs were first derived from blastocysts which were cultured in a mouse uterus *ex vivo* to expand the cell number by Martin Evans and Matthew Kaufman (Evans & Kaufman, 1981). In 1998, another revolution occurred in this field, when the first human ESC was isolated and cultured *in vitro* by Thomson et al. (Thomson et al., 1998). Initially in order to culture ESC *in vitro*, a layer of feeder cells (mouse embryonic fibroblasts, MEF) was needed to provide the leukemia inhibitory factor (LIF), which is indispensable in maintaining ESCs in an undifferentiated state (Doetschman, Eistetter, Katz, Schmidt, & Kemler, 1985; A. G. Smith et al., 1988). More recently it has been discovered that adding purified recombinant LIF into the culture medium is enough for mouse ESC to retain their pluripotency (Williams et al., 1988). For human ESCs, LIF in isolation does not have the same effect, however, a different factor basic fibroblast growth factor (bFGF) in the culture medium can keep human ESCs undifferentiated in the absence of MEF (Ludwig et al., 2006). Both human

and mouse ESC express transcription factors Nanog, Sox2, and Oct3/4, which play an important role in maintaining cell pluripotency, and which serve as markers to identify the undifferentiated state of stem cells. Additionally, these cells express species-specific stem cell markers, for example, stage-specific embryonic antigens SSEA-3 and SSEA-4 are expressed on human ESCs while mouse ESCs express SSEA-1 (Ginis et al., 2004; Richards, Fong, Chan, Wong, & Bongso, 2002).



J. Andrew Bradley et al., *Nature Reviews Immunology*, 2002

Figure 1.7 Differentiation of ESCs. Embryonic stem cells possess pluripotent differentiation potentiality. They can differentiate into all cell types belonging to lineages of the three primary germ layers. For example, neurons of the ectoderm, cardiac muscle cells of the mesoderm and pancreatic cells of the endoderm (Bradley, Bolton, & Pedersen, 2002).

Many studies have successfully driven ESCs to differentiate into specific cell type lineages, such as EC and SMCs in various conditions. More established methods, including treatment of ESCs with PDGF-BB when cultivated on collagen-IV coated flasks, will lead to SMC-differentiation (Xiao, Zeng, Zhang, Hu, & Xu, 2007); while, supplement ESC culture medium with VEGF for more than 21 days can induce EC-differentiation (Xiao et al., 2006). As SMCs and ECs are essential to cell types in the vascular wall, the ESC-derived SMC and EC will play crucial roles in the treatment of cardiovascular diseases.

1.4.1.2 Research into ESCs in cardiovascular diseases

In recent years, studies to develop new therapies for cardiovascular diseases have found that ESCs can be differentiated into specific lineages of vascular cells *in vitro* for potential use in vascular repair. In a mouse ischemia model, transplantation of ECs derived from human ESCs significantly increased neovascularization and blood perfusion in the ischemic hind limb, suggesting a promising therapy for new vessel generation in patients (Kane et al., 2010). Moreover, some studies have also demonstrated that ESCs have the ability to differentiate into SMCs. Within an ESC-derived embryonic body model system, the TGF- β -Smad signalling pathway is involved in the differentiation of SMCs, which provides very interesting evidence to suggest that different TGF- β signalling pathways may contribute to induction of early (α -SMA) (both Smad 2 and Smad 3 dependent) vs. late (SMMHC) (only Smad2 dependent) SMC markers (Sinha, Hoofnagle, Kingston, McCanna, & Owens, 2004). In a novel *in vitro* model, human ESC-derived mesenchymal cells (hES-MCs) can also differentiate into SMCs through TGF- β induced high level myocardin expression (Guo, Stice, Boyd, & Chen, 2013).

1.4.1.3 Challenges of ESC research

Human ESCs have various epigenetic profiles depending on the embryo they were derived from. This may lead to variation in their differentiation capacities. In order to remove this chance of variability, mouse ESCs are more extensively used, as mice have been bred selectively and have very similar genetic backgrounds (Lagarkova, Volchkov, Lyakisheva, Philonenko, & Kiselev, 2006). Human ESCs can also differentiate spontaneously and once transplanted *in vivo*

undifferentiated ESCs may form teratomas comprising diverse sorts of tissues of all three germ layers, which will be a major challenge of ESC-based therapy in patients (Baker, 2009). Because of these limitations, researchers are focusing on methods to control ESC differentiation and proliferation prior to transplantation into live organisms.

1.4.2 iPS cells

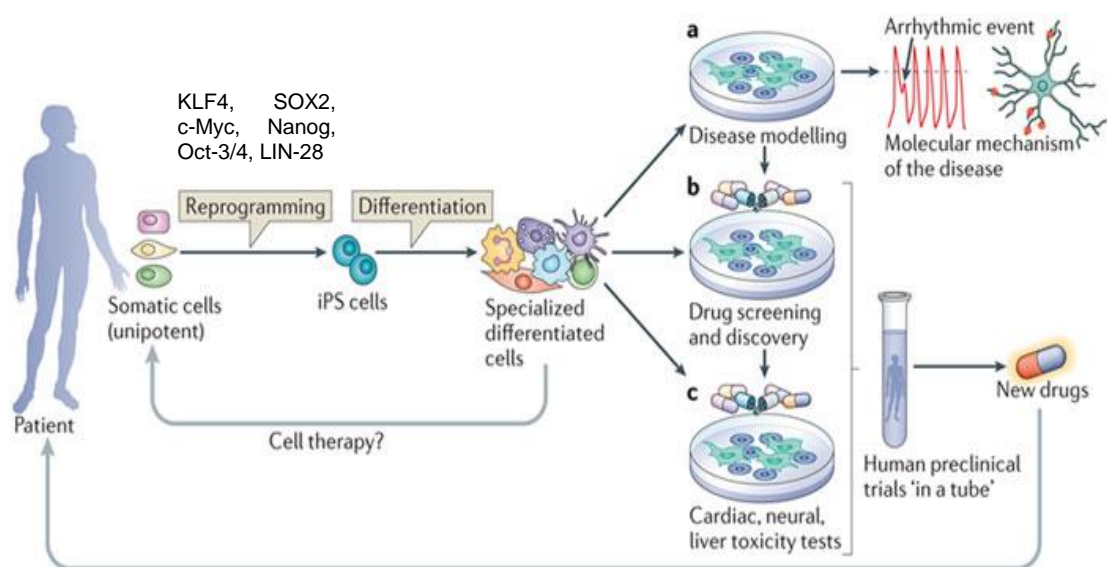
1.4.2.1 The generation of iPS cells

In 2006, Shinya Yamanaka made a revolutionary discovery in the stem cells field. In his study, both embryonic and adult mouse fibroblasts were induced into pluripotent stem cells by the introduction of Oct4, Sox2, Klf4, and c-Myc genes (Takahashi & Yamanaka, 2006). These are known as induced pluripotent stem cells (iPS cells). iPS cells can be obtained by reprogramming human or animal adult cells and then differentiating them into derivatives which constitute the three germ layers (endoderm, mesoderm, ectoderm). Subsequently, other research groups have successfully generated iPS cells from various mature mouse and human cell types by applying these four factors (Aoi et al., 2008; Hanna et al., 2008; Lowry et al., 2008; I. H. Park et al., 2008; Wernig et al., 2007; J. Yu et al., 2007). These iPS cells have similar morphologies and growth pattern and possess the ability, along with ESCs, to form teratomas after injection to mice. Recently, partially induced cells were prepared in a simpler way: researchers generated these partial-iPS (PiPS) cells by transferring four reprogramming factors to human fibroblasts for only 4 days. These PiPS cells can differentiate into ECs via SET translocation (myeloid leukemia-associated) (SET) similar protein (SETSIP) and VEGF without forming tumours *in vivo* (Margariti et al., 2012).

1.4.2.2 Research into application of iPS cells in cardiovascular diseases

It was found that when iPS cells are cultured on collagen IV-coated plates and stimulated with VEGF, they can differentiate into functional EC through the microRNA-21 and TGF- β 2 signalling pathway (Di Bernardini et al., 2014). Additionally, administering mouse iPS-derived

FIK-1⁺ cells, intravenously into mice after femoral artery injury, can effectively enhance re-endothelialisation and attenuate neointima formation, which suggests iPS may be useful in a therapeutic manner for the protection of vessels from post-angioplasty restenosis (Yamamoto et al., 2013). Besides differentiation to ECs, other studies show evidence that functional vascular SMCs can be derived from iPS cells. Human iPS cells can differentiate into contractile SMCs through mesenchymal stem cell (MSC) intermediates, indicating that it may be possible to acquire a limitless number of SMC for vascular regenerative medicine from iPS cells (Bajpai, Mistriotis, Loh, Daley, & Andreadis, 2012).



Milena Bellin, et al., Nature Reviews Molecular Cell Biology, 2012

Figure 1.8 Directed differentiation of iPS cells. The generation of iPS cells is performed by transfection of specific factors such as KLF4, SOX2, c-Myc, Nanog, Oct-3/4, LIN-28 into mature somatic cells isolated from patients. These reprogrammed iPS cells have the pluripotent potential to differentiate into a variety of specialised cells. These specific lineages of cells derived from iPS cells can be widely applied in construction of disease models (a), screening of drugs (b) and toxicity tests (c), which are ideal for both basic research into disease mechanisms and for preclinical trials for new drugs (Bellin, Marchetto, Gage, & Mummery, 2012)

As shown in Figure 1.8, due to iPS cells infinite self-renewal and pluripotency characteristics they are unique models for investigating various processes during embryonic development and establishing patient-specific cell and tissue growth for the study of the pathogenesis of diseases. Furthermore, as patient-derived iPS can be transplanted back into the same patient from whom they were derived, they can be generated to have the exact same genetic background and thus can evade immunological rejection and become a promising source for cell therapy with fewer ethical concerns (A. G. Smith, 2001; Takahashi et al., 2007).

1.4.3 Adult stem cells

Adult stem cells have been found in different tissues and organs around the whole body, not only in adults but also in infants. They can renew themselves and differentiate into specific lineages of cells found in the tissues they are located in. However, unlike ESC, which is known to be derived from blastocysts, the origins of some adult stem cells in specific tissues remain uncertain.

There are several cell types which have been identified as adult stem cells. The earliest discovery of adult stem cells can be traced back to the 1950s when researchers identified hematopoietic stem cells and bone marrow stromal stem cells (Thomas, Lochte, Lu, & Ferrebee, 1957) . The most mature clinical application of adult hematopoietic stem cells is bone marrow transplantation, which has been used for leukemia patients for many decades. A more recent discovery was made in the 1990s when scientists discovered stem cells in the adult brain, which had always been regarded as non-renewable. These cells are capable of differentiation into astrocytes, oligodendrocytes and neurons (Skoff & Knapp, 1991).

The advantages of adult stem cells are that they should be easier to harvest than ESCs and should not need the complex and time-consuming reprogramming of iPSCs. The derivation of blood, fat, and bone marrow-derived stem cells is much easier and without ethical complications of the destruction of an embryo to obtain ESCs and these adult cells are in much greater

abundance. In addition, as they are acquired from individuals, once they are transplanted back to the donor, they will not be rejected by the immune system.

The specific environment of the body which harbours the adult stem cells is called the “stem cell niche”. A specific niche is thought to exist in many tissues and organs, such as bone marrow, peripheral blood, skin, heart, brain, blood vessel wall. Usually, adult stem cells are maintained in a quiescent state for many years in the niche, but once they receive stimulation from adjacent injured tissues, the cells are activated to proliferate, migrate and differentiate to participate in a cascade of repair.

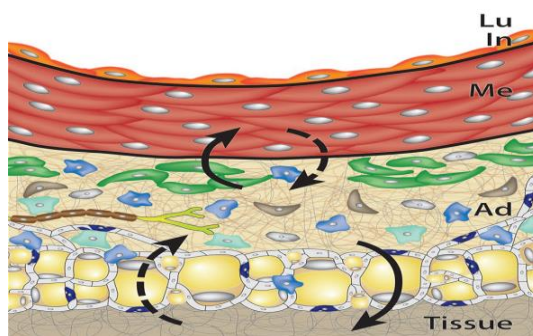
1.4.3.1 Adult stem cells within the vessel wall

For a long time, adult blood vessels were believed to be quiescent, and not thought to contribute to new blood vessel formation. This changed in the 1970s when, based on observations of tumour tissue vascularisation, Judah Folkman and colleagues hypothesised that new vessels were formed via angiogenesis - a process in which new vessels grow from pre-existing vessels (Folkman, 1971). The second tremendous discovery came in 1997 from Asahara et al., who found that CD34⁺ vascular endothelial growth factor receptor-2⁺ (VEGFR2) endothelial progenitor cells (EPCs) circulating in peripheral blood could participate in the formation of new vessels at the sites of active angiogenesis (Asahara et al., 1997). Subsequent studies indicated the bone marrow as a niche for EPCs, a source from which they are mobilised into the circulation and directed to the place of new vessel formation (Rafii, Lyden, Benezra, Hattori, & Heissig, 2002) (Barber & Iruela-Arispe, 2006). However, recent studies indicate that “EPCs” described in many published papers, especially early “EPCs” are monocytes/macrophages (Rehman, Li, Orschell, & March, 2003; S. J. Zhang et al., 2007). Thus, to date, the nature and function of “EPCs” in the circulating blood remain controversial.

In the last decade, breakthrough experiments in the field of stem cell research have taken place. Though there is still some debate, it is widely believed that new vessel formation is in part dependent on stem/progenitor cells that reside within the vascular wall (Hu, Davison, et al., 2002; Hu, Mayr, et al., 2002). The majority of experimental studies postulate that

stem/progenitor cells reside in two main zones within the vessel wall i.e. the subendothelial zone and the adventitia (Alessandri et al., 2001; Ergun, Hohn, Kilic, Singer, & Tilki, 2008; Ergun, Tilki, Hohn, Gehling, & Kilic, 2007; D. Klein, Hohn, Kleff, Tilki, & Ergun, 2010; Pasquinelli et al., 2007; Peault et al., 2007). The subendothelial zone in the microvessel wall corresponds to the adventitial zone in medium and large blood vessels.

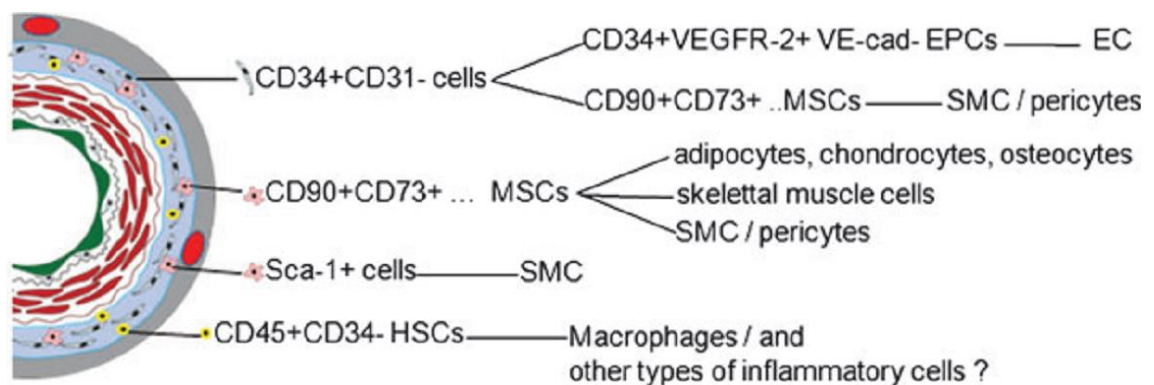
As mentioned previously, the adventitia is the outermost layer of a vessel, which is composed of connective tissue, collagen fibres, vasa vasorum, fibroblasts and quiescent inflammatory cells. The adventitia plays a key role in maintaining vessel wall homeostasis, including retrieval, integration, storage, and release of regulators such as nitric oxide (NO) (Gutterman, 1999; Scotland, Vallance, & Ahluwalia, 2000). It participates in the growth and repair of the vessel and mediates cross-talk between different cell types residing in the vessel wall (Gutterman, 1999; Haurani & Pagano, 2007; Sartore et al., 2001). In the adventitia of the aortic root, immunohistochemistry revealed the presence of progenitor cells that express a panel of progenitor cell markers such as Sca-1, c-kit, CD34, FLK-1 (Hu et al., 2004). The adventitia niche, the region of adventitia close to the media seems to fulfil the definition of such a niche environment and is believed to be a source of progenitors for vasculogenesis (Zengin et al., 2006)



Majesky et al., *Arterioscler Thromb Vas Biol.*, 2011

Figure 1.9 Cellular components of the adventitia of vessel wall. The adventitia interacts with other layers of the vessel wall (top) and the surrounding tissues in which the vessel is located (bottom). Because of their specific loci, adventitial cells can participate in vessel formation, repair and disease processes. Lu, lumen; In, intima; Me, media; Ad, adventitia.

As shown in Figure 1.9, Sca-1⁺ adventitial progenitor cells (green) are shown to differentiate into SMC-like cells to replace lost or damaged SMCs in the media (upward solid arrows). Furthermore, these Sca-1⁺ progenitors can also respond to injury or disease of surrounding tissues and participate in repair of damaged tissues (downward solid arrows). Dashed arrows indicate the theoretical direction of cell movement, either of SMCs in the media or of neighbouring tissue cells in the adventitia (Majesky, Dong, Hoglund, Mahoney, & Daum, 2011).



Suleyman Ergun et al., *Antioxidants & Redox Signalling*, 2011

Figure 1.10 Subsets of vessel wall stem cells and their differentiation capacities. Many studies have identified different populations of progenitor cells in the vascular adventitia using a variety of markers. These cells show a variety of differentiation capacities (D. Klein et al., 2011).

As shown in Figure 1.10, CD34⁺ cells are found in the vasculogenic zone, which potentially includes two subsets: CD34⁺VEGFR2⁺Tie⁺CD31⁻ cells that differentiate into ECs and CD34⁻CD3⁻CD44⁺CD90⁺CD105⁺CD73⁺ cells that differentiate into vascular SMCs. Additionally, vessel wall resident mesenchymal stem cells have also been identified within the native adventitial niche and can similarly differentiate into SMCs. Furthermore, adventitial Sca-1⁺ cells that reside within the vasculogenic zone from embryonic development to adulthood have the capacity to differentiate into SMCs *in vitro* under specific culture conditions. Finally, CD45⁺CD133⁺CD34⁻ cells have been found to give rise to macrophages and other inflammatory cells.

1.4.3.2 Vascular Wall Mesenchymal Stem/Stromal Cells

Mesenchymal stem cells (MSCs) are heterogeneous multipotent stem cells, which can differentiate into a variety of cells such as osteoblasts, adipocytes, chondrocytes and myocytes and play an important role in the development of organs and tissues derived from the mesenchyme (Abedin, Tintut, & Demer, 2004). Besides the bone marrow, which is the best-known storage site for MSCs, the vascular adventitia niche is suggested to be the reservoir for MSCs existence in nearly all organs (da Silva Meirelles, Caplan, & Nardi, 2008). Traditionally, MSCs were identified if cells showed the ability of plastic adherence in standard culture conditions and possessed fibroblast-like morphology. Recently, immuno-selection of specific markers to verify MSCs is more widely used. In 2006, the ISCT (international society for cell therapy) published the minimal criteria for defining MSCs which is CD105⁺, CD73⁺ and CD90⁺, and CD45⁻, CD34⁻, CD14⁻ or CD11b⁻, CD79α⁻ or CD19⁻ and HLA-DR⁻ (Dominici et al., 2006).

However, several studies have found that CD34, which is supposed to be a negative marker of MSCs according to the ISCT, can be expressed on uncultured bone marrow MSCs (Lin, Ning, Lin, & Lue, 2012) (Zimmerlin, Donnenberg, Rubin, & Donnenberg, 2013). Some groups have also reported CD34⁺ MSCs to be present on the vascular adventitia. In a study from Campagnolo et al, CD34⁺CD31⁻ cells isolated from human saphenous vein are found to express pericyte/mesenchymal antigens with Sox2 and to be endowed with clonogenic and multilineage differentiation capacities. In both *in vitro* and *in vivo* studies, they demonstrated that these cells interacted with the endothelial cell network and participated in angiogenesis (Campagnolo et al., 2010). In another study, CD34⁺CD31⁻ cells of three different human vessel walls: artery, vein and small vessels in adipose were compared with adipose-derived stem/progenitor cells. Although they displayed fibroblast-like morphologies, those cells exhibited different differentiation potentials, such as arterial stromal cells undergoing osteogenic processes and venous and small vessel stromal cells promoting angiogenesis (S. Yang et al., 2013). Furthermore, Klein et al. recently demonstrated a population of CD44⁺ vascular wall multipotent stem cells residing in the adventitial vasculogenic zone of human internal thoracic arteries that are capable of differentiating into pericytes and SMC (D. Klein et al., 2011).

As MSCs in the adventitia expressing a variety of surface markers have been reported by many different groups, it is unclear how many unique MSC populations exist and how many overlap with cell populations defined by other groups.

1.4.3.3 Pericytes

Pericytes were first described as contractile cells surrounding the endothelial cells of capillaries and venules by Rouget in 1873, however, description of their identifying features is still controversial (Armulik, Genove, & Betsholtz, 2011). The controversy exists due to the absence of a single specific marker which can distinguish them from other perivascular cells, such as SMCs or MSCs. Currently, several validated markers are involved in the definition of pericytes including PDGFR- β , NG2, CD13, α -SMA, and desmin (Armulik et al., 2011). Pericytes and microvascular ECs are thought to interact by direct contact or through paracrine molecular signalling and are separated by a basement membrane on their interface (Bergers & Song, 2005) (Fakhrejehani & Toi, 2012). They have been suggested to control blood flow and permeability through regulating vessel tone and maintenance of vascular homeostasis, playing an important role in angiogenesis (Gerhardt & Betsholtz, 2003). After de-endothelialisation of venous coronary bypass grafts, sub-endothelial pericytes in the venous intima are exposed to tissue factors in the blood stream, which induced their proliferation. An overabundance of these cells is implicated in plaque instability (Juchem et al., 2010). In addition, both *in vitro* and *in vivo*, pericytes have been demonstrated to contribute to vascular calcification by differentiating into adipocytes, chondrocytes or osteoblasts (Doherty et al., 1998; Johnson, Leopold, & Loscalzo, 2006).

Many similarities have been found between pericytes and MSCs. Besides the fact that they reside in the same compartment of the vessel, pericytes also express some MSC markers, such as CD44, CD73, CD90, CD105, and CD146 (Covas et al., 2008) (Dellavalle et al., 2007). Under *in vitro* culture conditions, pericytes have MSC-like properties, for instance: clonal proliferation capacity and multilineage differentiation potential for MSC-derived mesenchymal cells such as

adipocytes, osteoblasts, chondrocytes and smooth muscle cells, depending upon their specific tissue origin (Crisan et al., 2008; Farrington-Rock et al., 2004; Feng, Mantesso, De Bari, Nishiyama, & Sharpe, 2011). MSCs and pericytes appear to function similarly, both have been shown to promote angiogenesis and repair ischemic tissue (C. W. Chen et al., 2013) (Katare et al., 2011).

However, an increasing number of reports find differences in phenotype and genotype between MSCs and microvascular pericytes. The prevalent view of their distinction is that though both of them are CD31⁻, pericytes are CD34⁻CD146⁺, but MSCs are CD34⁺CD146⁻ (Zimmerlin et al., 2010) (Suga et al., 2009). In addition, not all cells defined as pericytes express MSC markers and possess multilineage differentiation capacity (Braun et al., 2013; Zimmerlin et al., 2013).

To address this controversy further, the evidence is required to determine whether pericytes and MSCs are just two different cell types residing in close proximity within the adventitia or whether one is the precursor of the other.

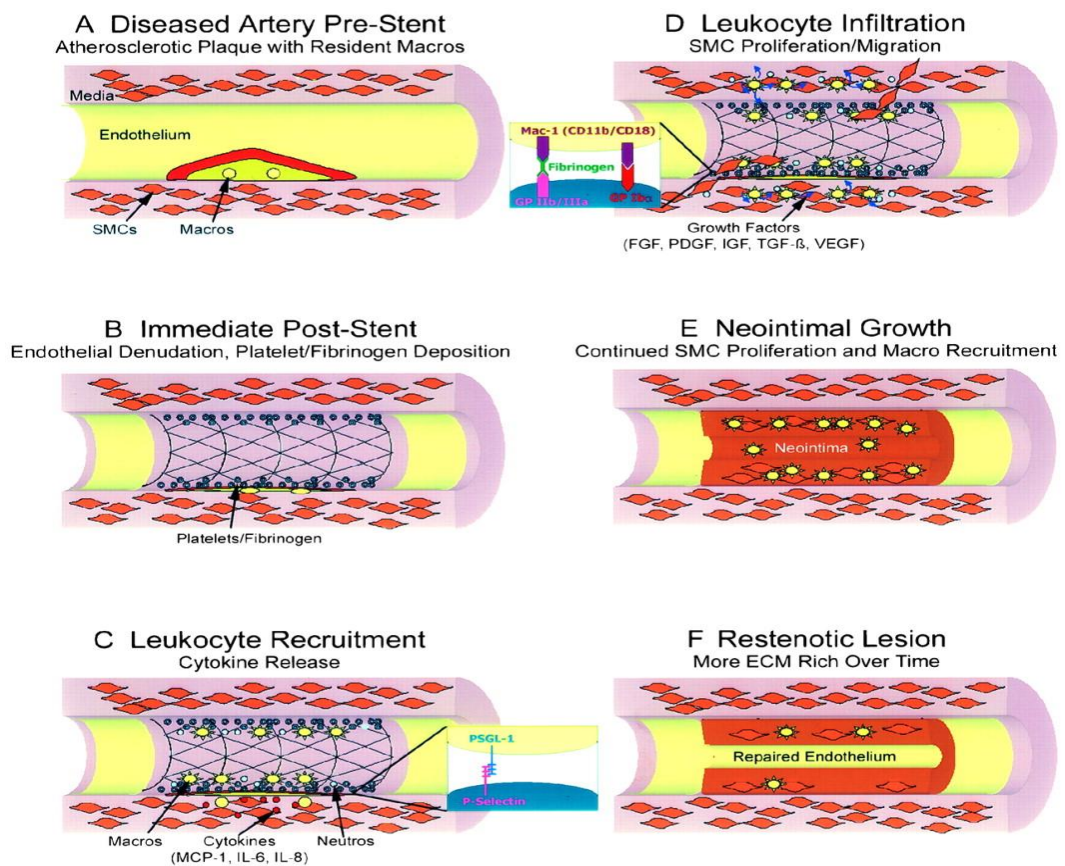
1.4.3.4 Sca-1⁺ vascular progenitor cells

Seminal studies by Hu et al. identified the existence of a population of cells that are Sca-1⁺, CD34⁺, Flk-1⁺ and c-kit⁺ in the adventitia of aortic roots of ApoE^{-/-} mice. Stimulated by PDGF-BB *in vitro*, these Sca-1⁺ cells can differentiate into SMCs. Sca-1⁺β-gal⁻ cells carrying the SMC-LacZ gene were seeded on the adventitia side of vein graft in ApoE^{-/-} mice, after 2-4 weeks, the β-gal⁺ cells can be found in the neointima, which indicates these Sca-1⁺ cells can migrate from adventitia to the neointima and differentiate into SMC *in vivo* (Hu et al., 2004). Subsequently, Passman et al. reported a sonic hedgehog (Shh) signalling domain restricted to the adventitia of the artery wall which is in close proximity to Sca-1⁺ progenitor cells. In Shh^{-/-} mice, the number of Sca-1⁺ cells in the aortic root is dramatically reduced. Similarly to the data showed by Hu, the Sca-1⁺ cells they characterised are also CD34⁺ and CD140b⁺ but c-kit⁻. They also found that *in vivo*, these Sca-1⁺ cells did not express SMC markers, but expressed transcription factors (e.g. serum response factor, myocardin family member) which are related to SMC differentiation. These Sca-1⁺ cells also expressed SRF-dependent transcription repressors to maintain their

progenitor properties *in vivo*. These data suggest Shh signalling is important to maintain Sca-1⁺ function as smooth muscle cell progenitors (Passman et al., 2008). Chen et al. reported a population of Sca-1⁺ cells, localised to the vasa vasorum in the adventitia of vein grafts, which possesses a differentiation potential to SMCs, adipocytes, osteoblasts or chondrocytes *in vitro*. In a bioreactor system, these Sca-1⁺ migrate into the decellularized vessel in response to CXCL12 circulating in the system (Y. Chen et al., 2013). Furthermore, Wong et al. demonstrated that sirolimus, a commonly used drug in DES, can induce Sca-1⁺ progenitor cells isolated from the adventitia of vein graft to migrate and differentiate into SMCs. The authors thus suggest a plausible mechanistic cause of restenosis following the application of sirolimus-eluting stents in patients (Wong et al., 2013).

1.5 Cellular contributions to post-angioplasty restenosis

Post-angioplasty restenosis is a long process, occurring due to a cascade of cellular responses to mechanical injury of the vessel wall (Figure 1.11), including endothelial denudation, thrombosis caused by platelets and fibrinogens, leukocyte-mediated inflammation, vessel wall cell migration and proliferation and ECM deposition in the neointima (Chaabane, Otsuka, Virmani, & Bochaton-Piallat, 2013).



Frederick G.P. Welt et al., *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2002

Figure 1.11 Schematic of the progress of post-angioplasty restenosis. **A.** Pre-stent atherosclerotic plaque in the artery. **B.** Endothelial cell impairment and platelet/fibrinogen deposition immediately after stent implantation. **C.** Leukocyte recruitment and cytokine release. **D.** Leukocyte infiltration into the subendothelium and SMCs proliferation and migration. **E.** Continuous SMC proliferation and macrophage recruitment contribute to neointima formation. **F.** More ECM replaces cells in the neointima, which results in stent-derived restenosis over a long period post angioplasty.

1.5.1 Endothelial cells

1.5.1.1 Endothelial cell injury

Vascular endothelium, as a crude restrictive barrier of the vessel wall, can protect vascular wall cells from inflammation; however, once ECs are impaired, they will become the initial sensors of a complex cascade of events, such as endothelial denudation, platelet activation, inflammatory cytokine and growth factor release and further neointima formation (Tesfamariam, 2008). The activation of ECs includes type I (transient response) and type II (more sustained response). Type I activation is rapid and mediated by ligands (e.g. histamine) binding to GPCRs (G protein-coupled receptors) directly, without protein synthesis, while type II activation is stimulated by TNF- α or IL-1 and keeps ECs in a persistently activated form, with production of adhesion molecules and cytokines (Pober & Sessa, 2007).

In vivo, disruption to shear stress is the major risk factor that activates the endothelium and induces cell migration, proliferation and apoptosis, as well as an increase in endothelium permeability, leukocyte and monocyte adhesion and cytokine release (Hahn & Schwartz, 2009). As mentioned above, GPCRs are involved in shear stress-induced EC activation. When a G protein is activated by a GPCR, both of the GTP-bound G α q/11 and released β and γ subunits can activate PLC β . Furthermore, β , γ also have the abilities to activate Ras (5-10min), ERK1/2 and JNK within 30min (Gudi et al., 2003), these signalling pathways then control the synthesis of new proteins such as E-selectin and VCAM-1 and MCP-1 release from ECs is increased (Chien, 2007). In another study, $\beta\gamma$ units of activated heterotrimeric G proteins were also identified as activators of a new guanine nucleotide exchange factor (GEF), p114RhoGEF, which in turn activates RhoA and Rac1, but not Cdc42 (Niu, Profirovic, Pan, Vaiskunaite, & Voyno-Yasenetskaya, 2003).

Post-angioplasty, ECs are injured and activated by the implanted stent, which recruits leukocytes and induces their adhesion by increasing expression of P-selectin, ICAM-1, VCAM-1, TNF- α and other inflammatory cytokines (e.g. IL-6, IL-8). Injured ECs are also associated with thrombosis through enhancement of tissue factors and inhibition of thrombomodulin

(Koppara et al., 2015). Subsequently, platelets adhere to adhesion proteins (e.g. vWF, collagen and fibrinogen) to promote platelet aggregation sub-endothelium and further thrombosis formation (Tesfamariam, 2008). Furthermore, injured ECs also cause dysfunction in the maintenance of vascular homeostasis and vasomotor tone. These dysfunctional ECs have reduced eNOS (endothelial nitric oxide synthase) activity but induce oxidative stress and increase reactive oxygen species, which further impairs ECs and continuously induces inflammatory signalling (Cai, 2005). The impaired ECs also have diminished NO (nitric oxide) activity, which together with decreased eNOS activity results in endothelium-modulated vasodilation. Another major factor inhibiting endothelial repair after stent implantation is the side effects of the drugs coating the stents. For example, sirolimus, which has been demonstrated to induce PAI-1 and inhibit tPA expression, also induced apoptosis and thrombosis, attributed to re-endothelialisation delay (Q. Ma et al., 2012; Muldowney et al., 2007). Sirolimus, as a lipophilic drug can easily be taken up by ECs, which induces NADPH oxidase-triggered superoxide production and aggravates oxidative stress in EC (Jabs et al., 2008). In terms of shear stress, the local blood flow is also disturbed by the implanted stent, and turbulent shear stress due to disturbed flow promotes the expression of pro-thrombotic and pro-adhesion molecules, such as vWF, ICAM-1 and VCAM-1, which also inhibit re-endothelialisation and thrombosis (Jimenez & Davies, 2009).

1.5.1.2 Endothelium repair

Re-endothelialisation is a self-repair process after an injury to maintain the protective role of endothelium in the vessel wall, which includes proliferation and migration of adjacent intact endothelial cells, resident vascular stem cells and recruited stem cells from the bone marrow. Resident vascular-derived endothelial cells have been proven to contribute to re-endothelialisation much more effectively in response to damaging stimulation than stem cells originating from the bone marrow.

The vasculogenic zone is located between the adventitia and the media, where cells identified as CD34⁺CD31⁻ are described as progenitors with the ability to differentiate into ECs, to participate in blood vessel formation and progression of atherosclerosis (Hu & Xu, 2011; Hu et al., 2004; Zengin et al., 2006). CD34⁺CD31⁻ adventitia cells can interact with ECs and promote capillary-like structure formation and stabilisation (Campagnolo et al., 2010). When adventitial cells were injected into a hind limb ischemia mouse model, a significant pro-angiogenic effect (a full blood flow recovery) was observed as early as 7 days after injection. This indicates that adventitial cells have therapeutic potential in angiogenesis/vasculogenesis (Campagnolo et al., 2010). Transplantation of adventitial cells can repair infarcted hearts through angiogenesis (Katare et al., 2011). Taken together, these recent data demonstrate that adventitial progenitor cells have the ability to repair ECs in ischemic tissue.

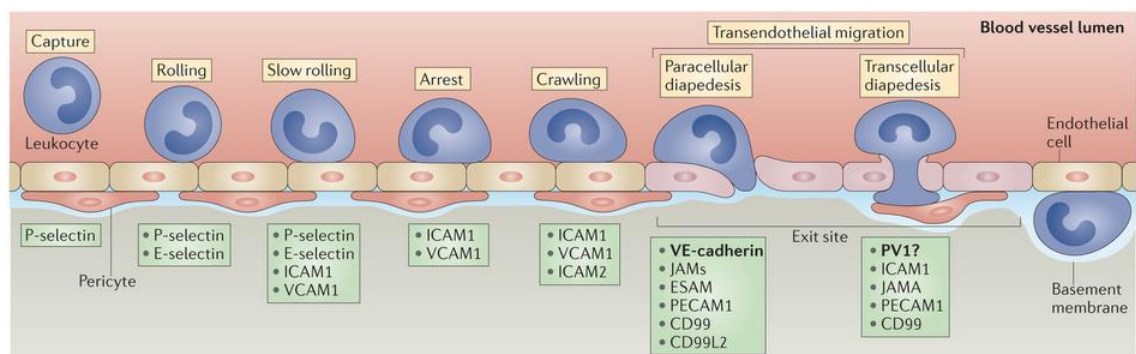
In response to injury or atherosclerotic plaques, resident stem or progenitor cells will have different structural and functional behaviours (Gutterman, 1999). In atherosclerosis, adventitial inflammation and angiogenesis are widespread. In particular, advanced plaques are vulnerable to rupture and thrombosis formation. In normal conditions, progenitor cells are involved in maintaining homeostasis of the physiological vasculature. On the other hand, progenitor cells may also supply cells that contribute to neointimal lesion formation and atherosclerosis.

It is widely accepted that the proliferation of SMCs significantly contributes to neointima formation. However, the debate over the origin of SMCs still persists. It is believed that the media and adjacent undamaged cells, such as mature ECs and SMCs can migrate into the intima to replace dead ECs (Ross, Glomset, & Harker, 1977). In addition, bone marrow-derived progenitors have also been postulated as another main source of SMCs (Saiura, Sata, Hirata, Nagai, & Makuuchi, 2001; Sata, 2003). Subsequently, experimental mouse models in which genes are over-expressed, deleted, or mutated have demonstrated that SMCs and ECs in atherosclerotic plaques are derived from local resident progenitor cells in the vessel wall rather than bone marrow-derived progenitor cells (Xu, 2004). In porcine coronary arteries and canine and rat carotid arteries, the increase in adventitial cell proliferation and migration in response to injury contributes greatly to neointimal lesion formation (Holifield et al., 1996; Mason et al.,

1999; Oparil et al., 1999; Scott et al., 1996; Shi et al., 1996). Transplanting cells to the adventitia of the injured artery and monitoring movement of these cells gives direct evidence for the ability of adventitial cell migration (Stenmark et al., 2013). Furthermore, Sca-1⁺ progenitor cells in the adventitia may be stimulated by injury to adopt a SMC-like phenotype before migration through the media and intima to form the neointima.

1.5.2 Leukocytes recruitment

Following angioplasty, platelets, leukocytes and monocytes are recruited by injured endothelial cells at the beginning of the inflammatory response (Furman et al., 1998; Ott, Neumann, Gawaz, Schmitt, & Schomig, 1996) (Figure 1.12). In both atherosclerosis and post-angioplasty neointima lesions, leukocytes and platelets have all been shown to co-localise in ischemic and reperfusion areas (Libby & Simon, 2001). Activated platelets interact with their recruited leukocytes through a cascade of adhesion involving P-selectin and $\beta 2$ integrin Mac-1 (CD11b/CD18) (Diacovo, Roth, Buccola, Bainton, & Springer, 1996; Evangelista et al., 1996).



Dietmar Vestweber, *Nature Reviews Immunology*, 2015

Figure 1.12 The cascade of leukocyte recruitment and transendothelial migration.

Post-angioplasty, the injured endothelial cells express a variety of adhesion receptors (shown in each panel as adhesion molecules for each step of leukocyte movement) at the initial stage of inflammatory response. These adhesion molecules mediate multiple steps of leukocytes movement including capture, rolling, slow rolling, arrest, crawling and trans-endothelial migration which leads to their recruitment and extravasation from the circulation into the injured vessel wall (Vestweber, 2015).

1.5.2.1 T cell infiltration

Recently, an abundance of studies has revealed that atherosclerosis is regulated by a series of immune responses. In these processes, immune cells play crucial roles in regulating atherosclerosis initiation and progression. Interestingly, either an increased or decreased number of lymphocytes is a documented risk for accelerating atherosclerosis (Dworacka et al., 2007; Nunez et al., 2011), which indicates that different lymphocyte subsets are acting in diverse ways during the progression of atherosclerosis. In the early stages of atherosclerosis, regulatory T cells can clear out injured cells and oxidized LDL from the vessel wall to prevent further inflammatory responses. Regulatory T cells increased markedly between 4-6 weeks and kept this high level until 12 weeks in ApoE^{-/-} mice fed a high-cholesterol diet. In contrast T helper 2 cells are recruited into the vessel wall between 4-10 weeks but after 10 weeks regulatory T cells decreased distinctly (Veillard, Steffens, Burger, Pelli, & Mach, 2004). As the disease progresses, T helper 1 cells play a dominant role against self-antigens, which exacerbate inflammation in the progression of atherosclerosis.

Similarly to the pathophysiology of original atherosclerosis, post-angioplasty restenosis is also attributed to a cascade of inflammatory events. A clinical investigation found that T lymphocytes were activated with an expression of the soluble interleukin-2 receptor in stable angina pectoris patients, but after coronary angioplasty, these cells developed a silent immunological status (Blum, Sclarovsky, & Shohat, 1995). In contrast, another study reported that there were significantly more T lymphocytes in restenotic lesions of patients with unstable angina (Piek et al., 2000). A further clinical study reported that in each stage of restenosis after stent placement, the neointimal area was infiltrated by CD3⁺CD20⁻ T lymphocytes (Grewe, Deneke, Machraoui, Barmeyer, & Muller, 2000). Neutrophils are observed at an early stage of restenosis, whereas lymphocytes and macrophages exist both in the early (3-7 days) and late (>6 months) stages (Farb et al., 1999). 6 months after PTCA, cytotoxic T lymphocytes CD3⁺CD56⁺ were found to be significantly increased in the circulation (Navarro-Lopez et al., 2003). In a porcine balloon injury model, lymphocyte infiltration into the stent was correlated with

increased neointimal formation and percentage of restenosis (Kornowski et al., 1998). Thus, the presence of T lymphocytes as immune cells in the in-stent neointima during both acute and chronic inflammation after PTCA indicates that there is a local immune response in post-angioplasty restenosis.

1.5.2.2 Monocyte/Macrophage recruitment

Increased macrophage infiltration is found in coronary plaques with restenosis, which implicates macrophages as a major indicator of restenosis after PCI (Moreno et al., 1996). At the sites of stent restenosis, there are abundant macrophages accumulating in the neointima (Komatsu, Ueda, Naruko, Kojima, & Becker, 1998). A possible mechanism suggested for restenosis relates to activation of monocytes and neutrophils in the circulation and adherence to thrombin-activated platelets after PCI (Mickelson, Lakkis, Villarreal-Levy, Hughes, & Smith, 1996).

In the early stages after vessel injury, monocyte recruitment is mediated by adherent platelet-derived adhesive substrates, such as selectins, integrins, and adhesion molecules (C. Weber, 2003). For example, the absence of platelet P-selectin on ApoE^{-/-} mice significantly inhibits macrophage infiltration and neointima formation after wire injury of the carotid artery (C. Weber, 2003). Besides the above, chemokines are also known to play a central role in monocyte recruitment in atherosclerosis. CCL2, as a potent monocyte chemoattractant, was found to be expressed on injured vessels within 24 hours of mechanical injury (Furukawa et al., 1999; Taubman et al., 1992). A clinical investigation reported that after PTCA patients with restenosis had significantly higher levels of CCL2 in their plasma, which correlated with elevated monocyte accumulation (Cipollone et al., 2001). This finding is also supported by various animal experimental models. In hypercholesterolemic rabbits, CCL2-mediated monocyte recruitment and neointimal hyperplasia were significantly diminished after anti-CCL2 gene therapy (Mori et al., 2002). Furthermore, the CCL2-CCR2 axis also contributed to monocyte accumulation and activation mediated neointima formation after perivascular cuff placement in normocholesterolemic mice and monkeys (Egashira et al., 2002). *In vitro*, exogenous CCL2

induced arrest of monocytes on adherent platelets, and this effect was abolished by pre-treatment with CCL2 antibody in an *ex vivo* perfusion of injured carotid arteries model. Moreover, deficiency of CCR2 also reduced macrophage accumulation in the neointima (Schober et al., 2004). These findings imply that CCL2 plays an important role in rapidly arresting monocytes to the denudated endothelium. In contrast, CXCL1 was immobilized on endothelial cells which enabled ECs to convert monocytes from a rolling phenotype to firmly adherent to the TNF- α activated endothelium, under physiological flow conditions (K. S. Weber, von Hundelshausen, Clark-Lewis, Weber, & Weber, 1999).

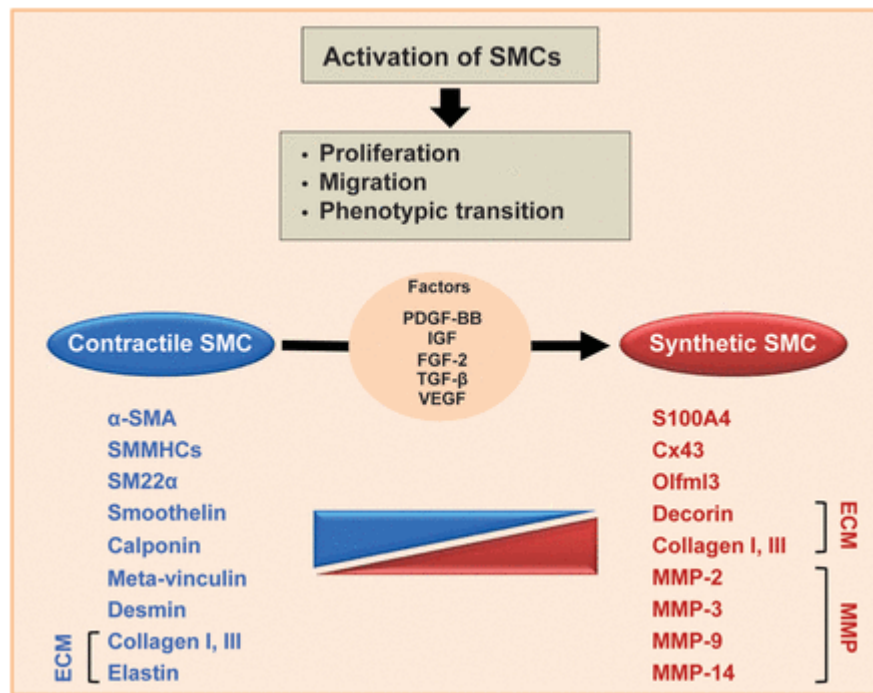
Beyond the early stage of monocyte infiltration, extended monocyte recruitment is induced by NF- κ B and I κ B- α released from SMCs and ECs in the neointima (Landry, Couper, Bryant, & Lindner, 1997; Lindner & Collins, 1996). Similar studies reported migration of macrophages into the neointima was significantly attenuated by inhibition of NF- κ B activation, using decoy oligodeoxynucleotides (Yamasaki et al., 2003; Yoshimura et al., 2001). P-selectin expressed on neointimal SMCs of an injured carotid artery in ApoE^{-/-} mice, mediated monocyte adhesion to SMCs in the neointima (Zeiffer et al., 2004). Chemokine CCL5 was detected on the luminal surface of neointima lesions 4 weeks after wire injury in ApoE^{-/-} mice and triggered circulating monocyte adherence to inflamed endothelium resistant to shear stress (Schober et al., 2002; von Hundelshausen et al., 2001).

In brief, many studies have established the role of monocyte and monocyte-derived macrophage infiltration in neointima, both in acute and chronic stages after vascular injury (Colombo & Sangiorgi, 2004; Fukuda et al., 2004). These monocytes/macrophages not only participate in neointimal hyperplasia but also activate injured endothelial cells and SMCs through the release of chemokines, cytokines and other growth factors (Libby, Schwartz, Brogi, Tanaka, & Clinton, 1992). This plays an important role in the process of restenosis after angioplasty.

1.5.3 Smooth muscle cells (SMCs)

1.5.3.1 SMC phenotype transition after vascular injury

After vascular injury and under the regulation of micro-environmental molecules, such as injured ECs, adherent platelets and recruited inflammatory cells, SMCs partially switch from a contractile to a synthetic phenotype and subsequently participate in neointima formation (Owens, Kumar, & Wamhoff, 2004) (Figure 1.13). Some of the growth factors and cytokines they produce are well characterised, such as TGF- β and PDGF-BB. TGF- β is one of the most potent growth factors that can induce contractile phenotypes in SMCs, and thus their functional differentiation (Tang et al., 2010) (S. Chen & Lechleider, 2004) (Masszi et al., 2003). Meanwhile, TGF- β inhibits SMC proliferation through regulating the cell cycle in late G1 phase (Reddy & Howe, 1993; Seay et al., 2005). PDGF-BB is shown to stimulate SMC proliferation and migration in both human and porcine SMCs (Hao et al., 2002; Madi et al., 2009). Furthermore, co-culture of ECs isolated from the porcine coronary artery with SMCs can induce proliferation of the latter and change their morphology from spindle- to rhomboid-shaped (Hao et al., 2002). Since coronary artery ECs are more heterogeneous in shape, these EC are maintaining activated status to mimic the dysfunctional endothelium after vascular injury.



Chaabane et al. Cardiovascular Research, 2013

Figure 1.13 Smooth muscle cell phenotypic transition after stent implantation. Schema shows the process of SMCs dedifferentiation from a contractile to a synthetic phenotype. A series of cellular responses after stent implantation initiates the activation of smooth muscle cell migration, proliferation, and phenotypic transition. From contractile to synthetic phenotype transition, the contractile markers in SMCs (blue) are decreased, while the synthetic markers in SMCs (red) are increased. In ECM, elastin is replaced by decorin. A group of MMP members is expressed in synthetic SMCs. The growth factors participating in this process are also shown here.

1.5.3.2 SMCs contribution to neointima formation

SMCs proliferation and migration play essential roles in post-angioplasty restenosis. In a rat carotid artery injury model, PDGF has been found to significantly induce SMCs migration towards neointima but did not affect proliferation. In contrast, bFGF markedly induced SMCs proliferation only in balloon-injured arteries but not in intact vessels (Reidy, Fingerle, & Lindner, 1992).

As part of the acute injury response, cells located around the stent start to proliferate, which prevents the stent from exposure to the flowing blood (Uchida et al., 2010). This exposure is necessary for vessel healing after stenting. However, in the late stages of injury, excessive proliferation of SMCs will cause intimal hyperplasia. Thus, these dedifferentiated and proliferative SMCs play an important role in the development of restenosis post angioplasty (Rzucidlo, Martin, & Powell, 2007).

SMC migration is another major cause of neointimal hyperplasia and involves the degradation of ECM, FAK activation and reorganisation of cytoskeletal related protein in SMCs. Studies show that high plasminogen activator activity correlates with SMC migration in rats (Bochaton-Piallat, Gabbiani, & Pepper, 1998), while in pigs, high urokinase activity is more important (Hao et al., 2002). Inhibition of FAK activity also attenuates SMC migration (Taylor et al., 2001). Reorganization of the cytoskeleton allows actin-based SMC movement by supplying a scaffold to generate force (Z. Wang & Newman, 2003).

1.5.4 Extracellular matrix degradation facilitates vascular cells migration

Extracellular matrix (ECM) is a mixture of molecules released from vessel wall cells which supports the vascular structure, mediates multicellular communication and maintains normal vessel function. The ECM exists in each layer of the vessel wall and has a diverse composition depending upon its origin. In the intima, a thin layer of subendothelial ECM is composed of proteoglycans and hyaluronan; in the media, ECM surrounding SMCs comprises elastic elements, collagen and proteoglycans; in the adventitia, fibrillary collagen is the main component of ECM.

Under pathological conditions, ECM also plays an important role in cell migration which contributes to both atherosclerotic and neointimal lesions post angioplasty. After vascular injury, ECM is degraded by matrix metalloproteinases (MMPs) at the margin and core of the lesions, which may contribute to lesion rupture (Nikkari et al., 1996). These MMPs have been shown to participate in SMC migration in response to vascular injury. Inhibition of MMP decreased

SMC migration in response to balloon injury in rat carotid arteries (Bendeck, Irvin, & Reidy, 1996). Overexpression of either tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) or TIMP-2 in SMCs significantly reduced migration in both migration assays *in vitro* and to a lesser extent in balloon-injured rat carotid artery intimal hyperplasia *in vivo* (Cheng et al., 1998; Forough et al., 1996). Overexpression of MMP9 in SMCs induced their migration and invasion *in vitro* and migration in an arterial matrix *in vivo*, which changed vascular remodelling (Mason et al., 1999). Similarly, SMCs isolated from MMP8^{-/-} mice exhibited lower migration *in vitro* compared with SMCs from WT mice (Xiao et al., 2014).

In addition, proteoglycans are involved in vascular cell migration (Wight, Kinsella, & Qwarnstrom, 1992). Hyaluronan (HA) and the HA-binding proteoglycan versican were found to be enriched in the pericellular matrix around SMCs, which facilitates SMCs migration by inhibition of cell adhesion (Evanko, Angello, & Wight, 1999). Furthermore, administering HA also diminishes monocyte influx and migration to the balloon injured carotid artery in rabbits (Ferns, Konneh, Rutherford, Woolaghan, & Anggard, 1995). Collagen type VIII contributes to neointimal lesion development due to induction of SMC migration and attachment (Hou, Mulholland, Gronska, & Bendeck, 2000). A soluble non-collagenous (NC1) domain of collagen type IV has also been demonstrated to regulate endothelial cells migration and adhesion, which may inhibit angiogenesis (Petitclerc et al., 2000).

1.5.5 Vascular progenitor cells contribute to neointima formation

SMCs are well established as the main source of cells contributing to restenosis. In recent years, many studies have demonstrated that a group of adventitia vascular progenitor cells participates in lesion formation. Some studies report that inflammatory cytokines or growth factors play a chemotactic role in progenitor cell migration. Endothelial cell-derived PDGF-B induced migration and proliferation of vascular progenitor cells with expression of PDGFR β (Hellstrom, Kalen, Lindahl, Abramsson, & Betsholtz, 1999). An *ex vivo* model showed that progenitor cells isolated from vein graft could migrate into decellularized vessels in response to SDF-1 via CXCR4 (Y. Chen et al., 2013). Subsequently, another study demonstrated that the migratory

ability of these vascular progenitor cells was significantly enhanced by Sirolimus via CXCR4 activation, which elucidated a new mechanism behind Sirolimus-eluting stent-induced restenosis (Wong et al., 2013). Furthermore, metalloproteinase-8 expressed on stem/progenitor cells facilitated their migration and subsequent contribution to atherosclerosis via the MMP8/ADAM10/E-cadherin signalling pathway (Xiao et al., 2013). EC-derived microparticles and non-microparticle secreted factors can induce perivascular mesenchymal stem cell proliferation and migration by activating NF- κ B signalling (Lozito & Tuan, 2014). However, the exact mechanism of vascular progenitor cells migration and subsequent neointima formation remain to be fully elucidated.

1.6 Molecular mechanisms mediate restenosis

Atherosclerosis and post-angioplasty neointima formation complicates progression of vascular disease, which contains three major stages: 1) endothelial cells activation and inflammatory cell infiltration to the sub-endothelial cell layer; 2) lesion development including neointima formation, collagen accumulation and core necrosis; 3) thrombosis. In each stage, the complex interactions between different cell types and molecules are transmitted through numerous signalling pathways at different times in different cell types. In this redundant signalling network, similar pathways may take different effects in another cell type and overlapping of different pathways may take over the same function. These redundant mechanisms are a product of evolution, which guarantees that critical biological functions can proceed without failure due to some deficiency in some stage of the signalling (Hopkins, 2013). This may bring difficulties for researchers wishing to explore the signalling mechanisms behind particular cell activities.

1.6.1 Inflammatory cytokines

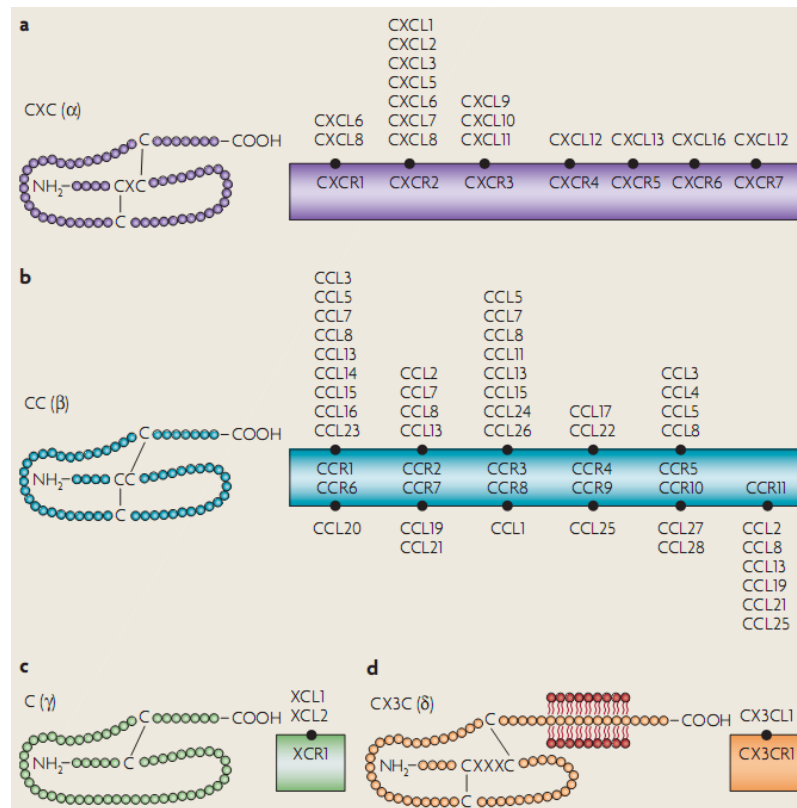
In the adventitia, progenitor cells may communicate with SMCs and ECs via soluble factors produced in the media and the intima and carried by the transmural fluid. This communication is driven by steep pressure gradients from the lumen to the adventitia (Alberding, Baldwin, Barton, & Wiley, 2005; Zerwes & Risau, 1987). In injured vessels, cytokines are produced by neutrophils, activated macrophages and SMCs in response to initial mechanical injury or acute thrombosis, in either an autocrine or paracrine manner, to maintain cells during the process of restenosis (Donners, Daemen, Cleutjens, & Heeneman, 2003). Accumulating data indicate that cytokines produced in inflammatory conditions can be categorised into two groups: pro-inflammatory cytokines, such as IL-1, IL-8, TNF- α , IFN- γ ; anti-inflammatory cytokines, such as IL-4, IL-10, TGF- β . Cytokines of both groups can stimulate the mobilisation of vessel wall cells. For example, TNF- α exacerbates vein graft neointima formation by an induction role in SMC and EC proliferation and migration (L. Zhang, Peppel, Brian, Chien, & Freedman, 2004). IL-8 is reported to increase EC proliferation and migration in the regulation of angiogenesis (A. Li, Dubey, Varney, Dave, & Singh, 2003). TGF- β , though classified as an anti-inflammatory

cytokine, has both pro- and anti- inflammatory effects. One study showed that TGF- β enhanced neointima formation through induction of SMC proliferation (J. D. Smith et al., 1999). However, another research group reported that TGF- β -increased collagen deposition in the adventitia was the main reason behind constrictive remodelling in angioplasty restenosis (Kingston et al., 2001). IL-10 is also reported to have significantly reduced SMCs proliferation and migration which exerts a protective effect from restenosis in balloon-injured rat aorta (Mazighi et al., 2004). Previous studies have shown that when adventitial inflammation occurs in injured vessels, a panel of cytokines such as TNF- α , TGF- β , granulocyte colony stimulating factor are produced (Michel et al., 2007; Mitchell & Libby, 2007; Wilcox, Okamoto, Nakahara, & Vinten-Johansen, 2001). These cytokines may be directly or indirectly related to the mobilisation of progenitor cells in the adventitia. TGF- β induced vascular wall resident stem cells differentiate into pericytes/SMCs and further facilitate neovascularization (D. Klein et al., 2011). In our group, Wong et al. reported that TNF- α mediated vascular progenitor cells to contribute to endothelial repair in vein grafts. Furthermore, adventitial cells can potentially release chemokines, cytokines or reactive oxygen species that can affect medial SMC tone and stimulate the recruitment and retention of inflammatory and progenitor cells from the circulation into the vessel wall. After activation, progenitor cells can potentially undergo proliferation, migration or differentiation that supports lesion formation.

1.6.2 Chemokines

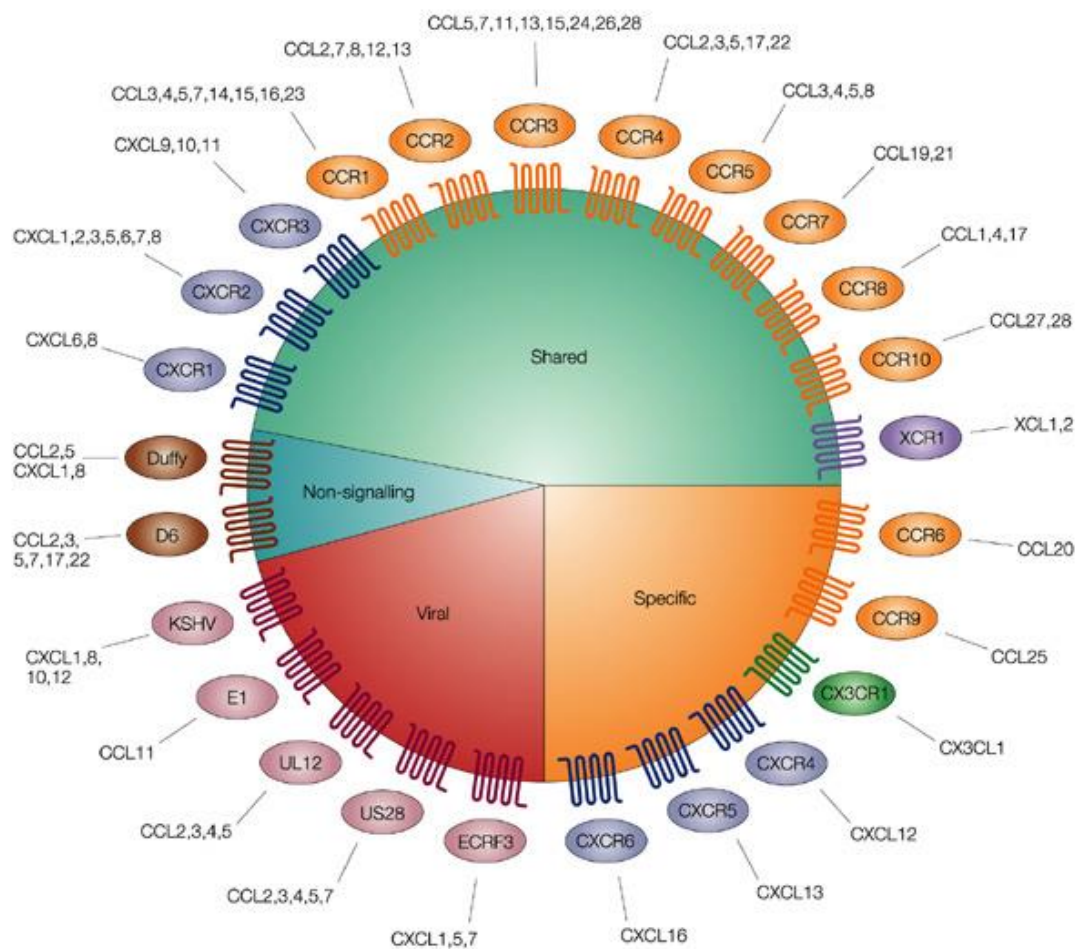
Chemotaxis is a movement of cells directly towards a gradient of chemical attractants (e.g. chemokines) (Majumdar, Sixt, & Parent, 2014). The initial research discovered that chemokines can be rapidly released from activated cells and attract immune cells such as lymphocytes and neutrophils to sites of inflammation, mediating various pathological process (Schall & Bacon, 1994). Subsequent research identified that they are also involved in fibrosis, tissue remodelling and angiogenesis (Lukacs, 2001) Most chemokines contain at least four cysteine residues, in a conserved position that is key to forming their 3-dimensional shape, and have three distinct domains. Chemokines are a group of small proteins with molecular weights ranging from 8 to 10KD. Based on their genetic organization and the position of two cysteine residues at the N-

terminus, members of the chemokine family are classified into four subfamilies namely the CC, CXC, C and CX3C (Moser & Loetscher, 2001) (Figure 1.14). As the region of the N-terminus can be modified, the integrity of the N-terminus is important for receptor binding and cell activity (Proudfoot et al., 1996).



William Rostène et al, Nature Reviews Neuroscience, 2007

Figure 1.14 Classification of chemokines. **a.** CXC chemokines have the distinct motif of one amino acid between the first two cysteine residues. Chemokines from this family are chemoattractants to neutrophils, T lymphocytes, B lymphocytes and natural killer cells. **b.** CC chemokines are shown to be identified by the first two cysteine residues being adjacent to one another. They attract monocytes, macrophages, basophils, T lymphocytes and eosinophils. **c.** the C chemokine subfamily is distinguished by containing only two conserved cysteine residues. The only members of this family are XCL1 and XCL2, which are known chemoattractants of T lymphocytes. **d.** the CX3C chemokine subfamily, which only has one member, CX3CL1, is characterized by a motif of three amino acids between the first two cysteine residues.



Nature Reviews | Cancer

Fran Balkwill et al. Nature Reviews Cancer, 2004

Figure 1.15 Classification of chemokine receptors and their corresponding ligands. The schematic shows the ligand-receptor binding pattern of different groups of chemokine receptors. Each receptor in the **Shared** group binds to several chemokines. In contrast, each receptor in the **Specific** group only binds to one chemokine. Particularly, receptors Duffy and D6 bind to some chemokines without transducing signalling of chemokine response.

All the chemokines exert their biological effects by interacting with 7 transmembrane spanning receptors coupled to a heterotrimeric G protein, known as chemokine receptors (Thelen, 2001) (Figure 1.15). Chemokines, as kinds of charged proteins, have low affinity to glycosaminoglycans expressed on the surface of some other cells, such as ECs. During

homeostasis, each chemokine can non-specifically bind to more than one receptor and vice versa. However, in inflammatory conditions, chemokines are constantly confined to their corresponding receptors (Rot, 1992). For example, CCL1 is a ligand for its respective receptor, CCR1. Some researchers have also shown that some other molecules which are not attributed to the chemokine family can bind to and activate chemokine receptors. For example, recently adrenomedullin was reported as a ligand for an atypical CXCR7 receptor, which modulates cardiac and lymphatic phenotypes (K. R. Klein et al., 2014). Another study demonstrated that HMGB1 (high mobility group box 1) can form a heterocomplex with CXCL12 and exert a function in recruiting inflammatory cells by binding to CXCR4 but not other HMGB1 receptors.

1.6.2.1 Chemokines play an important role in post-angioplasty restenosis

As chemokines play a vital role in recruiting inflammatory cells to the lesion, they are involved in nearly all kinds of disease processes, including cardiovascular diseases. Since atherosclerosis is a chronic inflammatory disease, chemokines have been reported to participate in nearly all stages of the disease. Among them, post-angioplasty restenosis, as a common complication after balloon angioplasty and stent implantation, has been extensively studied. Many researchers are focusing on the mechanisms behind excessive neointima formation, in which a large number of SMCs and inflammatory cells are orchestrated by a variety of chemokines. In an ApoE^{-/-} mouse model, CXCL12 and its receptor CXCR4 have been demonstrated to be crucial for neointimal hyperplasia via recruitment of bone marrow-derived c-kit⁻/PDGFR- β ⁺/lineage⁻/sca-1⁺ smooth muscle progenitor cells into the wire injured carotid arteries (Zernecke et al., 2005). Recently, another study showed that in a partial ligation of carotid artery in ApoE^{-/-} mouse model, intravenous injection of CXCL12 can stabilize the atherosclerotic plaque by mobilizing Sca-1⁺/Lin⁻/PDGFR- β ⁺ smooth muscle progenitor cells to the neointima lesion, which increases SMC and collagen content but reduces the number of macrophages (Akhtar, Gremse, Kiessling, Weber, & Schober, 2013). Furthermore, a recent study identified that endothelial-specific CXCR4 deficiency can significantly reduce injury-induced carotid artery neointima formation by decreasing SMCs and collagen but increasing number of macrophages. Simultaneously re-

endothelialisation, endothelial cell proliferation and Sca1⁺Flk1⁺Cd31⁺ cells mobilization were reduced in this Cxcr4^{EC-KO}ApoE^{-/-} mice model (Noels et al., 2014).

1.6.2.2 CCL2 and CCR2

CCL2 is a potent chemoattractant of monocyte/macrophages, and the recruited monocytes can further become lipid-laden macrophages (foam cells) in the vessel wall. These foam cells are major elements of the fatty streak, a hallmark of early atherosclerotic lesions (Fuster, Badimon, & Badimon, 1992). In early studies, elevated CCL2 was found in macrophage-rich atherosclerotic lesions in humans (Nelken, Coughlin, Gordon, & Wilcox, 1991) and nonhuman primates (X. Yu et al., 1992) and in other animal models (e.g. rabbit (Yla-Herttuala et al., 1991)) but not in healthy arteries (Nelken et al., 1991). Many studies have shown modified LDL can induce CCL2 expression in a variety of cell types. A dramatic increase in monocytes transmigration into the subendothelial region has been attributed to increased levels of CCL2, which is released from the human aortic endothelium when it is incubated with mildly oxidized LDL (Navab et al., 1991). In the late stages of atherosclerosis, CCL2 is also involved in neointima formation. Boring et al. presented the first evidence for the up-regulated expression of CCL2 in human atherosclerosis plaques and for lipid-activated endothelial and SMCs in the vessel wall. They generated a CCR2^{-/-}ApoE^{-/-} mouse to show that deficiency of CCR2 decreased lesion formation, which indirectly proves a role for CCL2 in the recruitment of monocytes to the atherosclerotic lesion, through the CCL2/CCR2 axis (Boring, Gosling, Cleary, & Charo, 1998). Recently, Grudzinska et al. performed a rat aortic-allograft model to investigate whether labelled adventitial tissue participated in transplant arteriosclerosis. Their data showed that fibroblasts and mesenchymal stem cells in the adventitia are the main source of cells which are stimulated by CCL2 and migrate into the neointima. Finally, they confirmed the importance of the CCL2-CCR2 axis in neointimal hyperplasia by using a vessel injury model in both CCL2^{-/-} and CCR2^{-/-} mice (Grudzinska et al., 2013). In a PVAT (perivascular adipose tissue) transplantation model, Manka et al. reported that transplantation of PVAT derived from the thoracic aortic of WT mice fed a high fat diet to LDLR^{-/-} mice. A high fat diet can also

accelerate neointimal formation, macrophage infiltration and angiogenesis in the adventitia. However, deletion of PVAT specific CCL2 can attenuate wire injury-induced neointimal hyperplasia and adventitial angiogenesis (Manka et al., 2014).

CCR2, as the only established receptor for CCL2, exerts most of its effects due to CCL2 and CCR2 interaction. CCR2^{-/-}ApoE^{-/-} mice showed a marked reduction in atherosclerosis lesion area compared to ApoE^{-/-} mice in response to high-fat diet, but no change in lipid or lipoprotein levels in plasma (Boring et al., 1998). In a similar study, in CCR2^{-/-}ApoE^{-/-} mice lesions were found to be less advanced, containing primary foam cells and fatty streaks, which indicates that CCR2^{-/-} mice are more resistant to the development of atherosclerosis (T. C. Dawson, Kuziel, Osahar, & Maeda, 1999). Notably, the phenotype of CCR2^{-/-} mice is not exactly the same as CCL2^{-/-} mice. Compared with WT mice, CCR2^{-/-} mice have a significant deficiency in delayed-type hypersensitivity responses and production of T helper 1-type cytokines (Boring et al., 1997). In contrast, CCL2^{-/-} mice have impaired T helper 2 responses (Gu et al., 2000). Moreover, Sarafi et al. discovered MCP-5, a new potent murine chemokine for recruitment of peripheral blood monocytes, which conducts its signals through CCR2 (Sarafi, Garcia-Zepeda, MacLean, Charo, & Luster, 1997). In humans, MCP-3 has also been identified and it can bind to CCR2b receptor (Kurihara & Bravo, 1996). Therefore, when CCR2 is deleted, some manifestation from these mutant mice may be due to the reduction of signalling pathways mediated by other chemokines but not only through CCL2.

1.6.2.3 CXCL1 and CXCR2

Besides CCL2, which has been established as a potent chemokine for recruitment of monocyte/macrophages, CXCL1 was also found to be expressed on activated ECs to arrest monocytes in the blood (K. S. Weber, P. von Hundelshausen, et al., 1999). In this important study, Weber et al. reported that both mRNA and protein levels of CXCL1 and CCL2 were significantly up-regulated in the TNF- α activated endothelium and that they take their effects through their respective receptors, CXCR2 and CCR2, expressed on monocytes. Subsequently,

the results of inhibition experiments revealed that CXCL1 was restricted to the endothelium, whereas MCP-1 was secreted as a soluble protein. Accordingly, CXCL1 arrested monocytes from rolling in the blood flow to adhere to the CXCR2 expressing endothelium. By contrast, CCL2 was involved in the spreading and trans-endothelial migration of the monocytes through CCR2. Two years later, another study by Huo et al. confirmed this conclusion (Huo et al., 2001). In the carotid arteries of ApoE^{-/-} mice, both CXCL1 and CCL2 were expressed on the endothelium. With blockage of CXCL1 or CXCR2, fewer monocytes were arrested in an *ex vivo* flow chamber system, which was not changed in the CCL2 or CCR2 related blocking groups. On the contrary, many more monocytes accumulated with pre-perfusion of CXCL1 on the carotid arteries compared with treatment of CCL2.

Interestingly, apart from this monocyte arrest in the early stage of atherosclerosis, in the more advanced atheroma, an elevated expression of CXCL1 has also been found to be responsible for recruitment of macrophages and lesion progression (Boisvert et al., 2006). Boisvert et al. generated double mutant mice by deletion of CXCL1 in LDLR^{-/-} mice, and found a significant reduction in atherosclerosis development in these mice. To further investigate in which stage of atherosclerosis CXCL1 take its effect, they generated leukocyte-specific CXCR2^{-/-} chimeric mice on an LDLR^{-/-} mice background and demonstrated a marked decline in macrophage accumulation in lesions, only in the advanced stage of atherosclerosis. Taken together, both CXCL1 in the vessel wall and CXCR2 on leukocytes are pivotal to macrophage infiltration in established atherosclerotic lesions.

The CXC family of chemokines show distinct and differing abilities in their angiogenic effects, owing to whether the ELR motif (Glu-Leu-Arg) is included in their structures (Strieter et al., 1995). As CXCL1 contains an ELR motif, it has been demonstrated to be a potent angiogenic factor by different studies. Caunt et al. found that thrombin can significantly induce expression of CXCL1 mRNA and protein in endothelial cells and the antibody of CXCL1 can ablate the angiogenic, chemotactic and proliferative effects induced by thrombin.

As the receptor of CXCL1, CXCR2 was also detected expressed in the intima of the human atherosclerotic plaque, Boisvert et al. further verified the role of leukocyte CXCR2 in the recruitment of macrophages and development of atherosclerosis in the mice. They generated chimeric mice on an atherosclerosis-prone LDLR^{-/-} background with either mIL-8RH^{+/+} (CXCR2 homologue) or mIL-8RH^{-/-} bone marrow and found CXCL1 was expressed in the intima of aortic atheroma lesions from both types of mice. In comparison with LDLR^{-/-} mIL-8RH^{+/+} BMT mice, the mIL-8RH^{-/-} BMT mice had reduced macrophage infiltration and less advanced atherosclerotic lesions, with restricted necrotic cores and diminished SMC proliferation (Boisvert, Santiago, Curtiss, & Terkeltaub, 1998).

In addition, CXCR2 also plays an important role in the homing of EPCs from the circulation to the injured sites of vessels and contributing to re-endothelialisation. In the adhesion assay, CXCL1 induced EPCs adhesion to platelet coated endothelial matrix can be markedly inhibited by blocking CXCR2. In a wire injured carotid artery model, injection of human KDR⁺CXCR2⁺ cells showed an improvement in endothelial recovery after denudation compared to the KDR⁺CXCR2⁻ injection into nude mice (Hristov et al., 2007). This effect of CXCR2 was further demonstrated in another study, which showed that blockage of CXCL1 using monoclonal antibody can delay re-endothelialisation and thus aggravate neointima formation via the CXCL1/CXCR2 axis (Liehn, Schober, & Weber, 2004).

However, there are several ligands binding to CXCR2 besides CXCL1 and its human orthologue GRO- α , such as CXCL8/IL-8, CXCL7, CXCL2, CXCL3 and CXCL5. Among them, CXCL8 has higher affinity with CXCR2 in humans (Richardson, Marjoram, Barak, & Snyderman, 2003). CXCL8 can trigger firm adhesion of rolling monocytes in the blood flow onto the endothelial cells, with an expression of E-selectin, which is associated with the acute inflammatory process (Gerszten et al., 1999). CXCL8 was also found to be present in human arterial atherosclerotic fibrous plaques (Rus, Vlaicu, & Niculescu, 1996). Detection of CXCL8 in carotid plaques of ischaemic stroke patients, but not in asymptomatic patients, revealed that CXCL8 was associated with stabilization of atherosclerotic plaques (Peeters et al., 2009). CXCL8 induces angiogenesis in human intestinal microvascular endothelial cells through

CXCR2 (Richardson et al., 2003). This evidence can be used to explain why the reduction of atherosclerosis lesions in CXCR2^{-/-} mice is much more robust than the one observed in CXCL1 deficient mice (Boisvert et al., 2006), as other ligands of CXCR2 can either mediate pro-atherogenic functions or even compensate the loss due to deficient CXCL1.

1.6.2.4 CCL5 and CCR5

Several studies have also demonstrated that CCL5 and its receptors CCR1 and CCR5 play important roles in atherosclerosis. In the early stage of atheroma, there is a continuous production of CCL5, which is a potent chemotactic molecule in the recruitment of Th1 lymphocytes and monocytes (Bursill, Channon, & Greaves, 2004). Hundelshausen et al. reported that CCL5 was released from thrombin-activated platelets and elicited arrest of monocytes in the blood and their adhesion to the surface of the inflamed endothelium (von Hundelshausen et al., 2001). In a study from Krohn et al., CCL5 was found to be expressed on SMCs, which contributed to neointima formation. With overexpression of transcriptional regulator YB-1 in SMCs, the expression of CCL5 is significantly up-regulated which leads to an increase in monocyte recruitment. In wire-injured carotid arteries of ApoE^{-/-} mice, intraluminal transfection of YB-1 shRNA significantly reduced neointima formation with decreased macrophage infiltration in the lesions. Furthermore, in both ApoE^{-/-} mice treated with a CCL5 antagonist and mice deficient in CCR5, the protective role of YB-1 knockdown in atherosclerosis was abrogated (Krohn et al., 2007). Another study with application of a CCL5 antagonist verified the role of CCL5 in monocyte recruitment. In this study, the chemokine variant [⁴⁴AANA⁴⁷]-RANTES, a potent inhibitor of endogenous CCL5, was shown to diminish atherosclerosis in LDLR^{-/-} mice, by decreasing numbers of T cells and macrophages infiltration in the plaque and inhibiting secretion of MMP-9. RANTES has also been shown to stabilise the atherosclerotic plaque and is associated with plaques with increased collagen and SMCs. (Braunersreuther et al., 2008).

As the putative receptor of CCL5, CCR5 has also been shown to participate in atherosclerotic development. A similar study using the CCL5 receptor antagonist Met-RANTES showed a significant reduction of atherosclerosis progression with less leukocyte infiltration and increased collagen deposition (Veillard, Kwak, et al., 2004). This finding demonstrated that CXCL5/CCR5 is involved in CCL5 attributed atherosclerosis. A study by Potteaux et al. further proved that CCR5 derived from bone marrow has the effect of promoting atherosclerosis. They generated reconstituted mice on an LDLR^{-/-} genetic background by transplantation of bone marrow from either CCR5^{+/+} or CCR5^{-/-} mice. In comparison to CCR5^{+/+} bone marrow control mice, CCR5^{-/-} bone marrow mice had enhanced IL-10, produced by peritoneal macrophages, and reduced TNF- α , released by T cells, which resulted in less recruitment of macrophages in lesions, smaller sizes of plaque, a 50% reduction in MMP-9 expression and double the amounts of collagen accumulation during 8-12 weeks (Potteaux et al., 2006).

1.6.3 GTPases

Small GTP-binding proteins, which have molecular weights ranging from 20kD to 40kD and can bind and hydrolyze GTP, are also called small GTPases. More than 100 small G proteins have been categorised in this superfamily. According to their primary amino acid sequences, they can be classified into five main subfamilies: Ras (Ras, Rap, Rad, Ral, Rin, and Rit), Rho (Rho, Rac, Cdc42, and Rnd), Rab, Sar1/ADP ribosylation factor (Arf, Arl, Ard, and Sarl), and Ran (Matozaki, Nakanishi, & Takai, 2000). These GTPases take their effects through a mechanism of GTP cycling to turn signal transduction on and off (Figure 1.16). GTPases play crucial roles in many aspects of cell functions, such as cell proliferation, migration and cytoskeletal dynamics.

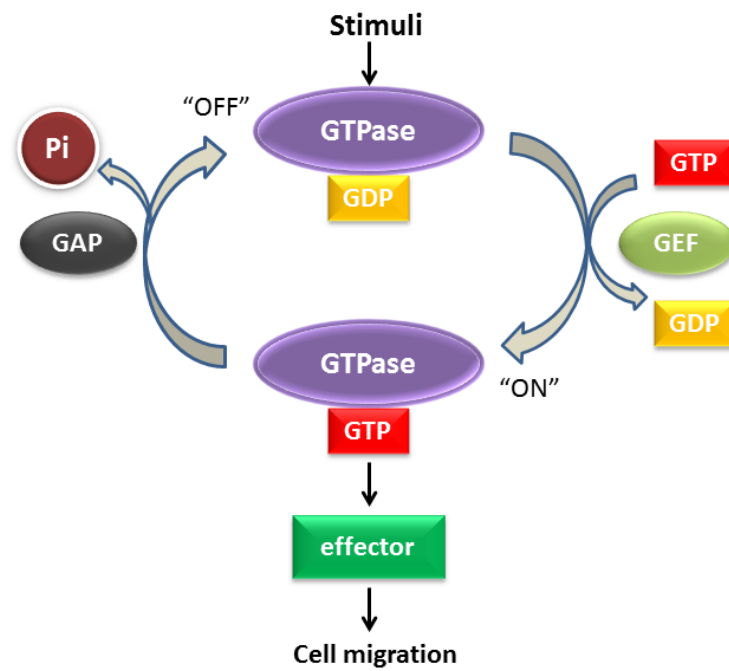
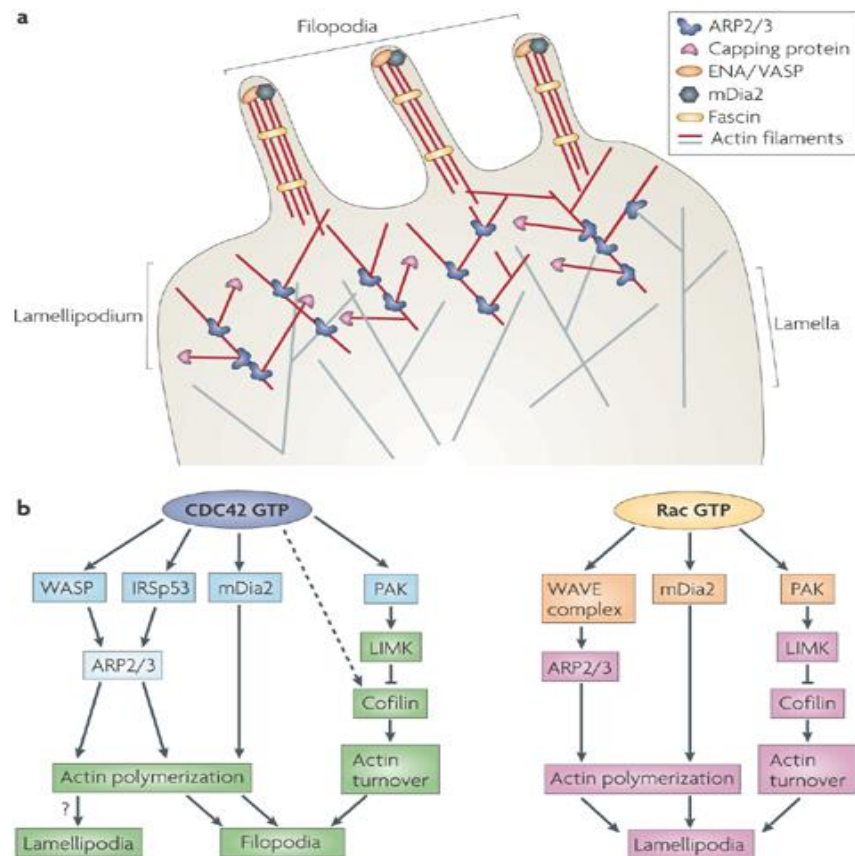


Figure 1.16 Schematic of the GTPase cycle. GTPases function as a molecular switch. GDP-bound forms serve as an “OFF” state. Once stimulated by upstream signals, they will switch into GTP-bound forms (“ON” state), activated by Guanine Nucleotide Exchange Factors (GEFs). Then, the GTP-bound proteins interact with their effectors and transduce signals downstream. GTPase-activating proteins (GAPs) catalyse the hydrolysis of GTP which turns the “switch” off, returning the GTPase to a GDP-bound form once again.

1.6.3.1 Rho GTPase

The Rho GTPase family is a subfamily of the small GTPase superfamily, which consists of 22 members of approximately 21 kD molecular weight, including Rho (A, B, C); Rac (1, 2, 3); Cdc42; TC10; TCL; Chp (1, 2); RhoG; Rnd (1, 2, 3); RhoBTB (1, 2); RhoD; Rif; and TTF. The well-established members: Rho, Rac1 and Cdc42 of the Rho family have been studied in humans, animals, plants and fungi (Boureux, Vignal, Faure, & Fort, 2007). In the last 20 years, they have been widely identified as key factors in arranging the cytoskeleton to orchestrate cell migration (Raftopoulou & Hall, 2004) (Figure 1.17).



Sarah J. Heasman & Anne J. Ridley, *Nature Reviews Molecular Cell Biology*, 2008

Figure 1.17 Cooperation of GTPase Cdc42 and Rac in the organisation of cell migration. **a.** Rho GTPase Cdc42 and Rac are in charge of different regions at the leading edge of a cell. Cdc42 manages cell polarity, the formation of filopodia and focal adhesions. Rac controls actin polymerization and formation of lamellipodia. **b.** GTP-bound Cdc42 and Rac (activated forms) induce cell actin polymerization and turnover to form filopodia and lamellipodia through similar signalling pathways (Heasman & Ridley, 2008).

1.6.3.1.1 Rac1/Cdc42 signalling pathway

Cdc42 is responsible for cell polarisation and for filopodia formation at the front of the migrating cell, while Rac1 is required for cell membrane extension and lamellipodia formation at the leading edge of the migrating cell (Raftopoulou & Hall, 2004). In the process of cell movement, cells first lose their attachment to the ECM and then form the filopodia and

lamellipodia to re-adhere to the ECM, thus, the coordination of the leading and rear edges of the cell results in cell migration (Van Aelst & D'Souza-Schorey, 1997). Activated Rac1 induces lamellipodia formation and subsequently activates actin-related protein 2/3 (ARP2/3) complexes leading to actin polymerization (Krause & Gautreau, 2014). In ECs Rac1 is responsible for regulating adherens junction distribution, to maintain the junctional integrity between adjacent cells (Bazzoni & Dejana, 2004). VE-cadherin is a component of adherens junctions and controls cytoskeleton rearrangement and endothelial migration, which plays an important role in endothelium permeability (Hordijk et al., 1999). A study showed that transformation of active mutant Rac1 (Tat-RacV12) into ECs enhanced cell migration, leading to an endothelial monolayer permeability increase by means of regulating VE-cadherin distribution (van Wetering et al., 2002). Activated Rac1 and Cdc42 can induce VEGF expression in ECs, and consequently promote angiogenesis (J. Ma et al., 2013). In vascular SMCs, NOX1 mediated proliferation and migration are dependent on Rac1 activation (Pescatore et al., 2012). In the wire injury femoral artery model, neointima formation was inhibited in *Nox1^{y/-}* mice in comparison with wild-type (WT) mice, which further demonstrates that Rac1 participates in neointima formation through Nox1 mediated SMCs proliferation and migration (Lee et al., 2009). Moreover, SMC-specific *Kalrn^{+/-}* mice show a significant reduction in neointima hyperplasia which is mediated by a decrease in Rac1 activation and further diminishment of SMC proliferation and migration (Wu et al., 2013).

1.6.3.1.2 RhoA signalling pathway

In contrast with Rac1 and Cdc42, RhoA promotes cell contraction and stress fibre formation at the rear of the cell. Furthermore, RhoA also induces focal adhesion maturation at the front edge of the cell but dissociation at the trailing edge (Raftopoulou & Hall, 2004). The effects of Rac1 and Cdc42 on Rho are either activation or inhibition depending on the cell type, stimulation and ECM. In mouse fibroblast cell line Swiss 3T3, they showed a sequence in activation, specifically, activated Cdc42 induces Rac activation which in turn activates Rho (Nobes & Hall,

1995). While in N1E-115 neuroblastoma cells, Tiam1 activated Rac1, which subsequently antagonised Rho signalling. This effect could be corrected by co-expression of an activated mutant of RhoA (Leeuwen et al., 1997). Thus, a cross-talk between different members of the Rho GTPase family keeps the balance of cell movement and allows cells to demonstrate a range of responses to specific stimuli (Evers et al., 2000).

1.6.3.2 Rho GTPases are involved in integrin signalling pathways

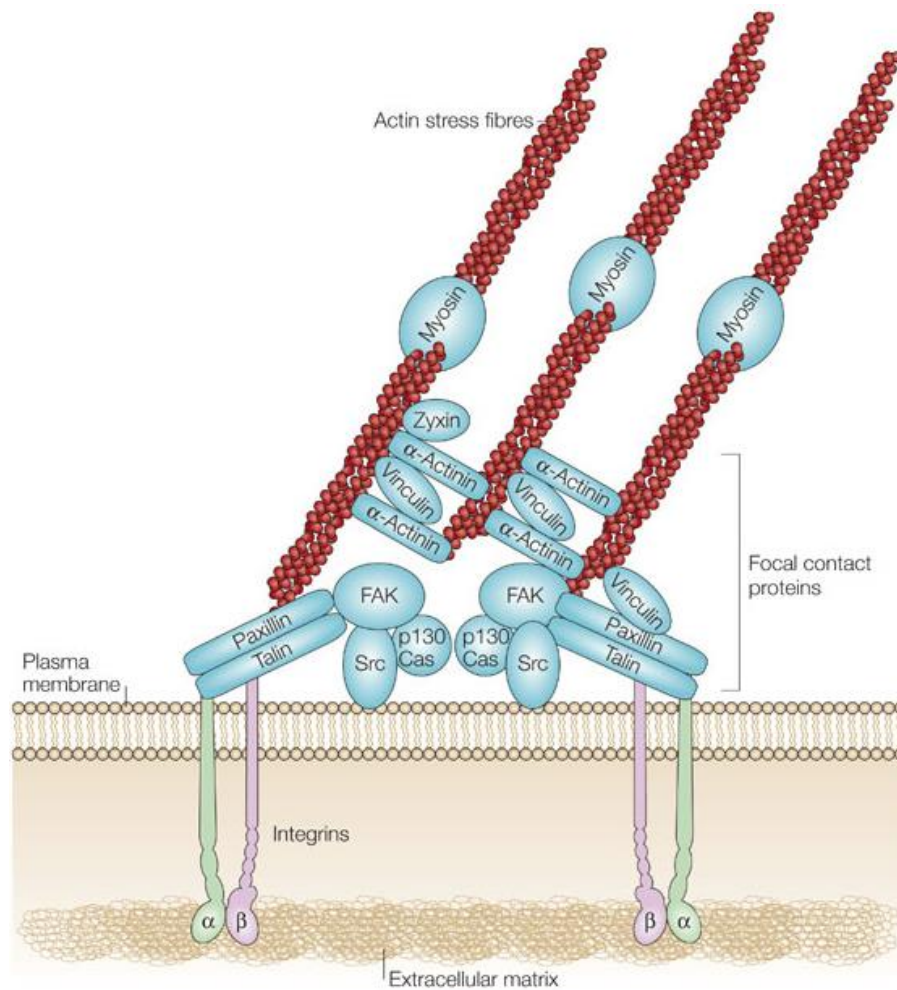
Endothelial integrins play pivotal roles in flow-induced signalling transduction. Integrins are heterodimeric transmembrane receptors, consisting of two different chains: α and β units. Interestingly, integrins are like a bridge, which can transmit signals bi-directionally between two cells or between a cell and the ECM. These integrins can not only transmit signals initiated by ECM binding to the cells (outside-in signal) but also control binding affinity of ECM with their domains through signalling from the inside of the cells (inside-out signal). For example, chemokines first bind to their receptors and “switch on” integrins by inside-out signals, then, the activated integrins further transmit the signals into the cell (outside-in signals) (Regelmann, Danzl, Wanjalla, & Alexandropoulos, 2006). As a result, the integrins either attach or release specific ECM to direct the cells migrating towards the chemotaxis. The process of cell migration is accomplished by binding a series of cytoskeleton adapter proteins to the cytoplasmic tails of α and β chains of integrins.

Talin, as a main adaptor protein, mediates activation of integrins through inside-out signalling by binding to the integrin β intracellular tail (Shattil, Kim, & Ginsberg, 2010). Vinculin, as another adapter protein, also participates in inside-out signalling transmission, but by binding to talin rather than binding to integrins directly. When the actin microfibers are stretched by physiological forces, the connected talin exposes more binding sites for vinculin, to increase activation of integrins and stabilise focal adhesions (del Rio et al., 2009). The intracellular tails of integrins can bind to adapters, G proteins, GEFs, GAPs, tyrosine kinases, serine-threonine kinases, and phosphatases. Among them, some mediate connections between focal adhesions

and cytoskeleton proteins: actin microfibers, intermediate filaments, and microtubules (Hopkins, 2013).

When integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ bind to fibronectin, vitronectin, or fibrinogen in the ECM, the activated integrins can produce inflammatory signalling through outside-in signals (Sechler, Corbett, Wenk, & Schwarzbauer, 1998). These inflammatory signals are transmitted mainly by GTPase Rac1 and Cdc42 and their downstream effector, tyrosine kinase PAK, to control NF- κ B activation (Orr, Hahn, Blackman, & Schwartz, 2008). Furthermore, this inflammatory signalling induces paxillin, which binds to the integrin α cytoplasmic tail, while FAK binds to talin and paxillin (H. C. Chen et al., 1995) (Turner & Miller, 1994). The tyrosine kinase Ack can specifically interact with activated Cdc42 and further inhibits its activity (Galisteo, Yang, Urena, & Schlessinger, 2006). PAK-interacting exchange factor β (β PIX) activates and interacts with Rac1, which is crucial for Rac1-mediated cell membrane spreading (ten Klooster, Jaffer, Chernoff, & Hordijk, 2006). Furthermore, β PIX mediates GTP exchange with Cdc42, by binding with it and triggering downstream signalling (Kepner et al., 2011).

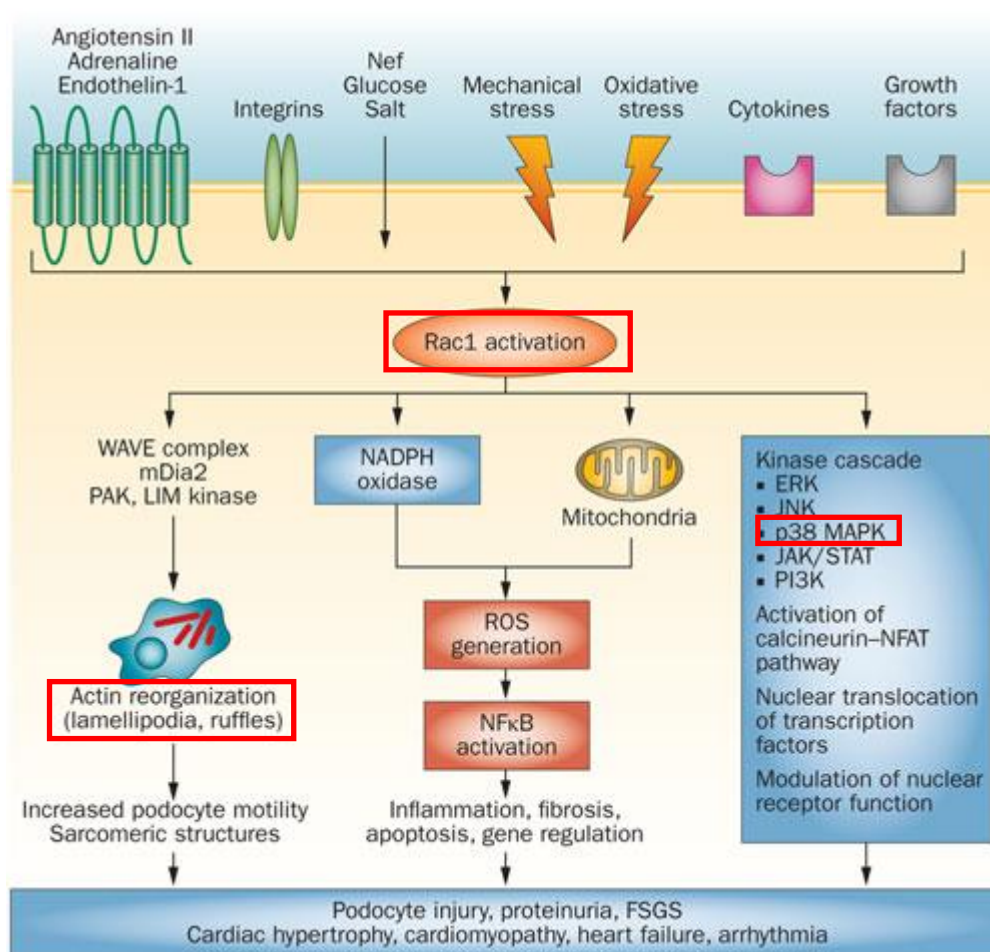
FAK is a remarkable promoter of directional cell migration. Integrin clustering leads to FAK activation (A. K. Mitra & Agrawal, 2006) and in turn FAK can further enhance integrins and ECM interaction (Michael, Dumbauld, Burns, Hanks, & Garcia, 2009). As Rho GTPase family members are important switches in cell migration, FAK also plays a key role in regulating their activity (Tomar & Schlaepfer, 2009). Rac1 and Cdc42 are involved in cell polarisation by regulating the actin cytoskeleton at the leading edge of migrating cells (Fukata, Nakagawa, & Kaibuchi, 2003). In contrast to RhoA, usually localises to the cytoplasm, mediating contraction of the cell membrane at the trailing edge via the ROCK1 and ROCK2 signalling pathway (Lock, Ryan, Poulter, Parsons, & Hotchin, 2012). FAK inactivates RhoA by interacting with RhoGAPs (Taylor, Macklem, & Parsons, 1999) during new adhesions assembly at the leading edge of the cell. FAK can also induce RhoA activation through RhoGEF, which is required for protrusion retraction (Figure 1.18).



Satyajit K. Mitra et.al, Nature Reviews Molecular Cell Biology, 2005

Figure 1.18 Schema of the molecular structure of focal contact proteins. The focal contact is an interaction site near the cell membrane where various cytoskeleton-related proteins are assembled, through which signals are transmitted between the ECM and interior of the migrated cell. For example, integrins bind to paxillin and talin and then activate FAK and vinculin to further control actin stress fibres. The focal contact is a dynamic group of focal adhesion proteins, in which the component proteins differ, depending upon a variety of external stimuli and internal responses (S. K. Mitra, Hanson, & Schlaepfer, 2005).

1.6.4 The P38 signalling pathway is involved in cell migration



Miki Nagase and Toshiro Fujita, *Nature Reviews Nephrology*, 2013

Figure 1.19 Schema of the potential downstream signalling pathway of activated Rac1 in cardiac disease. Rac1 can be activated by many kinds of stimuli (e.g. cytokines, growth factors) mediated by different types of receptors. Subsequently, the activated Rac1 stimulates various downstream signalling proteins, including activation of the WAVE complex, PAK kinase, ROS generation, kinases cascade such as p38 MAPK phosphorylation etc. These signalling pathways are involved in cell actin rearrangement, inflammation and regulation of nuclear factors in cardiac disease (Nagase & Fujita, 2013).

Mitogen-Activated Protein Kinases (MAPKs) are a family of Serine/threonine kinases including p38 MAPK, ERK and JNK. p38 MAPKs is one cascade of members of the MAPK family, which is activated by inflammatory cytokines and various extracellular stresses (Mittelstadt,

Salvador, Fornace, & Ashwell, 2005) (Figure 1.19). The p38 MAPK signalling pathway is involved in cell survival and apoptosis, migration and differentiation, and is related to many human diseases, such as cancer, cardiovascular dysfunction and Alzheimer's disease, etc (Wagner & Nebreda, 2009) (Cuenda & Rousseau, 2007). Among them, p38 MAPK plays a crucial role in chemotactic cell migration. VEGF mediates HUVEC migration through induction of rearrangement of the actin cytoskeleton via p38, but not via either the ERK nor JNK signalling pathways (Rousseau, Houle, Landry, & Huot, 1997). PDGF, IL-1 β and TGF- β induce migration of vascular smooth muscle cells and also activate p38, which can be blocked by its inhibitor SB203580 or through transfection of a p38 dominant negative mutant (Hedges et al., 1999). Migration of Hela cells in response to different chemoattractants, such as CXCL12, PDGF-BB or HGF, has been shown to be mediated by the p38 MAPK signalling pathway (Rousseau et al., 2006). p38 α -deficient mice were reported as embryonic lethal due to defects in placental angiogenesis, but once they were rescued, the embryonic cardiovascular development was normal (Mudgett et al., 2000) (Adams et al., 2000).

Many studies focused the on the association of p38 α -mediated cell migration with cytoskeleton reorganization. Either endothelial cells or SMCs migration induced by growth factors or cytokines were modulated by HSP27 phosphorylation and subsequent F-actin polymerization which regulates lamellipodia microfilaments regeneration (Hedges et al., 1999; Piotrowicz, Hickey, & Levin, 1998). Rac1 is involved in cytoskeleton organization and lamellipodia spreading which contributes to cell migration. TGF- β stimulates endothelial cell spreading on collagen I activated GTP-Rac1 and increases p38 phosphorylation (Varon et al., 2008). Ras/Rac1/p38 signalling pathway is also involved in biomechanical stress induced SMCs production of IL-6 (Zampetaki, Zhang, Hu, & Xu, 2005). Tissue factor enhanced cell migration dependent on Rac1 activation and p38 phosphorylation. The injured human keratinocyte upregulated MMP9 expression through either Rac1/Cdc42-controlled p38 phosphorylation or RhoA-stimulated JNK activation, which played an essential role in cell migration. As the downstream effector of Rac1, an inactive PAK1 mutant overexpression inhibited PDGF-induced p38 phosphorylation in tracheal SMCs migration (Dechert, Holder, & Gerthoffer, 2001).

A number of studies have shown p38 MAPKs are involved in vascular remodelling. Immunohistochemical staining revealed that activated p38 was expressed on medial cells in balloon-injured rat carotid arteries (Ohashi et al., 2000). Infection of artery tissue with a dominant-negative p38 mutant resulted in a reduction of intimal hyperplasia after balloon injury, which indicated the involvement of p38 in vascular smooth muscle cell migration and proliferation (Kim & Iwao, 2003). Furthermore, a doxycycline-inducible SMC-specific dominant-negative p38 α mice model showed significant downregulation of p38 activation in injured arteries and prevention of neointima formation (Proctor et al., 2008).

1.7 Hypothesis and aims of study

Recently, a population of progenitor cells has been identified within the adventitia of the vessel wall. These cells are found to express progenitor markers including Sca-1, c-kit, CD34, CD90, CD73 and CD45 and can either play a reparative role in atherosclerosis or exacerbate neointima formation. Previous studies have shown that these cells possess the high potential to differentiate into many cell lineages, including endothelial and smooth muscle cells (Campagnolo et al., 2015; Wong et al., 2013). However, the precise mechanism of migration and functional roles of these cells within the adventitia needs to be fully elucidated. It is known that these progenitor cells exist in close contact with SMCs, a population of cells that have been established to play important physiological and pathological roles within the media of the vessel wall. The hypothesis of the present study is that **SMCs are able to release specific chemokines to induce migration of vascular progenitor cells that further contribute to neointima formation.**

In order to demonstrate this hypothesis, the aim of the first part of the study was **to identify the migratory ability of vascular progenitor cells in response to SMC-co-cultures or its conditioned medium.** The **experimental design** included: isolation and identification of vein graft vascular progenitor cells and assessing progenitor cell migratory ability in response to SMCs and SMC-derived conditioned medium.

As these vascular progenitor cells have been shown to significantly increase migration towards SMCs, the next aim of the study was **to explore the underlying molecular mechanism that mediates the chemoattractive effects of SMCs in vitro.** The **experimental design** comprises: identify gene and protein levels of chemokines released in SMC-derived conditioned medium, investigate the specific chemokines responsible for VPC migration, detect the corresponding chemokine receptors expressed on vascular progenitor cells, and explore related signalling pathways involved in VPC migration.

Since CCL2 and CXCL1 were successfully identified as the main chemokines to take effects on vascular progenitor cell migration, the final part of this project aimed to **confirm the crucial role of vascular progenitor cell migration in contribution to neointimal development in response to CCL2 or CXCL1 *in vivo***. The **experimental design** contained: comparison of vascular progenitor cells migration towards intima (3 days) and contribution to neointima formation (2 weeks) after femoral artery wire injury in WT and CCL2^{-/-} mice, comparison of vascular progenitor cells contribution to neointima formation after femoral artery injury in the WT (BMT CCL2^{-/-}) and CCL2^{-/-} (BMT WT) chimeric mice, comparison of vascular progenitor cells migration in WT and local knock down of CXCL1 mice, exploration of Sca-1⁺ cells migration towards exogenous CXCL1 in WT mice.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Culture medium, recombinant proteins, antagonists, siRNA and shRNA

Cell culture medium was purchased from ATCC and Foetal Bovine Serum was purchased from Millipore. Recombinant murine JE/CCL2 and murine CXCL1 were obtained from Peprotech and added to cell culture medium at indicated concentrations. CCR2 antagonist ($C_{28}H_{34}F_3N_5O_4S$), CXCR2 antagonist Cpd-19 ($C_{18}H_{21}N_3O_4$), Rac1 inhibitor NSC23766 ($C_{24}H_{38}Cl_3N_7$) and Cdc42 inhibitor ML141 ($C_{22}H_{21}N_3O_3S$) were all purchased from Calbiochem. Rho Inhibitor I (CT04, C3 transferase active site) was purchased from Cytoskeleton. p38 inhibitor SB203580 ($C_{12}H_{16}FN_3OS$) was purchased from Merck Millipore. CCL2 siRNA (m) and Control siRNA (fluorescein conjugate)-A were purchased from Santa Cruz Biotechnology whereas Silencer[®] Select mouse CXCL1 siRNA and Negative Control siRNA were purchased from Ambion. The CCR2 and CXCR2 mission shRNA Bacterial Glycerol Stocks were purchased from Sigma-Aldrich. The shRNA Non-Targeting (NT) vector, SHC002 was used as a negative control.

2.1.2 Mice

C57BL/6J mice (WT mice) were purchased from Harlan, UK. Both GFP mice (C57BL/6-Tg (UBC-GFP) 30Scha/J) and CCL2^{-/-} mice (B6.129S4-Ccl2^{tm1Rol/J}) on C57BL/6J genetic backgrounds were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA). All the mice were housed 2-5 per cage in a temperature-controlled room under specific-pathogen-free conditions with a 12 hours light/dark cycle and given free access to autoclaved food and water. The mice were routinely screened for major pathogens to exclude any opportunistic infections that would possibly affect the experimental results. Mice were used in all procedures at 8 to 16 weeks of age, according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. The CCL2^{-/-} mice were paired with C57BL/6J mice and offspring genotypes were determined by standard PCR from ear punches.

2.1.3 Antibodies, qPCR primers and buffer recipes

Table 2.1 Antibody list and applications

Antibodies	Company	Application
Mouse anti-Cdc42	Millipore, 17-441	1:1000 WB
Mouse anti-Rac1	Millipore, 05-389	1:1000 WB
Rabbit anti-Phospho-p38 MAPK Thr180/Tyr182	Cell Signaling, 4511	1:500 WB
Mouse anti-p38 MAPK	Cell signaling, 9212	1:1000 WB
Rat anti-Sca-1/ Ly6A/E	Abcam, ab51317	1:100 IF
Mouse anti-Actin, α -Smooth Muscle-Cy3 (α -SMA)	Sigma, C6198	1:500 IF
Rabbit anti-CCR2	Abcam, ab21667	1:100 IF 1:100 Flow Cyt
Rabbit anti-vinculin	Abcam, ab73412	1:100 IF
Rabbit anti-paxillin	Abcam, ab32084	1:100 IF
Goat anti-PECAM-1	Santa Cruz, sc1506	1:50 IF
Goat anti-VE-cadherin	Santa Cruz, sc-6458	1:50 IF
Mouse anti-CCL2	R&D, AF-479-SP	1:100 IF 1:100 Neut

Mouse anti-CXCL1	R&D, AF-453-SP	1:100 IF 1:100 Neut
Rabbit anti-calponin	Abcam, ab46794	1:100 IF
Alexa Fluor[®] 488 Phalloidin	Life Technologies, A12379	1:50 IF
Mouse CXCR2 PE-conjugated Antibody	R&D, FAB2164P	10 μ l/10 ⁶ cells Flow Cyt

WB: Western Blot; IF: Immunofluorescence; Flow Cyt: Flow cytometry; Neut: Neutralisation

Secondary antibodies for immunostaining were anti-mouse Alexa Fluor 546, anti-rabbit Alexa Fluor 546 and anti-rat Alexa546 and were purchased from Life Technologies. Cells were also counterstained with 10 μ g/ml 4', 6-diamidino-2-phenylindole (DAPI). Secondary antibodies for Western Blotting for their corresponding primary antibodies were purchased from Dako.

Table 2.2 Primers sequence and parameters

	Primer	Sequence (5→3')	Start position	End position	Annealing Temp (°C)	Product size (bp)
qPCR	CCR2-F	TCCACTCTACTCCCTGGTATTC	310	331	58	119
	CCR2-R	TGGCCAAGTTGAGCAGATAG	428	409	57	
	CXCR2-F	TCTGGCCCTGCCCATCTTAATTCT	667	690	63	129
	CXCR2-R	AAAGTCTGAGGCAGGATACGCAGT	795	772	63	
	CCL2-F	AGTAGGCTGGAGAGCTACAA	221	240	57	123
	CCL2-R	GTATGTCTGGACCCATTCTTC	343	322	58	
	CXCL1-F	GCTGGGATTCACCTCAAGAA	184	203	57	82
	CXCL1-R	TGGCTATGACTTCGGTTTGG	265	246	58	
conventional PCR	GAPDH-F	TGTGATGGGTGTGAACCACGAGAA	638	661	64	130
	GAPDH-R	GAGCCCTTCCACAATGCCAAAGTT	767	744	64	
	CD133-F	TTAAACCAGGAGCTGCCCAAG	2203	2223	60	110
	CD133-R	AAGCCCAGGAAAAAGAAGGTCA	2312	2291	60	
	CD105-F	CACAACAGGTCTCGCAGAAA	489	508	58	200
	CD105-R	GCTTGGATGCCTGAAGAGTC	688	669	58	
	CD90-F	GCCAACTTCACCACCAAGGA	395	414	60	186
	CD90-R	GAGGAGGGAGAGGGAAAGCA	580	561	60	
	CD14-F	GCTTGTTGCTGTTGCTTCTG	341	360	58	197
	CD14-R	CGTGTCCACACGCTTTAGAA	537	518	58	

	Primer	Sequence (5→3')	Start position	End position	Annealing Temp (°C)	Product size (bp)
Conventional PCR	CD34-F	CCACAGACTTCCCCAACTG	464	482	57	394
	CD34-R	CAGGCCTAACCTCAGACTGG	857	838	59	
	Sca-1-F	TCTGAGGATGGACACTTCTC	186	205	56	384
	Sca-1-R	CTCAGGCTGAACAGAAGCAC	569	550	58	
	c-kit-F	TCCCAGAAACAGGCTGAGTT	1826	1845	58	204
	c-kit-R	TTCATGTGATTGCCCAGGTA	2029	2010	56	
	eNOS-F	TCACCATAGCTGTGCTGGCTTACA	2752	2775	64	169
	eNOS-R	AGGATGCAGGGCAAGTTAGGATCA	2920	2897	63	
	vWF-F	GGCTGTGCGGTGATTTTAACAT	422	443	60	127
	vWF-R	CGTTTACACCGCTGTTCCCTCA	548	528	60	
	Flk-F	TGAAATTGAGCTATCTGCCGG	654	674	58	102
	Flk-R	TTTGAAGGTGGAGAGTGCCAG	755	735	60	
	CD31-F	CAAACAGAAACCCGTGGAGAT	2086	2106	58	103
	CD31-R	ACCGTAATGGCTGTTGGCTTC	2188	2168	61	
	CD144-F	AAGAAACCGCTGATCGGCA	1332	1350	60	105
	CD144-R	TCGGAAGAATTGGCCTCTGTC	1436	1416	60	
	SM22-F	GATATGGCAGCAGTGCAGAG	601	620	58	140
	SM22-R	AGTTGGCTGTCTGTGAAGTC	740	721	57	
Calponin-F	GGTCCTGCCTACGGCTTGTC	160	179	62	161	
Calponin-R	TCGCAAAGAATGATCCCGTC	320	301	58		
α -SMA-F	TCCTGACGCTGAAGTATCCGAT	516	537	61	101	
α -SMA-R	GGCCACACGAAGCTCGTTATAG	616	595	61		
β -actin-F	TGGCACCACACCTTCTACAATGAGC	365	389	65	396	
β -actin-R	GCACAGCTTCTCCTTAATGTCACGC	760	736	64		

Table 2.3 Homemade buffers and composition

Buffer	Composition
Protein Lysis Buffer	25mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA (pH 8.0), 1% TritonX, cocktail of protease inhibitors (Roche; 1 tablet in 10ml) in distilled water
5x SDS Loading Buffer	10% sodium dodecyl sulphate, 50% glycerol, 0.05% bromophenol blue, 10mM β -mercaptoethanol, in 500mM Trishydrochloride (pH 6.8)
6x DNA Loading Buffer	30% glycerol, 0.3% bromophenol blue in distilled water
Phosphate Buffered Saline	137 mM NaCl, 2.7mM KCl, 8.1mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 1.76mM KH_2PO_4 in distilled water (pH 7.4)
Tris-Buffered Saline-Tween	50 mM Tris, 150 mM NaCl, 0.1% Tween 20 in distilled water (pH 7.6)
Transfer Buffer	5% NuPAGE [®] , 10% Methanol in distilled water

2.2 Methods

2.2.1 Cell isolation and culture procedures

2.2.1.1 Mouse vascular progenitor cell (VPC) culture

Mouse VPCs were derived from the outgrowth of adventitial tissues of vein grafts as previously described (Iakovou et al., 2005). Briefly, the vena cava from a C57BL/6J or GFP mouse was isografted between the two ends of the carotid artery of an isogenic mouse. After 2 weeks, graft tissues were carefully harvested and cut into pieces that were explanted on 0.04% gelatin (Solution Type B from Bovine Skin, Sigma, G1393 diluted to 0.04% with PBS)-coated flasks. The flask was incubated upside down without medium at 37°C, 5% CO₂ for 3 hours. The flask was then turned over and complete stem cell culture medium, comprised of Dulbecco's Modified Eagle's Medium (ATCC[®], 30-2002[™]) supplemented with 10% ES Cell Qualified Foetal Bovine Serum (EmbryoMax[®], Millipore, ES-009-B), 10 ng/ml leukaemia inhibitory factor (Millipore), 0.1 mM β-mercaptoethanol, 100U/ml penicillin/streptomycin and 2mM L-glutamine (Life Technologies), added and the flask incubated at 37°C, 5% CO₂ for 3 days. When the outgrowth cells reached 90% confluency, they were washed twice with PBS and trypsinized at 37°C for 2 minutes and then passaged every three days at a ratio of 1:3-1:4. The medium was changed every other day.

2.2.1.2 Isolation of single cell clones from mouse VPCs

Single cell clones were isolated by serial dilution of VPCs as described by Corning Incorporated. Briefly, 96 wells (two 48-well plates) were numbered by rows A to H and columns 1 to 12 (Figure 2.1). 200µl culture medium was added to each of the 96 wells apart from well A1. 400µl cell suspension was added to A1, and 200µl was transferred from A1 to B1. These 1:2 dilutions were repeated down the column until H1. 200µl was then discarded from H1. 200µl was next transferred from each of the wells in the first column (A1 to H1) to those in the second column (A2 to H2). This 1:2 dilution was repeated across the whole 96 wells. 200µl was discarded from each well in the last column, column 12. Once prepared, these plates were placed in a 37°C, 5% CO₂ incubator. The following day, wells containing only single cells were marked and in these

wells medium was changed every other day. 7 to 10 days later, single colonies from individual wells were sub-cultured into single wells of either a 24-well plate, a 12-well plate or a T25 flask. The number of clones that expanded successfully was then recorded. Clones were characterised using conventional PCR and immunofluorescent staining for different cell lineage markers.

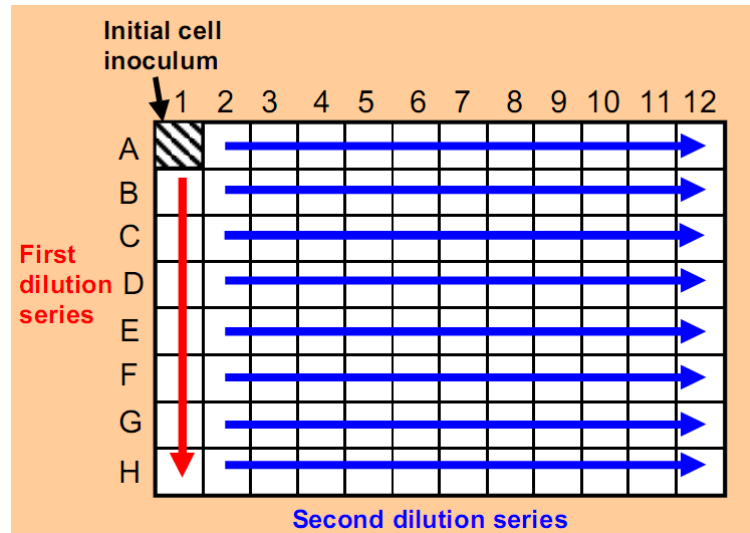


Figure 2.1 Single cell cloning by serial dilution.

2.2.1.3 Sca-1 positive cell sorting

As described in previous studies(X. Yang et al., 2006), heterogeneous VPCs explanted from vein grafts were sorted using magnetic cell sorting kits (MACS) with anti-Sca-1 immunomagnetic microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). According to the manufacturer's protocol for the Anti-Sca-1 MicroBead Kit, the cultured VPCs were trypsinized and centrifuged at 300g for 10 minutes. Supernatant was removed completely and cell pellets were resuspended in 90 μL of cold buffer (phosphate-buffered saline (pH 7.2), 0.5% FBS and 2 mM EDTA) and 10 μL of Anti-Sca-1-FITC for at most 10^7 total cells. The cells were then incubated in the refrigerator (4 $^{\circ}\text{C}$) for 10 minutes. After adding 1–2 mL of cold buffer, the cells were washed and centrifuged at 300g for 10 minutes. The cell pellet was then resuspended in 80 μL of the cold buffer with 20 μL Anti-FITC MicroBeads and incubated for 15 minutes at 4 $^{\circ}\text{C}$, followed by the same washing step as above. The cells were then resuspended in 500 μL of cold buffer and ready for magnetic separation. Briefly, a column filled with magnetic beads was placed in the magnetic field of a MACS Separator (Miltenyi Biotec).

The column was rinsed with 500 μ L cold buffer before applying the cell suspension. Unlabelled cells were passed through and collected for further analysis. After the column reservoir was empty, the fresh 500 μ l cold buffer was added to the column and this washing step was performed 3 times. Finally, the column was placed in a 15 ml collection tube and flushed with 1 ml cold buffer, by firmly applying the plunger, to harvest the magnetically labelled Sca-1⁺ cells. Sca-1 positive VPC populations were expanded for up to 5 population doublings.

2.2.1.4 Mouse vascular smooth muscle cell culture

Mouse vascular SMCs were isolated from aortas of C57BL/6 mice as described previously (Gallo et al., 1999). In brief, the aortas from the aortic arch to thoracic aorta were removed from mice and washed with DMEM. The intima and inner two-thirds of the media were carefully dissected from the aortas and cut into pieces, then positioned on a gelatin-coated (0.04%) plastic flask. The cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Inc.) supplemented with 10% foetal bovine serum (Gibico 10270), 100U/ml penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells were passaged at a ratio of 1:2 to 1:3 once they reached 90% confluence. The medium was refreshed every 2 days. The purity of SMCs was routinely confirmed by immunostaining with α -SMA, SM-22 α and SMMHC antibodies.

Smooth muscle cell conditioned medium (in serum free medium) was collected after an overnight incubation and centrifuged at 1500 rpm for 15 mins, at 4°C.

2.2.1.5 Isolation of mouse peritoneal macrophages

C57BL/6J mice were euthanised by cervical dislocation, and soaked in 70% ethanol for 5 minutes. The outer skin was cut using a sterile scissors and the intact peritoneum was exposed by manual retraction. 10 ml of cold sterile 3% FBS PBS was injected through the peritoneal wall into each mouse. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. Peritoneal fluid was then withdrawn using a syringe with a 19 gauge needle and centrifuged at 200g for 10 minutes at 4°C. Supernatant was discarded and cells were resuspended in buffer RLT (Qiagen) for RNA extraction.

2.2.2 Nucleic acid and protein study

2.2.2.1 RNA extraction

Total RNA extraction from vascular progenitor cells or smooth muscle cells was performed using the RNeasy[®] Mini Kit (Qiagen Cat. No 74106) according to the manufacturer's instructions. Briefly, cells from each well of a 6-well plate were washed with PBS twice before adding 350µl of RLT lysis buffer directly into each well. The cell lysate was pipetted and transferred into a QIAshredder spin column. After centrifugation at full speed for 2 minutes, a homogeneous lysate was acquired, by shearing the high-molecular-weight genomic DNA and other cellular components to reduce viscosity. An equal volume of 70% ethanol was added to the lysate and the sample mixed thoroughly by pipetting. The mixture was then transferred to an RNeasy mini column and placed in a 2 ml collection tube. Following centrifugation at full speed for 30 seconds, the flow through was discarded and 700µl of RW1 washing buffer was added to the column before centrifuging as above. The flow through was again discarded after washing with 500µl of RPE buffer. The sample was then washed again with 500µl of RPE buffer, followed by centrifugation for 2 mins to ensure that there was no ethanol carried over following the RNA elution step. The flow through was discarded and the column was centrifuged within a new 2 ml collection tube to remove any remaining contaminating solution that could be present on the outside of the column. Finally, the RNeasy column was placed in a new 1.5ml microcentrifuge tube and 25µl of RNase-free water was added to the column membrane followed by centrifugation at full speed for 1 minute to elute the RNA. The RNA concentration of each sample was determined by measuring absorbance at 280nm using a Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, UK).

2.2.2.2 Reverse transcription (RT)

1 µg of total RNA of each sample was used to synthesise cDNA in each RT reaction. RT was performed using the ImProm-II[™] reverse transcription system (Promega Cat. No A3800) in a 20µl reaction and the components are listed below (Table 2.4, Table 2.5). 10µl Mix A in a thin-walled PCR tube was heated in an RT-PCR machine (TECHNE TC-412, Bibby Scientific, UK) first to allow denaturation of RNA. After 5 mins incubation at 70°C, the machine was paused,

and the tube was removed and chilled on ice for 1 minute followed by the addition of 10 μ L Mix B. The solution in the tube was mixed briefly by pipetting, then placed back in the machine and continued on a pre-defined programme. The samples were annealed at 25°C for 5min and extended at 42°C for 1 hour and 30 minutes. Finally, a temperature of 70°C was applied for 15 mins in order for the reverse transcriptase to be deactivated. The cDNA in each 20 μ l reaction was diluted with 80 μ l of nuclease-free water to obtain a final concentration of 10ng/ μ l.

Table 2.4 Reverse transcription reaction mix

Reaction Component		Volume(μ l)	Final Concentration
Mix A	RNA	x	0.05 μ g/ μ l
	RNase-free water	9.9-x	-
	Random Primer	0.1	0.025 μ g/ μ l
Mix B	RNase-free H ₂ O	0.8	-
	Improm II 5x reaction buffer	4	1x
	25mM MgCl ₂	2.4	3mM
	25mM dNTPs	0.8	1mM
	RNasin ribonuclease inhibitor	1	1U/ μ l
	Improm II RT enzyme	1	1 μ l/reaction

Table 2.5 Reverse transcription program

Step	Temp(°C)	Time(min)	Note
1	70	5	Denature the RNA
2	4	5	Chill on ice prior to incubation
3	25	5	Anneal
4	42	90	Extend the first strand
5	70	5	Inactivate Improm II RT enzyme
6	4	-	-

2.2.2.3 Quantitative real-time polymerase chain reaction (Q-PCR)

Quantitative real-time PCR (qPCR) was performed to detect relative gene expression in each sample using SYBR green (Qiagen, Cat. No. 204057). The components of each reaction and program are shown below (Table 2.6, Table 2.7). First, 18 µl of master mix was loaded into the 96-well plate (Eppendorf White, Eppendorf, UK) and then 2 µL of sample cDNA was added to each appropriate well. The plate was centrifuged at 600rpm for 1 minute to ensure the contents were mixed thoroughly before running Q-PCR in the qPCR machine (Eppendorf Mastercycler[®] ep Realplex², Eppendorf, UK). The threshold cycle (Ct) values were automatically obtained and exported to Microsoft Excel. GAPDH was used as an endogenous control to normalise the relative expression of RNA in each sample. The fold change compared to control in each target gene was calculated using $2^{-\Delta Ct}$.

Each pair of primers for QPCR was designed using a software provided by DNA Integrated Technologies (IDT) (<http://eu.idtdna.com/scitools/Applications/RealTimePCR/>) and sequences are shown in Table 2.2.

Table 2.6 qPCR reaction mix

	Reaction Component	Volume (μ l)	Final Concentration
master mix	2x SYBR green	10	1x
	10 μ M Primers	1.5	0.75 μ M
	Nuclease-free water	6.5	-
	10ng/ μ l cDNA	2	20ng

Table 2.7 qPCR Program

Step	Temp($^{\circ}$ C)	Time(mm:ss)	Note
1	95	10:00	
2	95	00:15	repeat step2-3 for 40 cycles
3	60	01:00	
4	95	00:15	
5	60	00:15	establish the melting curve of the primers
6	-	20:00	
7	95	00:15	

2.2.2.4 Conventional polymerase chain reaction (PCR)

Conventional polymerase chain reaction was performed on 50ng cDNA using recombinant Taq DNA Polymerase (Invitrogen, 10342-53) to amplify the corresponding DNA. The PCR program and the components of the reaction are given below (Table 2.8, Table 2.9). The PCR products were analysed on 2% agarose gels and assessed using the BioSpectrum AC Imaging System and Vision- WorksLS software.

Specific primers for each PCR template were designed from corresponding mouse mRNA sequences using the Primer-BLAST tool (www.ncbi.nlm.gov.uk/tools/primer-blast). All primers were purchased from Integrated DNA Technologies, dissolved in DEPC water and

the stocks (100 μ M) stored at -80°C. The working solution (10 μ M) was made by mixing forward and reverse primers in DEPC water and stored at -20°C.

Table 2.8 Conventional PCR reaction mix

Reaction Component	Volume(μ l)	Final Concentration
10x PCR Buffer	2.5	1x
50mM MgCl₂	1.2	2.4mM
25mM dNTPs	0.2	0.2mM
10μM Forward primer	1	0.4 μ M
10μM Reverse primer	1	0.4 μ M
5U/μl Taq DNA Polymerase	0.2	1U
RNase-free H₂O	13.9	-
10ng/μl cDNA	5	50ng

Table 2.9 Conventional PCR program

Step	Temp(°C)	Time(mm:ss)	Note
1	94	03:00	-
2	94	00:45	repeat 2-4 steps for 35 cycles
3	62	00:30	
4	72	04:00	
5	72	10:00	-
6	4	-	hold

2.2.2.5 Agarose gel electrophoresis and gel purification

A 2% agarose gel was prepared by dissolving 2g agarose (Invitrogen, Cat. No. 16500-500) in 100ml 1x Tris-acetate-EDTA (TAE) buffer diluted from a 50x TAE (National Diagnostics, Cat. No, EC-872) stock solution (2.0M Tris Acetate + 100mM Na₂EDTA) and heated in a microwave for 2 minutes at 30-second intervals until it thoroughly melted. After the agarose solution cooled down to 60-70 °C, 10µl of SafeView nucleic acid stain (NBS biologicals, Cat. No, NBS-SV) was added to 100ml of liquid gel mixture. The gel mixture was swirled gently to avoid bubbles forming and poured into a Horizon[®] 11.14 horizontal gel cast electrophoresis apparatus (Biometra) with the well comb in place. After 30 minutes, the gel had solidified and therefore the comb could be carefully removed. 25µl DNA samples with 5µl of 6x Loading Buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) were loaded into each well after the gel had been immersed in 1x TAE buffer. Electrophoresis was performed at 180V for 30 minutes and the DNA fragments visualised under ultraviolet light using the Biospectrum[®] Imaging System 500 with image capture using Vision Works Software (Ultra-Violet Products Ltd).

2.2.2.6 Protein extraction

Harvested vascular progenitor cells in each 6-well were lysed with 30-50µl lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100 plus protease inhibitors (Roche), PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche)) . The lysate was then sonicated twice with a Branson Sonifier 150 at level 1 for 6 seconds, at 4°C before they were incubated on ice for at least 30 minutes. The lysate was then centrifuged at 15,000g for 10 min at 4°C. The supernatant was then transferred to a new cold 1.5 ml microcentrifuge tube and the concentration of protein in each sample was measured using the Bradford method. Briefly, 2µl of the protein lysate was added to 998µl of the Bio-Rad Reagent (diluted 1:5 with H₂O) and incubated for at least 5 minutes at room temperature. Duplicates were measured using the Bio-Rad Spectrophotometer 3000. 2µl of lysis buffer served as the blank measurement.

2.2.2.7 Western blot analysis

40µg of protein lysate was mixed with 5xSDS loading buffer and boiled at 96°C for 5 minutes before loading onto 4-12% Bis-Tris Protein gels (NuPAGE, Novex). 10 µl of protein ladder (Precision Plus Protein Ladder, Bio-Rad) was loaded simultaneously and electrophoresis performed in NuPage® MOPS SDS running buffer in an XCell SureLock™ Mini-Cell (Life technologies (Novex®) Cat. No. NP0335BOX) at 160-200V for 1.5 hours. The gel was then transferred onto a nitrocellulose (NC) membrane (Amersham Biosciences), with ice-cold transfer buffer (NuPAGE®, Novex) in the XCell™ Blot Module (Invitrogen) at 35-45V for 1.5 hours. The NC membrane was stained with Ponceau S solution (Sigma-Aldrich) and washed with TBS-T before blocking with 5% milk in TBS-T (0.1% Tween® 20 (Sigma-Aldrich) for 1 hour at room temperature and subsequently incubating with the primary antibody overnight at 4°C.

The corresponding secondary antibodies diluted in 5% milk TBS-T (1:3000) were incubated at room temperature for 45 mins after washing with TBS-T (10minutes x3). The membrane was incubated with Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare) for 3mins at room temperature. Exposure of films (Amersham, Kodak) was carried out using the Compact X4 (Xograph Imaging System).

2.2.3 Cell supernatant study

2.2.3.1 Mouse common chemokines multi-analyte ELISArray

The chemokines present in the SMC-conditioned medium were measured using the Mouse Common Chemokines Multi-Analyte ELISArray kit (MEM-009A, Qiagen) according to the manufacturer's protocol. As the plate map below (Figure 2.2) indicates, columns 1 to 12 were coated with a panel of twelve common mouse chemokine specific capture antibodies (RANTES, MCP-1, MIP-1a, MIP-1b, SDF-1, IP-10, MIG, Eotaxin, TARC, MDC, KC, 6Ckine). 50µl samples of Serum-free DMEM medium (control) and different batches of SMC-conditioned medium samples diluted in reaction buffer were pipetted into appropriate wells along each row and incubated with 50µl assay buffer for 2 hours at room temperature. Sample Dilution Buffer

and Antigen Standard Cocktail (the mixture of all 12 of Antigen Standards with 1:500 dilution from provided antigen standards) served as negative controls and positive controls respectively. Following the incubations, the plate was inverted and washed with 1x Wash Buffer 3 times and blotted on absorbent paper to remove any residual buffer. 100µl of the diluted Detection Antibody solution was added to the appropriate rows and incubated for 1 hour at room temperature. After incubation, the plate was washed, as above, before adding Avidin-HRP solution to each well and incubating for 30 minutes at room temperature in the dark. After repeating the washing steps, Development Solution was added and the plate incubated for 15 minutes at room temperature in the dark before adding 100 µl Stop Solution. The absorbance was read at 450 nm subtracting readings at 570 nm for correction within 30 minutes of stopping the reaction.

The change in levels of each chemokine in the different batches of the SMC-conditioned medium was then compared with positive and negative controls on the plate. Concentrations of chemokines CXCL1 and CCL2 were then confirmed using corresponding Quantikine® ELISA kits.

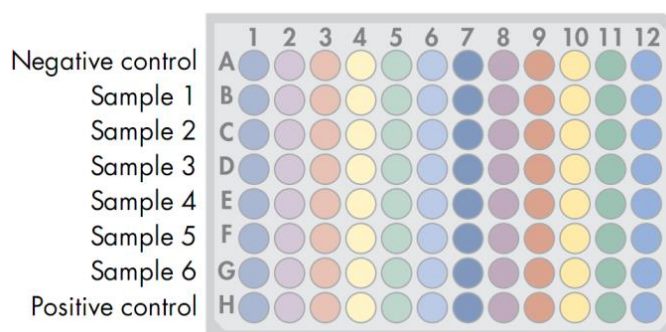


Figure 2.2 Layout of multi-analyte ELISArray plate

2.2.3.2 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of CXCL1 and CCL2 released into the SMC-conditioned medium were detected by mouse CXCL1 (MJE00) and mouse CCL2 (MKC00B) Quantikine® ELISA kits, both purchased from R&D Systems. As this was a sandwich ELISA Microwells in the kit arrived pre-coated with polyclonal antibody either for mouse CXCL1 or CCL2. Briefly, either

50µl of mouse CXCL1, CCL2 Standards or SMC-conditioned medium samples were added to corresponding wells. This was followed by the addition of 50 µl of Assay Diluent. Samples and standards were diluted in an appropriate carrier Diluent. Standards comprised mouse CXCL1 or CCL2 in seven 2-fold serial dilutions with a peak concentration of 1000pg/ml peak and were used for the generation of a standard curve.

After sample or standard addition the plate was mixed by gentle tapping and covered with an adhesive strip before 2 hours of incubation at room temperature. After incubation, the wells were aspirated and washed with Wash buffer 5 times. Following the final wash, the plate was inverted and blotted against clean paper towels. 100µl of an anti-Mouse CXCL1 or CCL2 HRP conjugate was then pipetted into each well and the wells incubated for 2 hours at room temperature. After incubation, the plate was washed 5 times followed by the addition of 100µl Substrate Solution (1:1 mixture of Colour Reagent A (H₂O₂) and Colour Reagent B (Tetramethylbenzidine) used within 15 mins of preparation) to each well and incubated for 30 mins at room temperature protecting from light. Finally, the reaction was stopped by adding 100 µl of Stop Solution to each well. The optical density of each well was determined within 30 mins using a Tecan microplate reader set to 450 nm with wavelength correction at 570 nm. The average O.D. of the blank was subtracted from average of the duplicate readings for each standard and sample. A four parameter logistic (4-PL) curve-fit was subsequently generated to create a standard curve and the concentration of CXCL1 or CCL2 of each sample was calculated in ng/ml.

2.2.3.3 RT² profilerTM PCR arrays for mouse chemokines and receptors

Total RNA (0.5 µg) was extracted from either mouse peritoneal macrophages, VSMCs, Sca-1⁺ VPCs or single VPC clones cultured *in vitro*. Reverse transcription was performed using QuantiTect[®] Reverse Transcription Kit (Qiagen) in a 20µl reaction and the components are listed below (Table 2.10). Mix A was incubated in an RT-PCR machine for step 1 followed by immediate chilling on ice. During this time, Mix B was prepared, before it was added into Mix A for each sample. Then samples were placed back into the machine for the continuation of the following steps (Table 2.11). After first-strand cDNA synthesis, the cDNA in each 20µl reaction

was diluted with 180µl of RNase-free water to get a final concentration of 2.5ng/µl. TR² Profiler PCR Arrays (Qiagen, PAMM-022Z) were used to screen mouse chemokine receptors according to the manufacturer's instructions. The PCR components (Table 2.12) for a 96 well plate were prepared in a loading reservoir. 25 µl of reaction mix well was loaded into each well using an 8-channel multi-channel pipette and tips were changed following each pipetting step to prevent cross contamination. The 96-well plate (Table 2.14) was centrifuged at 1000RPM for 1min to remove bubbles which may interfere with results. Reactions were run in an Eppendorf[®] Mastercycler[®] ep Realplex model 4S using the program shown below (Table 2.13) and acquired data were analysed using the $2^{-(\text{average}\Delta\text{CT})}$ method to determine the expression level of each transcript (Table 2.15) normalized to the expression level of housekeeping gene controls.

Table 2.10 Reverse transcription reaction mix

	Reaction Component	Volume (µl)	Final Concentration
Mix A	Template RNA	x	0.5 µg
	gDNA Wipeout Buffer 7x	2	1x
	RNase-free water	12-x	-
Mix B	Quantiscript Reverse Transcriptase	1	-
	Quantiscript RT Buffer, 5x	4	1x
	RT Primer Mix	1	-

Table 2.11 Reverse transcription program

Step	Temp(°C)	Time(min)	Note
1	42	2	
2	-	-	pause
3	42	15	
4	93	3	inactivate Quantiscript Reverse Transcriptase
5	4	-	hold

Table 2.12 RT² Profiler™ PCR Arrays reaction mix for a 96-well plate

Reaction Component	Volume (µl)	Final concentration
2x RT ² SYBR Green Mastermix	1350	1x
cDNA synthesis reaction	102	-
RNase-free water	1248	-

Table 2.13 RT² Profiler™ PCR arrays program

Cycles	Temp(°C)	Time(mm:ss)	Note
1	95	10:00	Activate DNA Taq Polymerase
40	95	00:15	Perform fluorescence data collection
	60	01:00	

Table 2.14 Layout of RT² Profiler™ PCR Arrays mouse chemokines and receptors plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	C5	C5ar1	Ccbp2	Ccl11	Ccl12	Ccl17	Ccl19	Ccl2	Ccl20	Ccl21	Ccl22	Ccl24
B	Ccl25	Ccl28	Ccl3	Ccl4	Ccl5	Ccl6	Ccl7	Ccl9	Ccr1	Ccr10	Ccr11	Ccr2
C	Ccr3	Ccr4	Ccr5	Ccr6	Ccr7	Ccr8	Ccr9	Ccr12	Cklf	Cmklr1	Cmtm2a	Cmtm3
D	Cmtm5	Cx3cl1	Cx3cr1	Cxcl1	Cxcl10	Cxcl11	Cxcl12	Cxcl13	Cxcl14	Cxcl16	Cxcl2	Cxcl3
E	Cxcl5	Cxcl9	Cxcr2	Cxcr3	Cxcr4	Cxcr5	Cxcr6	Cxcr7	Fgf2	Fpr1	Gpr17	Hif1a
F	Ifng	Il10	Il16	Il1b	Il4	Il6	Il8ra	Itgam	Itgb2	Kdr	Mapk1	Mapk14
G	Pf4	Plau	Ppbp	Prkca	Slit2	Tgfb1	Tlr2	Tlr4	Tnf	Tymp	Vegfa	Xcl1
H	Actb	B2m	Hprt1	Ldha	Rplp1	RGDC	RTC	RTC	RTC	PPC	PPC	PPC
	Housekeeping Genes					Genomic DNA Control	Reverse Transcription Control			Positive PCR Control		

Table 2.15 List of genes in RT² Profiler™ PCR Arrays

	Position	GenBank	Symbol	Description
chemokine and chemotactic cytokines	A03	NM_011329	Ccl1	Chemokine (C-C motif) ligand 1
	A04	NM_011330	Ccl11	Chemokine (C-C motif) ligand 11
	A05	NM_011331	Ccl12	Chemokine (C-C motif) ligand 12
	A06	NM_011332	Ccl17	Chemokine (C-C motif) ligand 17
	A07	NM_011888	Ccl19	Chemokine (C-C motif) ligand 19
	A08	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2
	A09	NM_016960	Ccl20	Chemokine (C-C motif) ligand 20
	A10	NM_009137	Ccl22	Chemokine (C-C motif) ligand 22
	A11	NM_019577	Ccl24	Chemokine (C-C motif) ligand 24
	A12	NM_009138	Ccl25	Chemokine (C-C motif) ligand 25
	B01	NM_001013412	Ccl26	Chemokine (C-C motif) ligand 26
	B02	NM_020279	Ccl28	Chemokine (C-C motif) ligand 28
	B03	NM_011337	Ccl3	Chemokine (C-C motif) ligand 3
	B04	NM_013652	Ccl4	Chemokine (C-C motif) ligand 4
	B05	NM_013653	Ccl5	Chemokine (C-C motif) ligand 5
	B06	NM_009139	Ccl6	Chemokine (C-C motif) ligand 6
	B07	NM_013654	Ccl7	Chemokine (C-C motif) ligand 7
	B08	NM_021443	Ccl8	Chemokine (C-C motif) ligand 8
	B09	NM_011338	Ccl9	Chemokine (C-C motif) ligand 9
	C12	NM_027022	Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A
	D01	NM_024217	Cmtm3	CKLF-like MARVEL transmembrane domain containing 3
	D02	NM_153582	Cmtm4	CKLF-like MARVEL transmembrane domain containing 4

D03	NM_026066	Cmtm5	CKLF-like MARVEL transmembrane domain containing 5
D04	NM_026036	Cmtm6	CKLF-like MARVEL transmembrane domain containing 6
D05	NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1
D07	NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1
D08	NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10
D09	NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11
D10	NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12
D11	NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13
D12	NM_019568	Cxcl14	Chemokine (C-X-C motif) ligand 14
E01	NM_011339	Cxcl15	Chemokine (C-X-C motif) ligand 15
E02	NM_023158	Cxcl16	Chemokine (C-X-C motif) ligand 16
E03	NM_009140	Cxcl2	Chemokine (C-X-C motif) ligand 2
E04	NM_203320	Cxcl3	Chemokine (C-X-C motif) ligand 3
E05	NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5
E06	NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9
F06	NM_008337	Ifng	Interferon gamma
F07	NM_010551	Il16	Interleukin 16
F08	NM_008361	Il1b	Interleukin 1 beta
F09	NM_021283	Il4	Interleukin 4
F10	NM_031168	Il6	Interleukin 6
G03	NM_019932	Pf4	Platelet factor 4
G04	NM_023785	Ppbb	Pro-platelet basic protein
G06	NM_011577	Tgfb1	Transforming growth factor, beta 1
G09	NM_013693	Tnf	Tumour necrosis factor
G11	NM_008510	Xcl1	Chemokine (C motif) ligand 1

chemokines and chemotactic cytokines receptors	A01	NM_007577	C5ar1	Complement component 5a receptor 1
	A02	NM_021609	Ackr2	Chemokine binding protein 2
	B10	NM_009912	Ccr1	Chemokine (C-C motif) receptor 1
	B11	NM_007721	Ccr10	Chemokine (C-C motif) receptor 10
	B12	NM_007718	Ccr11	Chemokine (C-C motif) receptor 1-like 1
	C01	NM_009915	Ccr2	Chemokine (C-C motif) receptor 2
	C02	NM_009914	Ccr3	Chemokine (C-C motif) receptor 3
	C03	NM_009916	Ccr4	Chemokine (C-C motif) receptor 4
	C04	NM_009917	Ccr5	Chemokine (C-C motif) receptor 5
	C05	NM_009835	Ccr6	Chemokine (C-C motif) receptor 6
	C06	NM_007719	Ccr7	Chemokine (C-C motif) receptor 7
	C07	NM_007720	Ccr8	Chemokine (C-C motif) receptor 8
	C08	NM_009913	Ccr9	Chemokine (C-C motif) receptor 9
	C09	NM_145700	Ackr4	Chemokine (C-C motif) receptor-like 1
	C10	NM_017466	Ccr12	Chemokine (C-C motif) receptor-like 2
	C11	NM_008153	Cmklr1	Chemokine-like receptor 1
	D06	NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1
	E07	NM_178241	Cxcr1	Chemokine (C-X-C motif) receptor 1
	E08	NM_009909	Cxcr2	Chemokine (C-X-C motif) receptor 2
	E09	NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3
	E10	NM_009911	Cxcr4	Chemokine (C-X-C motif) receptor 4
	E11	NM_007551	Cxcr5	Chemokine (C-X-C motif) receptor 5
	E12	NM_030712	Cxcr6	Chemokine (C-X-C motif) receptor 6
	F01	NM_007722	Ackr3	Chemokine (C-X-C motif) receptor 7
	F02	NM_010045	Darc	Duffy blood group, chemokine receptor

	F03	NM_013521	Fpr1	Formyl peptide receptor 1
	F04	NM_001025381	Gpr17	G protein-coupled receptor 17
	G11	NM_011798	Xcr1	Chemokine (C motif) receptor 1
other chemotaxis	F05	NM_010431	Hif1a	Hypoxia inducible factor 1, alpha subunit
	F11	NM_008401	Itgam	Integrin alpha M
	F12	NM_008404	Itgb2	Integrin beta 2
	G01	NM_011949	Mapk1	Mitogen-activated protein kinase 1
	G02	NM_011951	Mapk14	Mitogen-activated protein kinase 14
	G05	NM_178804	Slit2	Slit homolog 2 (Drosophila)
	G07	NM_011905	Tlr2	Toll-like receptor 2
	G08	NM_021297	Tlr4	Toll-like receptor 4
	G10	NM_138302	Tymp	Thymidine phosphorylase
		H01	NM_007393	Actb
H02		NM_009735	B2m	Beta-2 microglobulin
H03		NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H04		NM_010368	Gusb	Glucuronidase, beta
H05		NM_008302	Hsp90ab1	Heat shock protein 90 alpha(cytosolic), class B member 1
H06		SA_00106	MGDC	Mouse Genomic DNA Contamination
H07-H09		SA_00104	RTC	Reverse Transcription Control
H10-H12		SA_00103	PPC	Positive PCR Control
G10		NM_138302	Tymp	Thymidine phosphorylase

2.2.4 *In vitro* study of cell properties

2.2.4.1 Transwell chemotaxis assay

Migration assays were performed using Transwell inserts with 8.0 μm pore-sized membrane filters (Corning). Vascular progenitor cells were loaded into the upper chamber at a density of 5×10^4 cells in 200 μl serum free media, and the bottom chamber contained 800 μl SMC conditioned medium or serum-free medium with indicated concentrations of recombinant murine CCL2 or CXCL1; serum free medium served as the negative control. After an overnight incubation, non-migrating cells on the upper side of the filters were carefully washed with PBS and removed using a swab. Vascular progenitor cells on the underside of the membrane were fixed with 4% PFA in PBS for 10 mins at room temperature followed by 0.1% crystal violet solution staining for 15 mins at room temperature. Data were expressed as the fold of migrated VPCs compared to the control in 9 fields (Figure 2.3) of each insert (at 20x magnification). For experiments that involved inhibitors (i.e. Cdc42 inhibitor and P38 inhibitor), VPCs were pre-treated with the respective inhibitors for an hour before the cells were transferred to Transwells.

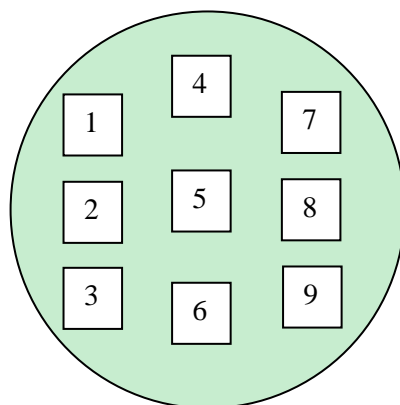


Figure 2.3 Layout of fields in each view of a transwell insert at 20x magnification

2.2.4.2 Scratch wound assay

Vascular progenitor cells were seeded into 12-well tissue culture plates at a density of 5×10^4 cells/well. After 24 hours of growth, when cells had formed ~90% confluent monolayers, a straight scratch was made using a clean 1ml pipette tip across the centre of the well. While scratching across the surface of the well, the long-axial of the tip was kept perpendicular to the

bottom of the well and force applied with the same dynamics and speed. Another straight line was then scratched perpendicular to the first line to create a “+” in each well (same force maintained). Following scratching, photos of the wells were taken under the microscope immediately to record the initial distance of the gap for comparison after migration. The wells were then gently washed with PBS twice, to remove cell debris, prior to treatment with SMC conditioned medium or serum-free medium with or without (positive control) recombinant murine CCL2 or CXCL1. After an overnight incubation, the cells were washed with PBS and fixed with 4% PFA for 10 mins. The migration of vascular progenitor cells was quantified as the mean number of the cells which migrated into the “wound” area (the area was decided by comparison to the initial distance of the gap) in 8 different views (as shown in Figure 2.4) of each well using a phase contrast microscope with a 10x objective.

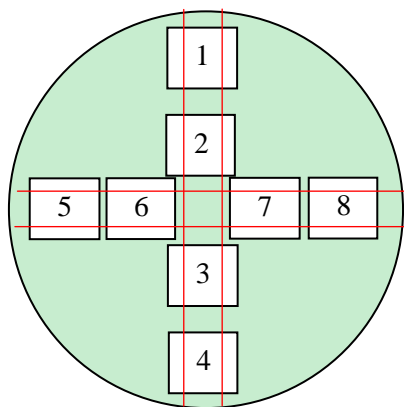


Figure 2.4 Layout of fields in each view of 12-well at 10x magnification

2.2.4.3 Time-lapse microscopy

5×10^3 vascular progenitor cells/well were seeded in 6-well plates. After 4 hours the cells had adhered to the bottom of the plate and were treated with either serum-free medium (negative control) or SMC conditioned media. The 6-well plate was then placed into the microscope chamber, with conditions at 37°C and 5% CO₂, of a fully motorized, multi-field time-lapse microscope (Eclipse TE 2000-E; Nikon) with a charge-coupled device camera (ORCA; Hamamatsu Photonics). Images were acquired from 10 random fields in each well, every 5 minutes for a period of 20 hours, using a 10x objective with bright field settings. Images were acquired using Volocity software (PerkinElmer), and 10 random single cells in each field were

tracked using ImageJ software (National Institute of Health). Analysis of cells speed and persistence of direction during their migration was performed using the Chemotaxis plugin from Integrated BioDiagnostics.

2.2.4.4 CCL2 and CXCL1 gene knockdown in SMCs

Mouse smooth muscle cells were infected with CCL2 siRNA (Santa Cruz Biotechnology, Sc-43914) or CXCL1 siRNA (Ambion 4390771, ID S67079) by electroporation using an Amaxa™ Basic nucleofector™ Kit (Lonza, Cat No: VPI-1004). Control siRNA (FITC Conjugate)-A (Santa Cruz Biotechnology, sc-36869) was used to infect SMCs as a negative control. Each sample required 1×10^6 SMC cells and 300nM of control, CCL2 or CXCL1 siRNA. Cells were harvested by trypsinization and the cell density determined before centrifugation at 100 g for 10 mins. The cell pellet (1×10^6) was then resuspended in 100 μ l of room temperature Nucleofector™ solution containing the indicated siRNA, followed by transfer into a certified cuvette provided by the company. The sample covered the bottom of cuvette without air bubbles. The cuvette was inserted into the Lonza Nucleofector™ device and a specific program for SMCs applied. After the program was completed, 500 μ l of pre-warmed culture medium was added immediately into the cuvette and the mix was then gently transferred into the prepared 6-well plate (each well containing 1ml culture medium was pre-equilibrated in a 37°C, 5% CO₂ incubator). Following incubation at 37°C overnight (approx. 18 hours), the supernatant and SMC cells were harvested separately for analysis of gene ablation using ELISA and qPCR respectively.

2.2.4.5 Lentiviral particle generation

Gene ablation of CCR2 or/and CXCR2 was carried out using short hairpin RNA (shRNA) lentiviral plasmid transfer. The bacterial glycerol stock of CCR2 MISSION shRNA (SHCLNG-NM_009915, TRCN0000001680) and CXCR2 MISSION shRNA (SHCLNG-NM_009909, TRCN0000026605) were purchased from Sigma-Aldrich. The bacterium from each tube was picked up and amplified by incubating at 37°C with shaking at 220RPM overnight, in 20ml of

autoclaved 2.5% LB (Fisher Scientific, LB Broth, Miller) medium supplemented with 100 µg/ml Ampicillin in individual 50 ml conical tubes. The bacterium was then harvested by centrifugation at 4000 RPM for 15 min. Plasmid purification was performed using a QIAprep Spin Miniprep kit (Qiagen, Cat. No. 27016) as described in the instructions of the manufacturer. The concentration of the plasmid was determined by measuring absorbance at 260nm using a Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, UK). The plasmid was stored at -20°C.

For the generation of Lentiviral particles 27 µl of FuGENE® 6 Transfection Reagent (Promega, Cat. No. E2691) was mixed with the 4.5 µg lentiviral plasmids (CCR2 shRNA or CXCR2 shRNA) and packaging plasmids in serum-free DMEM medium. The mixture was incubated at room temperature for 5 minutes before it was added to 60% confluent 293T cells, which were seeded one day prior to transfection in a T75 flask with 5 ml serum-free DMEM medium. After an overnight incubation, the medium was replaced with 1% FBS DMEM medium. The supernatant containing the lentivirus was harvested 48 hours later and filtered through a 0.45 µm PVDF filter (Millex®-HV, SLHV033RS) before storing at -80°C. The transduction unit (TU) was calculated as previously described. (Margariti et al., 2010). A non-targeting shRNA vector (SHC002) was used to generate the control particles.

2.2.4.6 CCR2 and CXCR2 gene knockdown in vascular progenitor cells

To transfect vascular progenitor cells, 2×10^5 VPCs were plated in a T25 flask coated with 0.04% gelatin one day prior to transfection. Upon reaching 30% confluency, the cells were incubated with shCCR2 or/and shCXCR2 lentiviral supernatant in complete culture medium supplemented with 10 µg/ml of Polybrene Infection / Transfection Reagent (Millipore, TR-1003-G) for 24 hours. Subsequently, the medium was replaced with complete culture medium and the cells incubated for another 48 hours. The infected VPCs were further selected in culture medium containing 500ng/ml puromycin for 3-4 days. The gene and protein expression of CCR2 and CXCR2 in VPCs was assessed using real-time RT-PCR and flow cytometry analysis after gene ablation.

Non-targeting shRNA plasmid that was used as negative controls was kindly generated by Dr Mei Mei Wong.

2.2.4.7 BrdU proliferation Assay

Vascular progenitor cells were treated with/without SMC-conditioned medium for 18 hours on a gelatin-coated 96-well culture plate. The VPC proliferation assay was performed using a Cell Proliferation ELISA, BrdU (colourimetric) (Roche). According to the manufacturer's instructions, BrdU labelling solution (final concentration of 10 μ M) was incubated with cells for 2 hours at 37°C after they had been treated. After removing the labelling medium, 200 μ l of FixDenat was added to each well for 30 mins at room temperature. The solution was then removed thoroughly before incubating with BrdU conjugated antibody for another 90 mins. After 3 washes with PBS, 100 μ l of substrate solution was added and incubated at room temperature. Once a change in colour was detected, 25 μ l of 1M H₂SO₄ was added to stop the reaction. The absorbance was measured at 450nm with correction at 690nm within 5mins of adding the stop solution. A mixture of BrdU labelling solution and its antibody in culture medium was used as blank to identify the unspecific binding of BrdU and antibody to the plate. The wells in which cells had not been incubated with BrdU but were incubated with antibody were used as background controls to evaluate the unspecific binding the antibody to VPCs.

2.2.4.8 Rac1/Cdc42 GTPase activation assay

The activation assay of GTP-bound Rac1/Cdc42 was performed using the Rac1/Cdc42 Activation Assay Kit (Upstate, Millipore) according to the protocol provided. VPCs were serum starved overnight upon reaching 90% confluency in 100mm culture dishes. The cells were then stimulated with different treatments at indicated time points before they were rinsed twice with ice-cold TBS and placed on ice. Cells were scraped using a cell scraper in 500 μ l 1x MLB provided in the kit and transferred into a 1.5ml Eppendorf tube. The cell lysate was precleared by adding Glutathione Sepharose 4B beads (GE Healthcare) with gentle rocking for 10mins at 4°C. The agarose beads were then collected, following centrifugation at 14,000g for 5 seconds, before transferring the supernatant into new Eppendorf tubes. Aliquots containing 30 μ g protein were prepared for either total Rac1, Cdc42 (input), GTP γ S (positive) or GDP (negative) control

analysis. For the preparation of GTP γ S or GDP controls, cell lysates were incubated with EDTA (final concentration 10mM) and either GTP γ S (final concentration 100 μ M) or GDP (final concentration 1mM) separately in two tubes at 30°C for 15 mins with agitation. To perform GTP-Rac1/Cdc42 pull-down assay, each 0.5ml cell lysate (including GTP γ S and GDP controls) was mixed with 20 μ g of PAK-PBD agarose beads at 4 °C for 1 hour with gentle rotation. The agarose beads were collected after centrifugation at 4,000g for 3 mins and washed thrice with MLB. After thoroughly removing MLB solution, the beads were resuspended in 25 μ l 2X Laemmli sample buffer with reducing agent and boiled for 5 minutes. Finally, samples were separated by 4–12% Bis-Tris gels, transferred to a nitrocellulose membrane and blotted with Rac1 or Cdc42 antibodies (Upstate, Millipore).

2.2.5 Mouse genotyping and models

2.2.5.1 Isolation of DNA from mouse ear punch

Pieces of ear punch from each mouse were lysed in Eppendorf tubes containing 700 μ l of lysis buffer (Table 2.16) and 350 μ l 10mg/ml Proteinase K, following an overnight incubation in the buffer at 55 °C until the tissues dissolve. Tubes were inverted periodically during the incubation. After the addition of 250 μ l of 6M NaCl, the tubes were placed on a rocker at 40 RPM for 5 minutes before they were centrifuged at maximum speed for 10 minutes. The supernatant was then transferred to a new tube and 500 μ l isopropanol was added, the tubes were then shaken at 40 RPM once more before they were centrifuged at maximum speed for 10 minutes. 500 μ l of 70% ethanol was added to the pellets following the careful removal of the supernatant. After thorough vortexing, the supernatant was removed by centrifugation and the pellets air dried for approximately 30 minutes. Finally, DNA was rehydrated by adding 20 μ l TE buffer (Table 2.17) and incubating at 37 °C for 1 hour. Samples were then stored at 4 °C for up to 1 week before PCR.

Table 2.16 DNA Lysis Buffer

Reaction Component	Volume (ml)	Final concentration
1M Tris-Hcl pH8.0	10	50 mM
0.5M EDTA	40	100 mM
3M NaCl	6.6	99 mM
20%SDS	10	1% SDS
ddH₂O	133.4	-
Total	200	-

Table 2.17 TE Buffer

Reaction Component	Volume (ml)	Final Concentration
1M Tris-HCl pH7.4	1	10 mM
0.5M EDTA	0.2	1 mM
ddH₂O	98.8	-
Total	100	-

Genotyping for CCL2^{+/-} and CCL2^{-/-} mice using standard PCR (Table 2.18) was performed according to a protocol (Table 2.19) provided by The Jackson Laboratory. The following primer sequences were used:

oIMR7415 (CCL2^{-/-} forward) 5'- GCC AGA GGC CAC TTG TGT AG-3',

oIMR9219 (CCL2^{+/+} forward) 5'- TGA CAG TCC CCA GAG TCA CA'-3'

oIMR9220 (common reverse) 5'- TCA TTG GGA TCA TCT TGC TG -3'.

Table 2.18 PCR Reaction Mix

Raction Component	Volume (μ l)	Final Concentration
ddH ₂ O	3.33	-
10 X AB PCR BufferII	1.20	1X
25 mM MgCl ₂	0.96	2mM
2.5 mM dNTP	0.96	0.2 mM
20 μ M oIMR9219	0.60	1 μ M
20 μ M oIMR9220	0.60	1 μ M
20 μ M oIMR7415	0.60	1 μ M
5 mM DNA Loading Dye	1.66	0.69 mM
5 U/ μ l Taq DNA Polymerase	0.09	0.04 U/ μ l
DNA	2.00	-
Total	12	-

Table 2.19 PCR program

	Temp($^{\circ}$ C)	Time(min)	Note
1	94	3	-
2	94	0.5	repeat 2-4 steps for 35 cycles
3	61	1	
4	72	1	
5	72	2	-
6	10	-	hold

2.2.5.2 Creation of bone marrow chimeric mice

Bone marrow transplantation was carried out on both CCL2^{+/+} mice and CCL2^{-/-} mice. Recipient CCL2^{-/-} mice or CCL2^{+/+} mice (6-8 weeks of age) received lethal irradiation (950 rads) by whole body X-Ray. Either CCL2^{+/+} or CCL2^{-/-} donor mice were euthanized before removing femurs and tibias aseptically. Their bones were rinsed with DMEM medium and bone marrow cells were flushed from their marrow cavities using a 27 gauge needle. Preparation of single cell suspensions was performed by passing the marrows through the needle repeatedly, followed by the application of a cell strainer to remove large clumps. Cells were then spun down at 1000RPM for 5 mins and resuspended in DMEM to give a final concentration of 5×10^7 cells/ml. Within 24 hours of receiving irradiation, the recipients were warmed up, until their tail veins could easily be identified, before they were placed in a restraint with their tails sticking out. 200 μ l (1×10^7 cells) of cell suspension was injected into their tail vein with a 30 gauge needle for each mouse. If an injection was missed (control) or failed, irradiated recipients were observed to die after 7-10 days. Femoral artery wire injury was performed on surviving recipients, 3 weeks after bone marrow transplantation.

2.2.5.3 Femoral artery wire injury

Mice were weighed and anaesthetized by intraperitoneal injection of ketamine (75 mg/kg; Ketalar[®], Pfizer) combined with medetomidine hydrochloride (1 mg/kg; Domitor[®], Orion). A pinch test was performed on the tail of mouse to confirm that it was fully anaesthetized. The lubricating ointment was applied to the eyes of mice to prevent corneal desiccation. Hair was removed by application of a depilatory cream from both legs to the lower abdomen. The mouse was then put on an aseptic operation board and covered with an aseptic drape. The depilated area of skin was sterilized with 70% ethanol and dried with sterile cotton swabs just before surgery. An incision into the skin over the femoral artery was made before locating femoral artery by blunt dissection of surrounding tissues. The tissues were moistened periodically using saline for irrigation. The femoral artery was isolated by gently separating the femoral nerve and vein from the femoral artery, in sequence using micro-forceps. The anterior branch of the

femoral artery was looped with a 10-0 silk suture before performing an arteriotomy using a 30 gauge needle at the distal part of the bifurcation. The opening of the arteriotomy was lifted with micro-forceps before a 0.25 mm guide wire (CROSS-IT 100XT, HI-TORQUE) was introduced into the artery until it could be inserted no further. The wire was inserted and retracted 5 times before it was allowed to remain in the femoral artery for 3 mins. After gently and slowly removing the wire, the artery was immediately ligated. The same procedure was applied on the other femoral artery of each mouse. One injured artery was seeded with Sca1⁺ vascular progenitor cells (1x10⁶ cells) in the presence of 25 µl Matrigel[®] Basement Membrane Matrix (Corning), saline was also used as a negative control. The wound was closed after the matrigel had solidified. Anaesthesia was reversed with atipamezole hydrochloride (5 mg/kg; Antisedan, Orion) after surgery. Mice were allowed to recover in a warm container. In all experiments, mice were monitored continuously during recovery and periodically for 2 days.

For siRNA knockdown experiments *in vivo*, either 5 µg CXCL1 or Negative Control siRNA dissolved in 30% pluronic Gel-127 was perivascularly delivered to the femoral arteries immediately after wire injury. After 3 days, GFP-Sca1⁺ vascular progenitor cells (1x10⁶ cells) within 25 µl Matrigel[®] Basement Membrane Matrix were seeded onto the adventitia of both siRNA treated femoral arteries. Arteries were harvested for either RNA extraction or for *en face* staining after 72 hours.

2.2.6 Immunofluorescence and histology

2.2.6.1 Immunofluorescent staining of cells

Cells were seeded at a density of 1x10⁴/well in 500µl culture medium in individual chambers of a 4-well chamber slide coated with 0.04% gelatin. After 24 hours, the culture medium was replaced with serum-free medium. Following an overnight serum starvation, the cells were treated with relevant stimuli (details will be described in corresponding experiments) and then washed twice with warm PBS before staining. First, the cells were fixed with 4% paraformaldehyde (PFA) for 15 mins at room temperature and washed with PBS, before permeabilising with 0.1% Triton X-100 (Sigma-Aldrich) for 5 mins at room temperature. After

washing with PBS 3 times, the cells were blocked with 5% normal donkey serum (Dako) in PBS for 1 hour at room temperature. Incubation of cells with primary antibodies or blocking solution only (negative control) was performed at 4°C overnight. The primary antibodies used and the dilutions in blocking solution are shown in Table 2.1. The next day, cells were washed with PBS 3 times, each time for 5 minutes. Secondary antibodies (Corresponding fluorescent-conjugated IgG antibodies were used as secondary antibodies (Invitrogen)) were applied to cells for 45 mins at 37°C and protected from light. Following three 5 minute washes in the dark, the cells were counterstained with DAPI (1:1000 in PBS) for 3 minutes at room temperature. They were then mounted using fluorescent mounting media (Dako) and images were acquired using either an Olympus IX81 microscope with Volocity software (PerkinElmer) or a Leica SP5 confocal microscope with Leica LAS AF software.

2.2.6.2 FACS analysis

The protein expression of CCR2 or CXCR2 on the membrane of vascular progenitor cells was analysed by flow cytometry. The VPCs from each well (sample/well) of a 6-well plate after different treatments were harvested and transferred into BD falcon tubes (2×10^5 /tube). Samples were pooled together for IgG control staining. Cells in each tube were fixed with 4% paraformaldehyde at room temperature for 15 mins. The cells were then washed with 3 ml PBS and centrifuged at 1500RPM for 5 mins, followed by incubation with either anti-CCR2 antibody (Abcam) or PBS (IgG control tube) on ice for 1 hour. After further washing with PBS, the cells were incubated with either anti-CXCR2 PE-conjugated antibody (R&D) or mouse IgG PE (BD biosciences) (IgG control tube) on ice for 30 mins in the dark. Following washing and centrifugation, the cells were analysed using BD AccurTM C6 Flow Cytometer with a 488nm laser. Data analysis was carried out using FlowJo_V10 (FLOWJO, LLC) software.

2.2.6.3 Harvesting femoral arteries for histology

Euthanasia was performed on each mouse using cervical dislocation. After death, a small incision was made in the right atrium to create an outlet for blood. The mouse was perfused slowly by injecting 20 ml of saline from left ventricle using a syringe with a 25 gauge needle.

The clearing of blood in the liver served as an indicator of a good perfusion. Fixation was then performed by replacing saline with 10ml of 4% formaldehyde. The fixation tremors of the whole body were observed within seconds. An incision into the skin was made over the femoral artery, and the distal end of the injured artery was located by identification of the silk suture for ligation. The artery was excised from surrounding tissues from the distal part, near the suture, to the proximal part, beside the abdominal aorta. The artery was then transferred into 4% formaldehyde solution at 4°C for the further process of histology. For paraffin section preparation, the fixed artery was transferred to 70% ethanol at 4°C 24 hours before embedding in paraffin blocks. For the preparation of frozen sections, dehydration of the fixed arteries was performed in 30% sucrose solution at 4°C overnight followed by embedding them within Bright Cryo-m-Bed (Bright Instruments). The samples were then placed in liquid nitrogen for fast and adequate cooling before being cut into 5 mm thick sections. Paraffin sections were stored at room temperature while frozen sections were stored at -20°C before performing histological and immunofluorescence staining to assess the extent of the injury and neointima formation.

2.2.6.4 Haematoxylin and Eosin (HE) staining of paraffin section

Deparaffinization and rehydration were performed on paraffin sections before H&E staining. The slides were first immersed in xylene for 10mins and this was then repeated in fresh xylene solution. Rehydration was then performed using the following concentrations of ethanol: 100% ethanol for two 5 minute immersions; 70% ethanol for one 5 minute immersion. The slides were washed briefly in tap water before H&E staining. After blotting excess water from the slides with a paper towel, the slides were stained in haematoxylin solution for 5 mins, followed by washing under running tap water for 5 mins. Differentiation of the staining was performed by repeatedly dipping the slides into 1% acid ethanol 5 times. Subsequently, the slides were washed under running tap water for 5-10 mins and it was confirmed that the nuclei had turned blue under the microscope. Next, counterstaining with eosin solution was performed for 5 mins before dehydration. Following brief washing in water, the slides were immersed in first 70% ethanol and then 100% ethanol twice, 2 minutes for each step. The excess ethanol was blotted and dried before the slides were immersed in xylene twice, each time for 5 mins. Finally, the

slides were mounted with xylene based DPX mounting medium (Fisher Scientific) and dried overnight. All steps took place in a flow hood.

2.2.6.5 Immunofluorescent staining of frozen sections

The slides containing frozen sections were thawed at room temperature for 30 minutes before staining. Rehydration of frozen sections was performed in PBS for 5 mins to remove tissue freezing matrix. To fix the tissue sections, the slides were immersed in pre-cooled acetone (-20 °C) for 10 min at room temperature. After briefly washing twice with PBS, the tissue sections were blocked with 5% normal donkey serum (Dako) in PBS for 1 hour at room temperature. Following removal of the block buffer, incubation of cells with primary antibodies or blocking buffer (negative control) was performed at 4°C overnight in a humidified chamber. The slides were rinsed thrice in PBS, 5 mins each time before the application of appropriate secondary antibodies for 45 mins at 37°C protected from light. Following repeated 5mins washes, tissue sections were counterstained with DAPI for 5 mins at room temperature. The slides were mounted with fluorescent mounting media (Dako) and images were acquired using an Olympus IX81 microscope with Volocity software (PerkinElmer) or an SP5 confocal microscope with Leica LAS AF software.

2.2.6.6 *En face* staining

After fixation with 4% paraformaldehyde overnight, the femoral arteries were permeabilized and blocked with 0.5% Triton X-100 and 5% donkey serum together for 2 hours. Incubation of vessels with primary antibodies was performed at 4°C overnight, followed by incubation with corresponding fluorescent-conjugated secondary antibodies (Invitrogen) or IgGs as negative controls for 2 hours at room temperature. Then nuclei were counterstained with DAPI for 5 mins at room temperature before dissection of the arteries. One end of the artery was pinned on rubber and the other end was opened, allowing micro-scissors to be inserted into the lumen of the artery. The artery was split continuously from one end to the other end, avoiding injury in the inner layer of the vessel. Subsequently, the opened artery was cut transversely into several segments and these cuts allowed the unfolded artery segments to be flattened. The segments were mounted on poly lysine-D-coated slides (Thermo Scientific) with the intima layer facing

the 1.5 cover glass. Images were taken using a Leica TCS SP5 confocal microscope and assessed using LAS AF Lite software.

2.3 Statistical analysis

Data for this study are presented as the mean \pm standard error of the mean (S.E.M.) of at least three separate experiments. The analysis was performed using GraphPad Prism V.6 (GraphPad Software, San Diego CA) using analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparison test. Significance was considered when $p < 0.05$.

Chapter 3

Results

3.1 Characterization of vascular progenitor cells (VPCs)

Previous work from our group has described a population of progenitor cells identified within pathological vessels in a mouse vein graft model (C. W. Chen et al., 2013; Margariti et al., 2012). This earlier work showed that 2 weeks after implantation of vein grafts, a large heterogeneous population of cells migrated from the adventitia of vessels and contributed to neointima formation (Hu et al., 2004). To further characterise the migrated progenitor cells in this heterogeneous population in the current study, single cell clones were isolated and cultured in stem cell medium. 2 days after serial dilution of VPCs to obtain individual cells, 4 single cell clones showing different morphologies were seen (Figure 3.1).

Clones were characterised using conventional PCR and immunofluorescent staining. Clones were evaluated for their gene expression of a panel of lineage markers for a range of cell types such as hematopoietic stem cells (CD133), mesenchymal stem cells (CD105, CD90), monocyte/macrophages (CD14), progenitor cells (Sca-1, c-kit, CD34), endothelial cells (EC) (eNOS, vWF, Flk-1, CD31 and CD144) and smooth muscle cells (SMC) (SM22, Calponin, SM α -actin) using conventional PCR. The clones expressed a high level of progenitor cell marker Sca-1 and a panel of SMC markers (Calponin, SM22 α and α -SMA), very low levels of CD34, CD105, CD90, CD14 but no EC markers (Figure 3.2).

The clones were further characterised by staining for cell surface markers, using fluorescent antibodies, and were consistently found to show high levels of Sca-1 expression, but no expression of endothelial or smooth muscle cell markers (Figure 3.3). Although the expression of SMC markers was present at the gene level, the cells did not show positive staining for either calponin or SM-MHCII at the protein level. While the clones had different morphologies (Figure 3.1) they expressed a similar panel of cell markers at the gene (Figure 3.2) and protein levels (Figure 3.3).

In order to purify the Sca-1⁺ progenitor cell population from the heterogeneous population derived from vein graft outgrowth, anti-Sca-1 immunomagnetic microbeads were used. Approximately 15% of total cells collected were identified as Sca-1⁺ progenitor cells after

sorting. The purity of cells sorted for Sca-1 via this method was shown to be 98% in a previous study (Wong et al., 2013). Clones and Sca-1⁺ progenitor cells were further characterised using a qPCR array, showing gene expression of chemokines and chemokine receptors. Data are shown in Figure 3.4 and Table 3.1, Table 3.2, Table 3.3 indicates the presence of a variety of chemokine receptors in VPCs and importantly, CCR2 and CXCR2 (receptors of CCL2 and CXCL1, respectively).

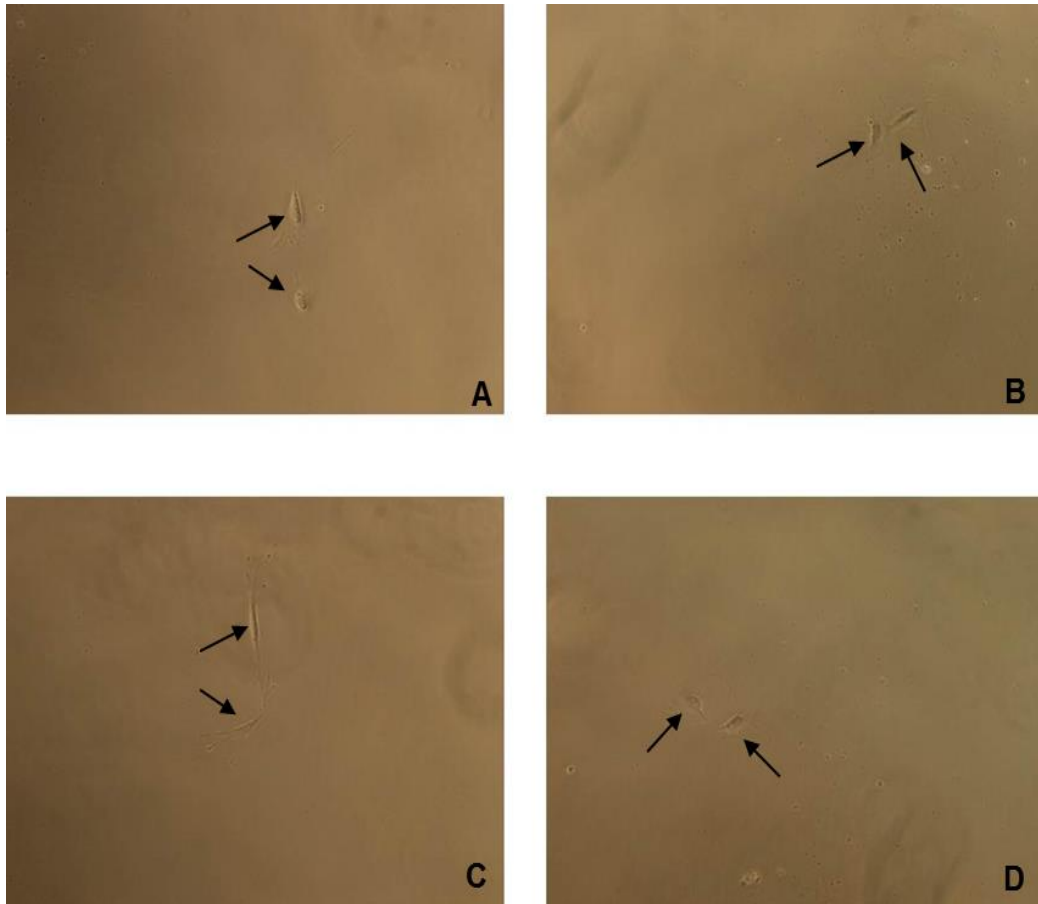


Figure 3.1 Morphology of different single cell clones. Cells were cloned by serial dilution and single cell clones were picked 2 days after seeding cells in 48 well plates. Approximately 3 to 5% of cells formed clones that could be gradually expanded. Images A, B, C, and D represent four different single cell clones, two cells (arrows) from each clone are shown in the represented images. Images were taken using a 10× objective.

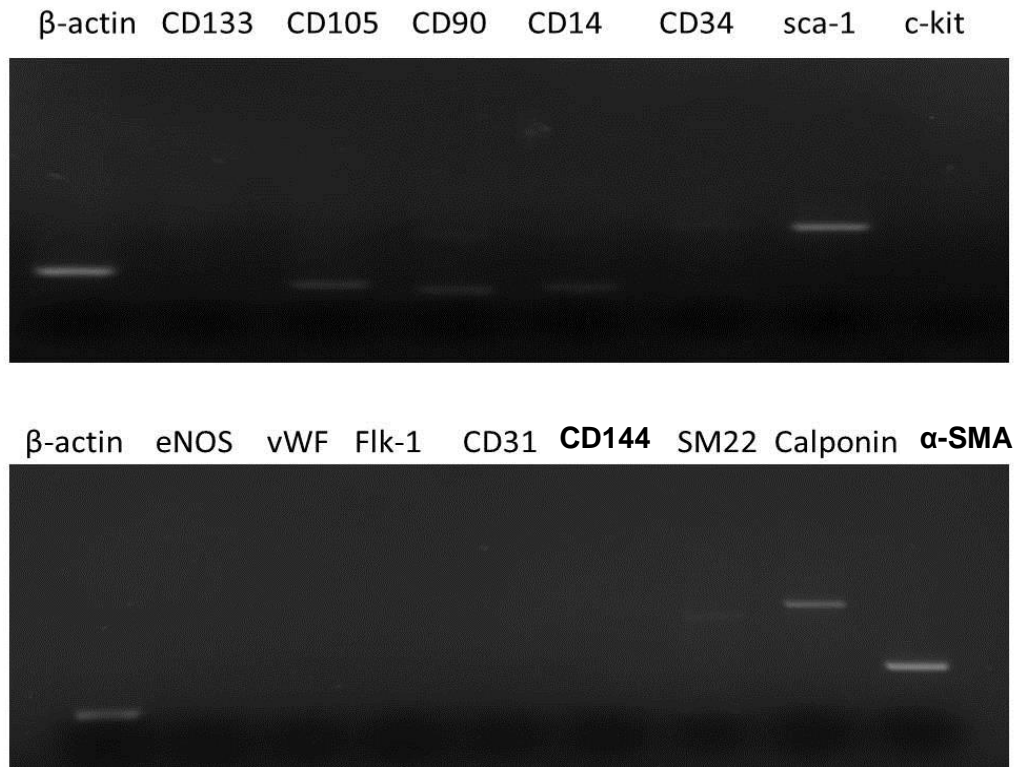


Figure 3.2 Characterisation of vascular progenitor cells at the gene level. Representative images of conventional PCR show that clones express high levels of Sca-1 (progenitor marker), SM22, calponin, α -SMA (SMC markers), and also lower levels of CD90, CD105 (mesenchymal stem cell markers) and CD14 (monocyte/macrophage marker), but no EC markers. The result shown is representative of different clones from the three independent experiments performed.

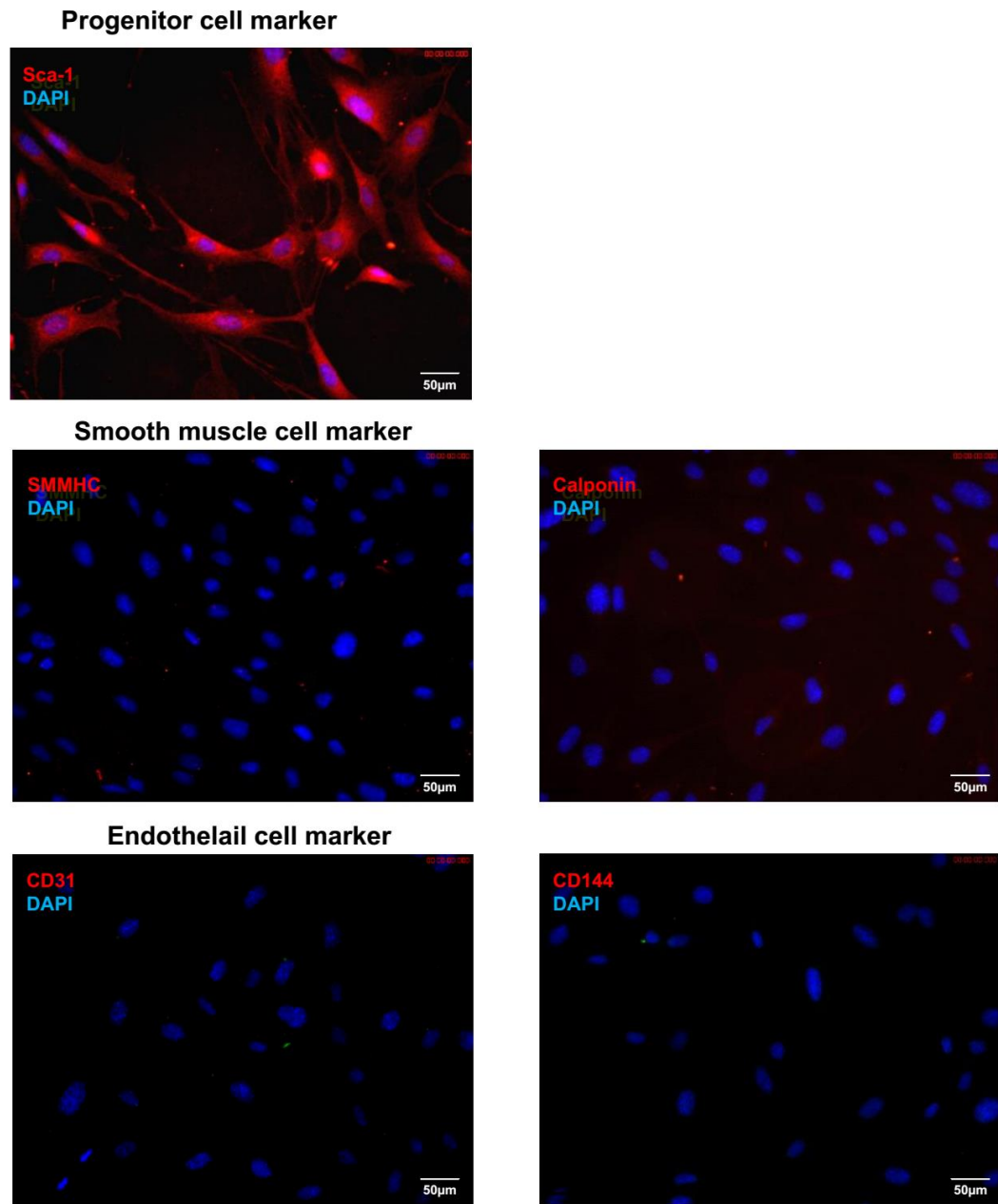


Figure 3.3 Characterization of vascular progenitor cells at the protein level. Clone colonies were characterised using immunofluorescence staining for progenitor cell marker (Sca-1), SMC markers (SM-MHCII, Calponin) or endothelial cell markers (CD31, CD144). The nuclei were counterstained with DAPI. Images of indirect immunofluorescent staining are representative of at least 3 experiments.

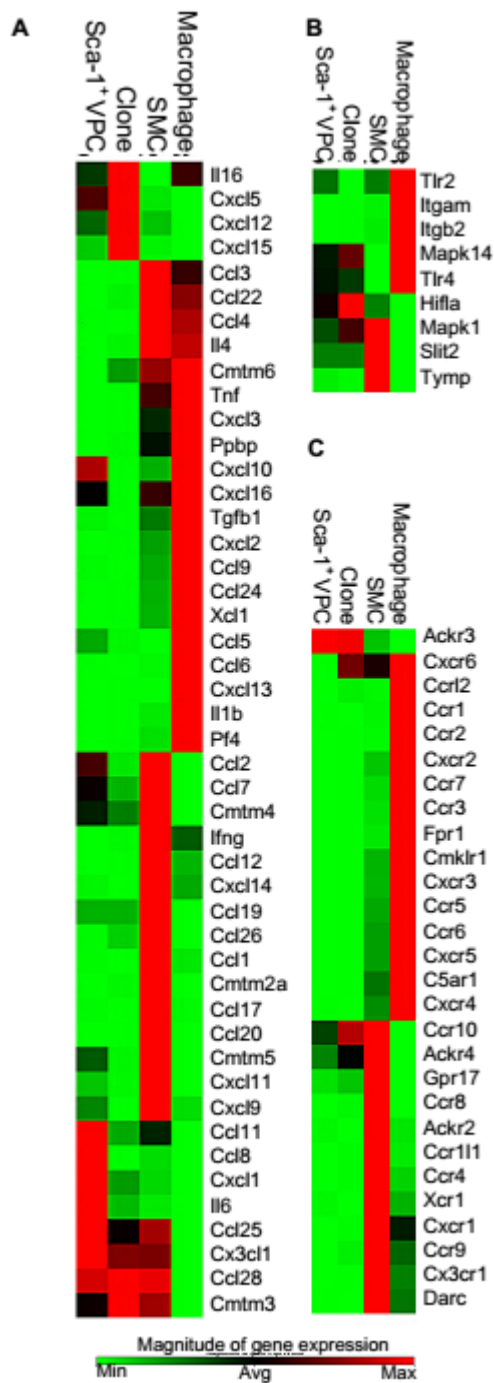


Figure 3.4 The gene expression of chemokines and chemokines receptors in different cell lines. The cluster grams show the expression of chemokines (A), and other chemotactic cytokines (B) on macrophages, smooth muscle cells, clones and Sca-1⁺ VPCs individually. The magnitude of gene expression is indicated on a scale from green to red.

Table 3.1 Gene expression of chemokines and chemotactic cytokines in different cell lines ($2^{-\text{Avg}\Delta\text{Ct}}$)($\times 10^{-4}$)

Refseq	Symbol	Description	M ϕ	SMC	clone	Sca-1 ⁺ VPC
NM_011329	Ccl1	Chemokine (C-C motif) ligand 1	1.06	7.16	0.8	0.83
NM_011330	Ccl11	Chemokine (C-C motif) ligand 11	0.68	7.16	3.14	15.81
NM_011331	Ccl12	Chemokine (C-C motif) ligand 12	78.92	560.56	0.46	0.42
NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	3.12	175.55	2.05	2.95
NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	0.65	7.16	1.6	1.6
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	1225.41	11916.8	1677.83	7981.14
NM_016960	Ccl20	Chemokine (C-C motif) ligand 20	0.41	309.91	0.67	1.21
NM_009137	Ccl22	Chemokine (C-C motif) ligand 22	5.61	7.16	0.58	0.45
NM_019577	Ccl24	Chemokine (C-C motif) ligand 24	46.28	7.16	0.61	0.47
NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	28.69	100.48	73.64	116.09
NM_0010134 12	Ccl26	Chemokine (C-C motif) ligand 26	0.58	7.16	1.15	0.62
NM_020279	Ccl28	Chemokine (C-C motif) ligand 28	0.57	7.16	7.42	6.88
NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	363.48	600.79	0.5	0.42
NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	161.16	191.44	0.79	1.17
NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	1484.45	26.1	38.74	263.02
NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	64591.01	617.68	0.89	6.35
NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	527.26	25105.4	4055.64	13329.9
NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	7.62	34.43	7.98	417.52

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_011338	Ccl9	Chemokine (C-C motif) ligand 9	937.31	156.03	3.76	9.6
NM_027022	Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A	0.41	7.16	0.46	0.42
NM_024217	Cmtm3	CKLF-like MARVEL transmembrane domain containing 3	1143.35	1464.01	1536.8	1350.28
NM_153582	Cmtm4	CKLF-like MARVEL transmembrane domain containing 4	10.87	193.44	56.33	91.29
NM_026066	Cmtm5	CKLF-like MARVEL transmembrane domain containing 5	0.41	7.26	0.49	2.62
NM_026036	Cmtm6	CKLF-like MARVEL transmembrane domain containing 6	185.56	158.21	78.02	52.8
NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	0.55	82.47	84.59	111.61
NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	208.76	5598.06	965.89	4179.3
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	111.87	24.1	8.59	95.39
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	0.41	7.16	0.46	1.13
NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	0.85	1222.58	10752.5	3211.53

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13	9062.62	7.16	0.46	0.42
NM_019568	Cxcl14	Chemokine (C-X-C motif) ligand 14	42.79	233.25	5.32	8.33
NM_011339	Cxcl15	Chemokine (C-X-C motif) ligand 15	0.55	9.82	450.6	44.19
NM_023158	Cxcl16	Chemokine (C-X-C motif) ligand 16	322.33	264.24	175.14	249.41
NM_009140	Cxcl2	Chemokine (C-X-C motif) ligand 2	720.26	133.04	3.83	3.22
NM_203320	Cxcl3	Chemokine (C-X-C motif) ligand 3	69.67	29.56	0.75	0.54
NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	5.18	1351.84	34058.2	22058.4 8
NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	0.94	8.4	0.46	2.33
NM_008337	Ifng	Interferon gamma	2.57	7.16	0.46	0.42
NM_010551	Il16	Interleukin 16	145.25	7.57	231.64	95.17
NM_008361	Il1b	Interleukin 1 beta	882.66	34.79	1.02	0.74
NM_021283	Il4	Interleukin 4	6.4	7.16	0.63	0.54
NM_031168	Il6	Interleukin 6	109.32	183	366.85	2128.63
NM_019932	Pf4	Platelet factor 4	2620.66	149.16	2.99	1.26
NM_023785	Ppbbp	Pro-platelet basic protein	12.26	7.16	2.72	2.65
NM_011577	Tgfb1	Transforming growth factor, beta 1	884.7	386.87	210.7	221.69
NM_013693	Tnf	Tumour necrosis factor	66.06	42.1	0.46	0.42
NM_008510	Xcl1	Chemokine (C motif) ligand 1	47.91	7.16	0.46	0.42

Table 3.2 Gene expression of other chemotactic proteins in different cell lines

$$(2^{-\text{Avg}\Delta\text{Ct}})(\times 10^{-4})$$

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_010431	Hif1a	Hypoxia inducible factor 1, alpha subunit	485.18	1209.93	3514.36	2109.05
NM_008401	Itgam	Integrin alpha M	4286.88	89	0.46	0.42
NM_008404	Itgb2	Integrin beta 2	6695.82	226.87	17.62	15.13
NM_011949	Mapk1	Mitogen-activated protein kinase 1	322.33	910.62	694.12	526.05
NM_011951	Mapk14	Mitogen-activated protein kinase 14	230.04	159.87	208.76	191.66
NM_178804	Slit2	Slit homolog 2 (Drosophila)	0.47	1105.68	276.1	276.74
NM_011905	Tlr2	Toll-like receptor 2	131.82	59.33	34.59	61.21
NM_021297	Tlr4	Toll-like receptor 4	157.12	50.76	91.92	100.13
NM_138302	Tymp	Thymidine phosphorylase	0.52	7.16	0.55	0.73

**Table 3.3 Gene expression of chemokine receptors and chemotactic cytokine receptors
in different cell lines ($2^{-\text{Avg}\Delta\text{Ct}}$)($\times 10^{-4}$)**

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_007577	C5ar1	Complement component 5a receptor 1	437.27	118.66	0.46	0.42
NM_021609	Ackr2	Chemokine binding protein 2	0.89	7.16	0.59	0.65
NM_009912	Ccr1	Chemokine (C-C motif) receptor 1	1836.04	30.5	2.13	3.99
NM_007721	Ccr10	Chemokine (C-C motif) receptor 10	0.72	7.16	6.23	3.11
NM_007718	Ccr11	Chemokine (C-C motif) receptor 1-like 1	0.64	7.16	0.46	0.42
NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	892.92	7.29	0.51	0.42
NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	854.56	45.75	0.46	0.42
NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	0.96	7.16	0.46	0.42
NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	756.07	126.74	0.47	0.42
NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	36.99	7.16	0.48	0.42
NM_007719	Ccr7	Chemokine (C-C motif) receptor 7	99.44	7.16	0.78	0.45
NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	0.41	7.16	0.46	0.42

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_009913	Ccr9	Chemokine (C-C motif) receptor 9	2.76	7.16	1.03	0.88
NM_145700	Ackr4	Chemokine (C-C motif) receptor-like 1	0.41	7.16	3.72	2.02
NM_017466	Ccr12	Chemokine (C-C motif) receptor-like 2	224.78	7.16	10.26	4.99
NM_008153	Cmklr1	Chemokine-like receptor 1	203.52	29.67	0.72	0.42
NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1	20.05	80.77	0.46	0.42
NM_178241	Cxcr1	Chemokine (C-X-C motif) receptor 1	3.42	7.16	0.46	0.42
NM_009909	Cxcr2	Chemokine (C-X-C motif) receptor 2	63.52	7.16	0.46	0.42
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	48.36	7.16	0.46	0.42
NM_009911	Cxcr4	Chemokine (C-X-C motif) receptor 4	283.86	63.59	0.46	0.59
NM_007551	Cxcr5	Chemokine (C-X-C motif) receptor 5	36.99	7.16	0.46	0.42
NM_030712	Cxcr6	Chemokine (C-X-C motif) receptor 6	11.95	7.16	9.02	1.31
NM_007722	Ackr3	Chemokine (C-X-C motif) receptor 7	60.23	145.08	779.13	795.5
NM_010045	Darc	Duffy blood group, chemokine receptor	2.85	9.42	0.48	0.48

Refseq	Symbol	Description	Mφ	SMC	clone	Sca-1 ⁺ VPC
NM_013521	Fpr1	Formyl peptide receptor 1	806.6	33.72	1.16	0.91
NM_001025381	Gpr17	G protein-coupled receptor 17	0.52	7.16	1.24	0.87
NM_011798	Xcr1	Chemokine (C motif) receptor 1	1.42	7.16	0.46	0.54

3.2 SMC-conditioned medium induced VPC migration

To investigate whether SMCs affect the migratory ability of VPCs, a transwell migration assay was performed. Increasing numbers of SMCs were seeded on the lower chamber while a fixed number of VPCs were seeded on the 8.0 μm pore membrane in the upper chamber. Using an increasing ratio of VPCs to SMCs (from 1:0 to 1:5), and allowing an overnight incubation, we found that the migration of VPCs increased as the number of SMCs increased and that this reached its peak when the ratio was 1:2 (Figure 3.5). Next, the SMC-conditioned medium was collected from SMCs cultured overnight in serum-free medium and used for migration assays. The addition of conditioned medium resulted in consistent VPC migration in both transwell and wound healing assays. VPCs treated with conditioned medium demonstrated a 3 to 5 fold increase in migration compared to the serum free medium control (Figure 3.6).

Time-lapse microscopy was used to track the speed and the persistence of single progenitor cells during their migration when treated overnight. Intriguingly, the persistence, which is the capacity of cells to maintain the direction of migration towards targets, was increased in response to SMC conditioned medium compared to the control (Figure 3.7). Subsequent BrdU cell proliferation assays confirmed that the increased migration was not due to VPC proliferation (Figure 3.8 A). Further qPCR analysis revealed that the mRNA levels of SMC markers (calponin and α -SMA) and endothelial marker (CD31) in VPCs did not significantly change (Figure 3.8 B, C, D). VPCs also maintained progenitor characteristics, as Sca-1 expression did not significantly change compared to the control (Figure 3.8 E). Additionally, immunofluorescence staining for paxillin, vinculin and phosphorylated FAK showed that relocation of cytoskeleton-related proteins was increased (Figure 3.9). Taken together, the results support the notion that both co-culture with SMC and SMC-derived conditioned medium can induce VPC migration.

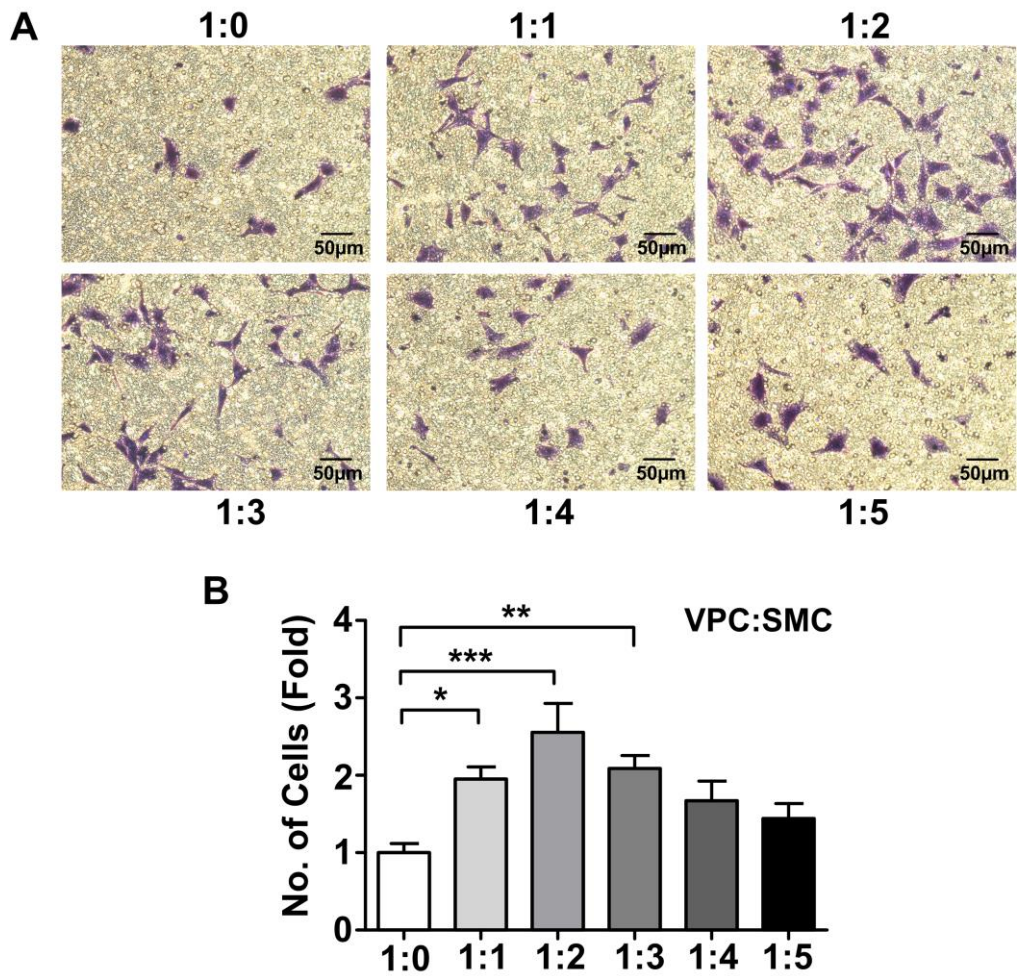


Figure 3.5 SMCs induce vascular progenitor cell migration. A, B. Chemotaxis of vascular progenitor cells (5×10^4 cells/well) toward an increasing number of SMCs (the ratios of VPCs to SMCs were shown in the figure) in $8.0 \mu\text{m}$ transwells after 18 hours was documented after 0.1% crystal violet staining. Serum-free culture media was used as a control for all migration experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with serum free control.

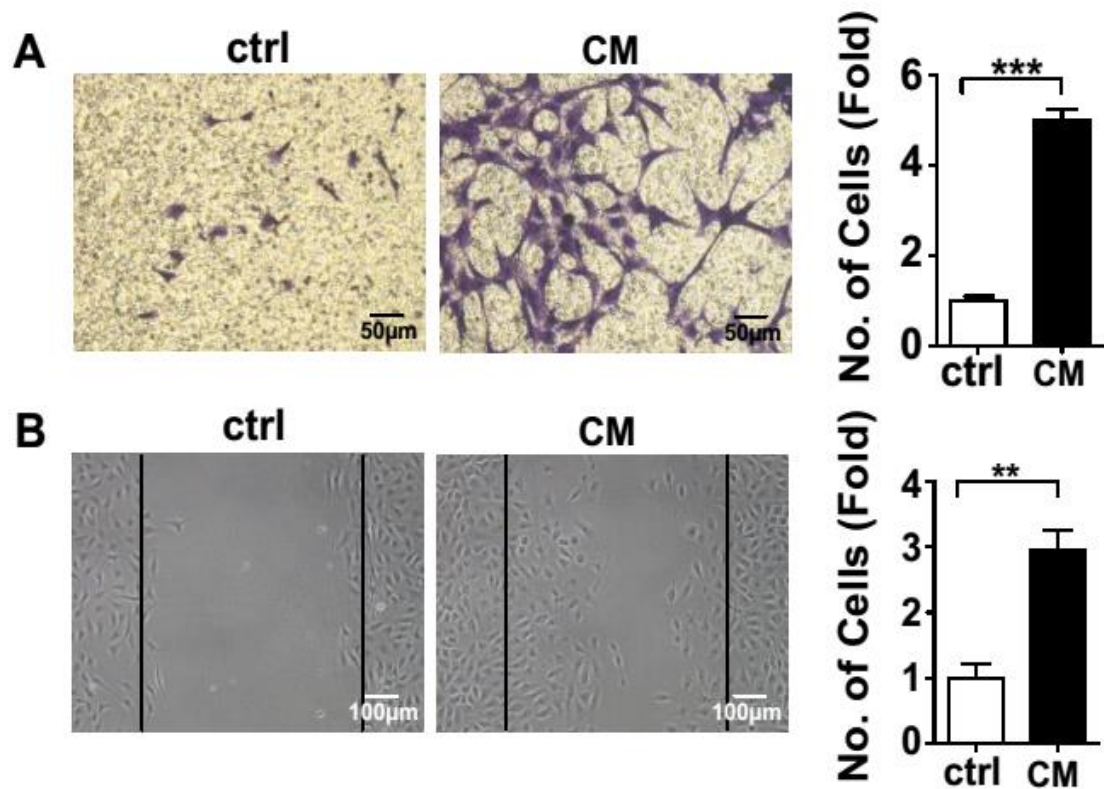


Figure 3.6 SMC conditioned medium induces vascular progenitor cell migration.

Migration assays using transwell (A) and wound healing (B) show a significant increase in VPC migration towards SMC conditioned medium (CM). Representative images were taken from transwell and wound healing assays with a 20× objective. Chemotaxis index was defined by counting 10 fields of view and is presented as fold increase compared to the control. Graphs are shown as mean ± SEM of at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$. Serum-free culture medium served as the control.

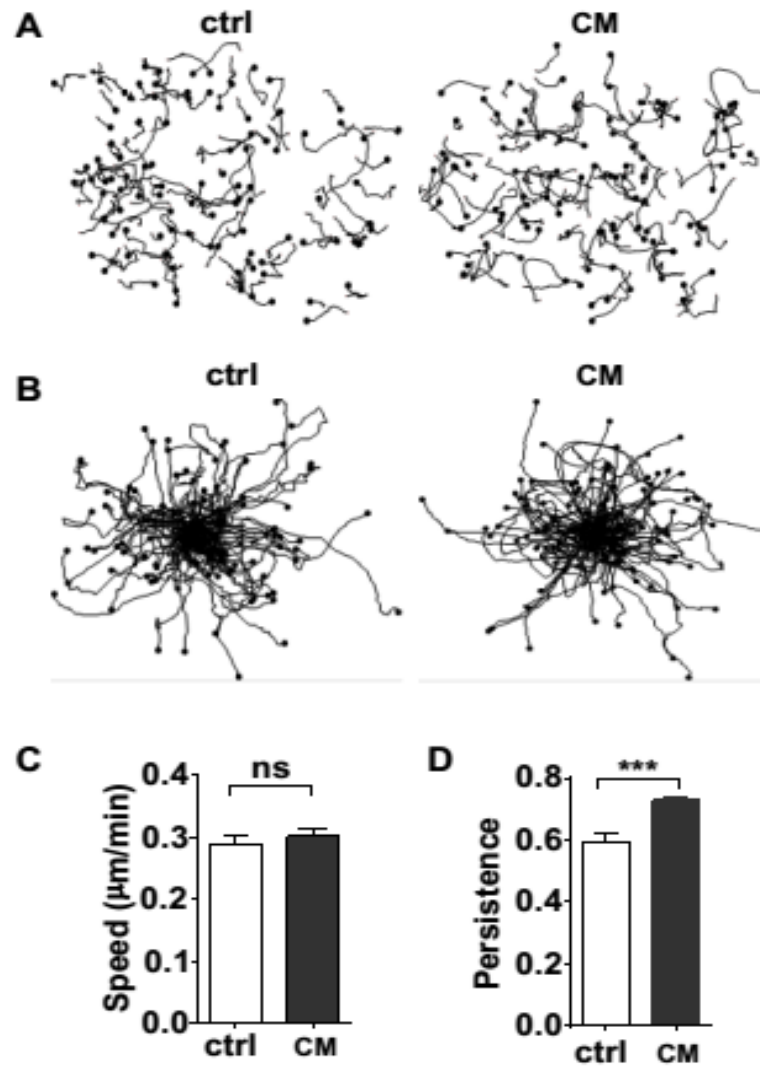


Figure 3.7 SMC conditioned medium increases vascular progenitor cell persistence during its migration but makes no difference to migration speed. Vascular progenitor cells were incubated with SMC conditioned medium or control medium for 18 hours. Time-lapse microscopy was performed to observe the movement of single cells. Trajectory plots of migrating VPCs are shown per cell in (A) and centred in (B), 100 tracked single cells in each group are displayed. Quantification analysis of the speed (C) and persistence (the capacity of cells to maintain their migration direction from the start point to the end point) (D) of VPCs was performed in 3 independent experiments. All graphs are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, $P > 0.05$. ctrl, control. CM, SMC conditioned medium.

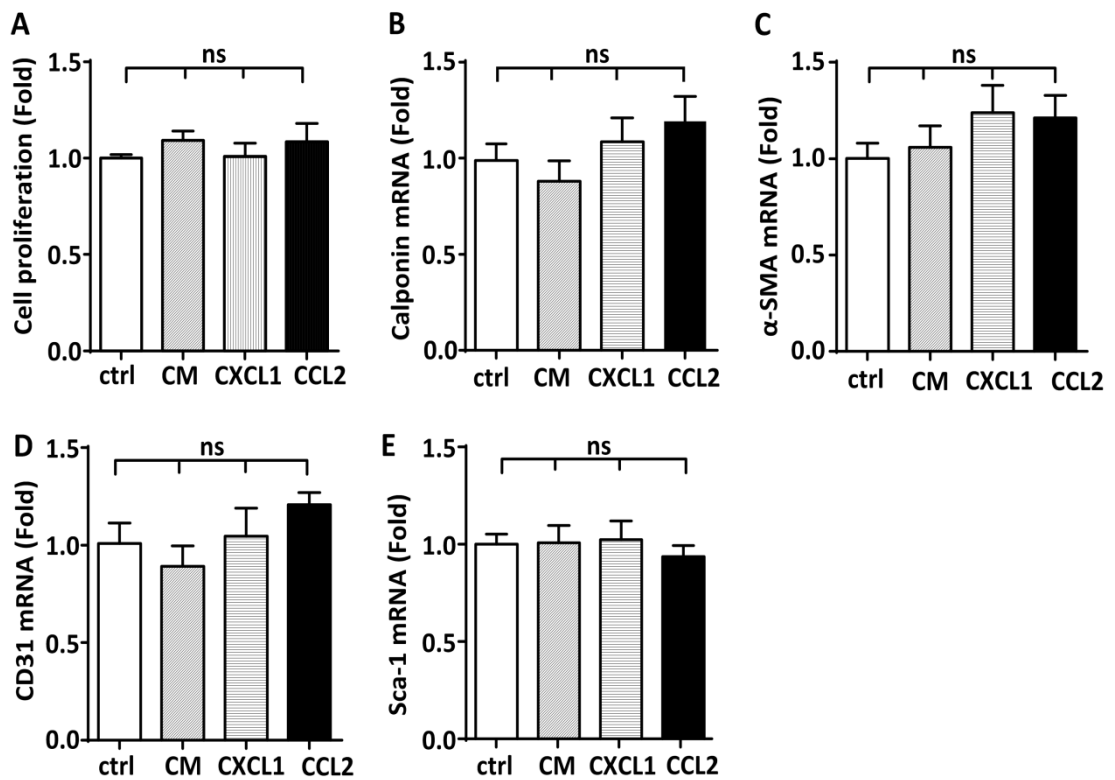


Figure 3.8 SMC conditioned medium has no effect on VPC proliferation and differentiation. A. Evaluation of vascular progenitor cell proliferation after 18 hours in response to SMC conditioned medium, CXCL1 or CCL2 treatments in comparison to an untreated control was by BrdU incorporation assay. B, C, D, E. Total RNA from vascular progenitor cells was harvested and subjected to qPCR for analysis of expression of SMC markers (α -SMA, SM-MHCII), an endothelial cell marker (CD31) and a progenitor cell marker (Sca-1). All graphs are shown as mean \pm SEM of n=3. ns, P>0.05. ctrl, control. CM, SMC conditioned medium.

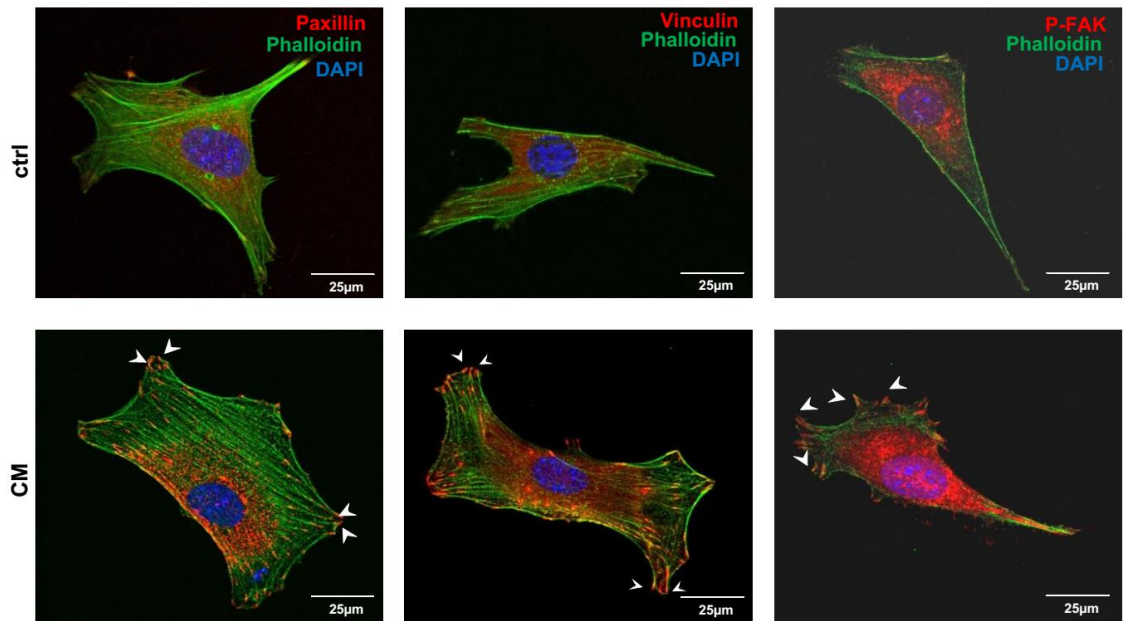


Figure 3.9 SMC conditioned medium increases paxillin, vinculin and phosphorylated FAK expression. Vascular progenitor cells were treated with SMC conditioned medium for 5 minutes before immunofluorescence staining for paxillin, vinculin and phosphorylated FAK. ctrl, control. CM, SMC conditioned medium. p-FAK, phosphorylated FAK. White arrows indicate paxillin, vinculin, FAK respectively. n=3.

3.3 SMC-released CCL2 and CXCL1 induced VPC migration

To further investigate which mediators released by SMCs that were responsible for inducing VPC migration, we performed a chemokine multi-ELISA array. This revealed that CXCL1 and CCL2 levels were markedly increased in the SMC-conditioned medium when compared to serum-free medium (Figure 3.10 A). Further experiments using murine CCL2 or CXCL1 Quantikine kits confirmed that the amounts of CCL2 and CXCL1 in the conditioned medium were 4.63 ± 1.29 ng/ml and CXCL1 3.08 ± 0.99 ng/ml, respectively (Figure 3.10 B, C). While the level of CCL5 was also up-regulated in comparison to the control, subsequent transwell migration assays found that it did not affect VPC migration (Figure 3.13). To confirm the roles of CCL2 and CXCL1 in mediating VPC migration, we performed transwell and wound healing assays using exogenous mouse recombinant CCL2 and CXCL1 proteins. VPC migration was enhanced in response to CCL2 and CXCL1 treatment and peaked at 5 ng/ml (a 6 fold increase in CCL2 (Figure 3.11 A) and a 2 fold increase in CXCL1 (Figure 3.12 A) treated cells). These were similar concentrations of CCL2 and CXCL1 to those measured in the SMC-derived conditioned medium. That a 5ng/ml CCL2 (Figure 3.11 B) or CXCL1 (Figure 3.12 B) treatment enhanced VPC migration was confirmed by wound healing assay. Silencing of CCL2 or CXCL1 in the SMCs using siRNAs resulted in significant down-regulation of CCL2 and CXCL1 at both the mRNA and protein levels (Figure 3.14). Importantly, CCL2 or CXCL1 depleted SMCs failed to stimulate VPC migration in transwell assays (Figure 3.15). To further investigate the relationship between CCL2 and CXCL1 in SMC conditioned medium induced VPC migration, CCL2 and/or CXCL1 concentrations were depleted using their corresponding neutralising antibodies. The antibodies were found to be effective and selective as they significantly reduced the availability of their target chemokine, while they had no effect on the other chemokine studied. Depletion of either CCL2 or CXCL1 did not result in a compensatory release of the other chemokine and there appeared to be no interactions between the two antibodies (Figure 3.16). Having confirmed the effectiveness of the neutralising antibodies they were used to study the role of CCL2 and CXCL1 in VPC migration. Depletion of either CCL2

or CXCL1 in SMC conditioned medium inhibited migration of VPCs by nearly 50%. However, simultaneous depletion of CCL2 and CXCL1 in SMC conditioned medium did not further reduce this migration level (Figure 3.17). Finally, incubation with various concentrations of CCL2 or CXCL1 did not stimulate VPCs autocrine production of the other chemokine (Figure 3.18). Therefore, these results suggest that CCL2 and CXCL1 derived from SMCs are responsible for SMC-induced VPC migration. Furthermore, their interaction is neither cumulative nor redundant but both are required for migration to be induced.

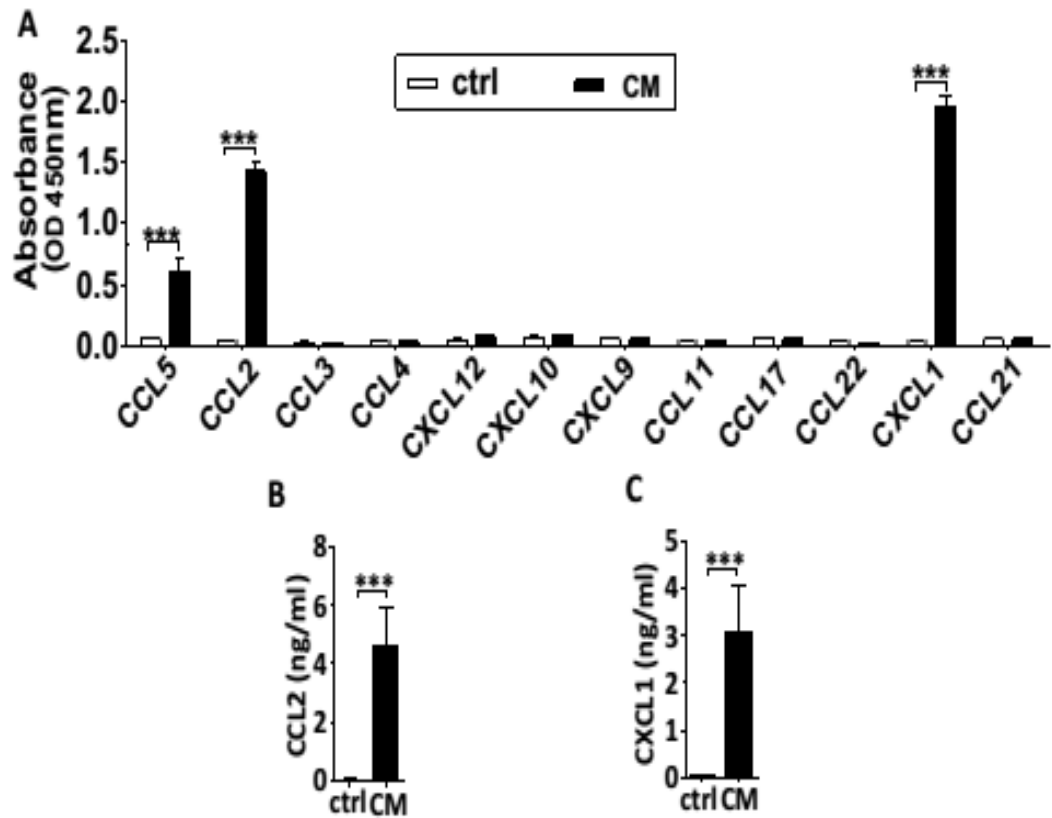


Figure 3.10 Chemokine profiling in SMC conditioned medium. The identification of chemokines in SMC conditioned medium using a Chemokine Multiarray ELISA kit. The graph shows the absorbance at 450nm of each chemokine, with the serum-free culture medium as a control. B, C. This ELISA is not quantitative, but merely identifies the presence of the chemokine. The concentrations of CCL2 and CXCL1 in SMC conditioned medium were precisely quantified using the murine CCL2 or CXCL1 Quantikine ELISA kit. The quantification was from at least three independent experiments. *** $p < 0.001$

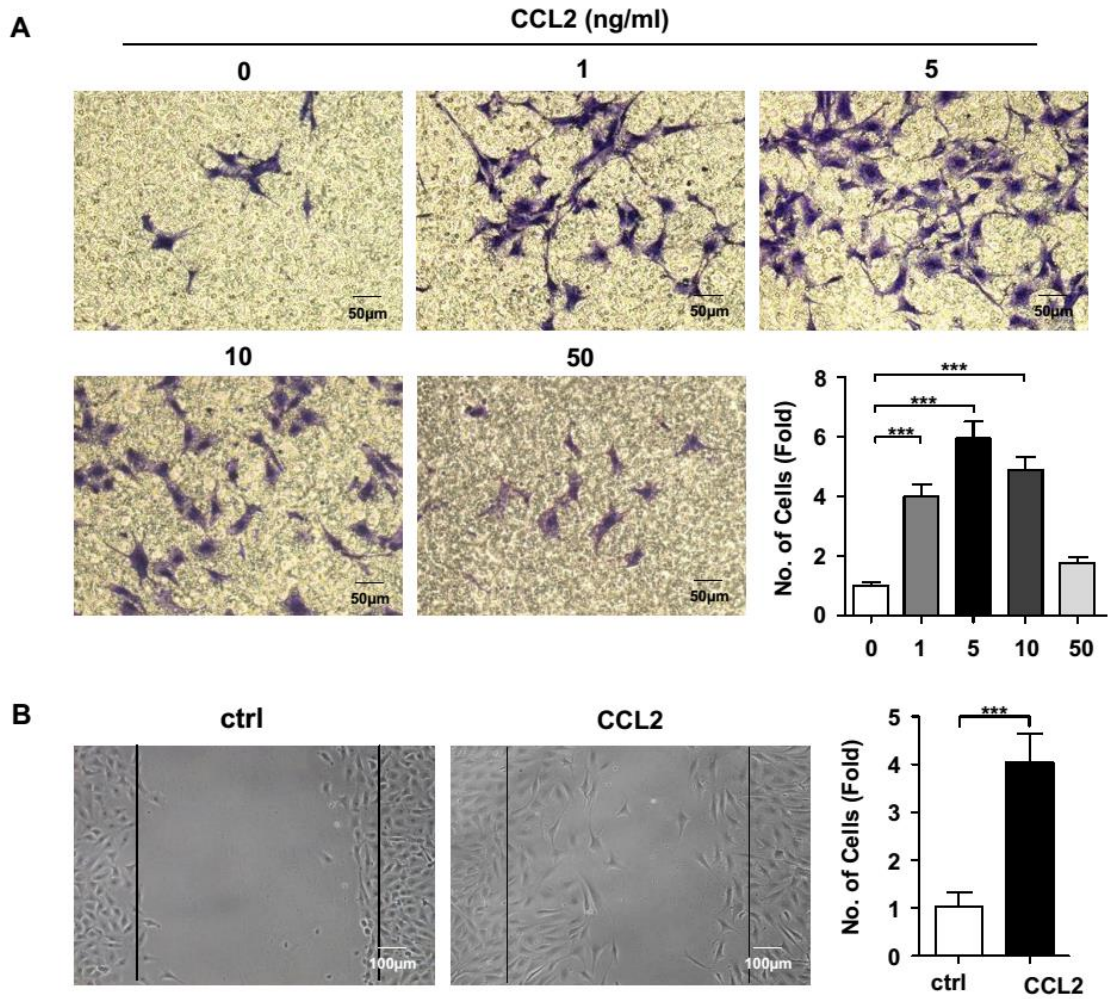


Figure 3.11 CCL2 induced vascular progenitor cell migration. **A.** Changes in vascular progenitor cell migration in response to a gradient of CCL2 in serum-free culture medium were evaluated using a transwell assay. **B.** The wound healing assay was performed on vascular progenitor cells with or without treatment with CCL2 (5ng/ml). Graphs show the fold increase with untreated cells as the controls. *** $p < 0.001$

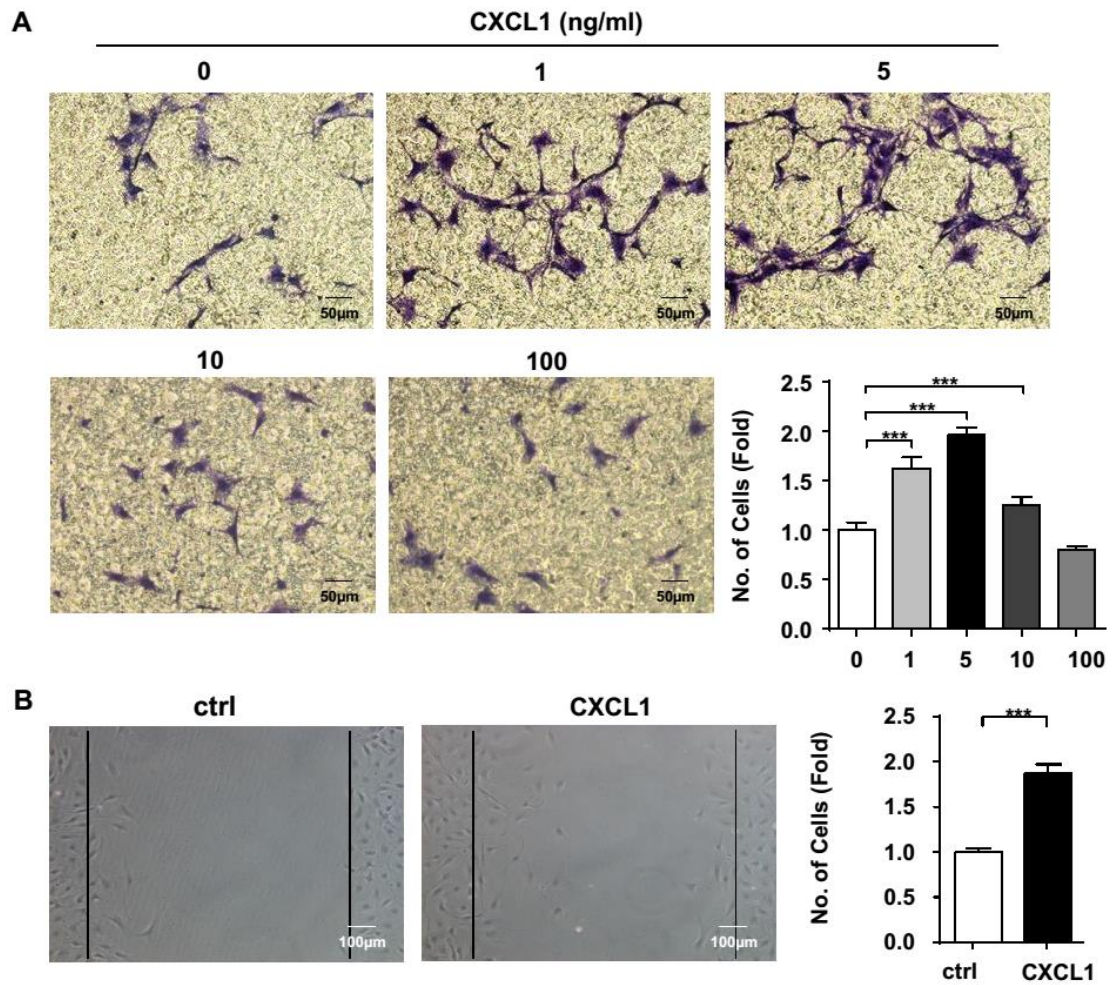


Figure 3.12 CXCL1 induced vascular progenitor cell migration. **A.** Changes in vascular progenitor cell migration in response to a gradient of CXCL1 in serum-free culture medium were evaluated using a transwell assay. **B.** The wound healing assay was performed on vascular progenitor cells with or without treatment with CXCL1 (5ng/ml). Graphs show the fold increase between treated cells and untreated cell controls. *** $p < 0.001$

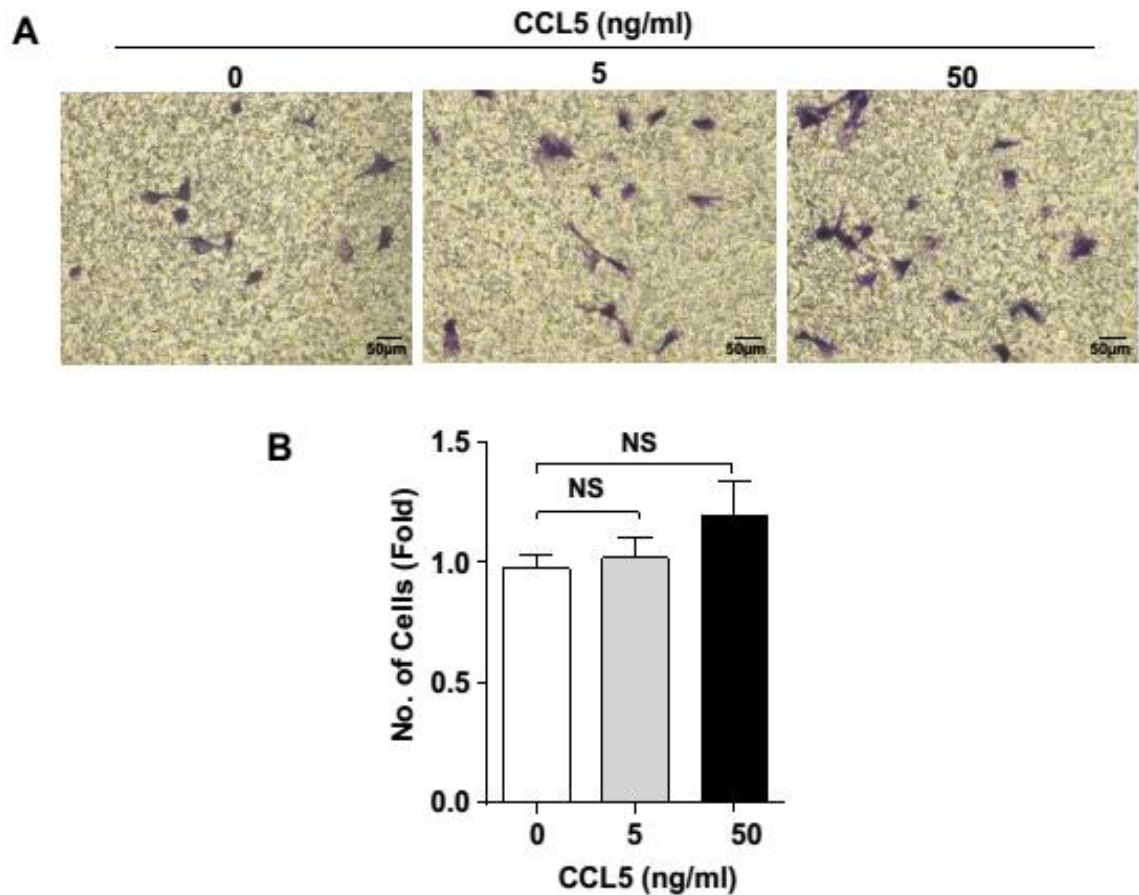


Figure 3.13 CCL5 has no effect on VPCs migration. **A.** The transwell assay was performed on vascular progenitor cells that migrated toward either serum-free medium (control) or serum free media containing murine recombinant CCL5 (5ng/ml, 50ng/ml). Transwell migration assays show VPCs migrated towards serum-free medium with or without mouse recombinant CCL5 (5ng/ml, 50ng/ml). **B.** The graph is presented as mean \pm SEM of $n\geq 3$. ns, $P>0.05$.

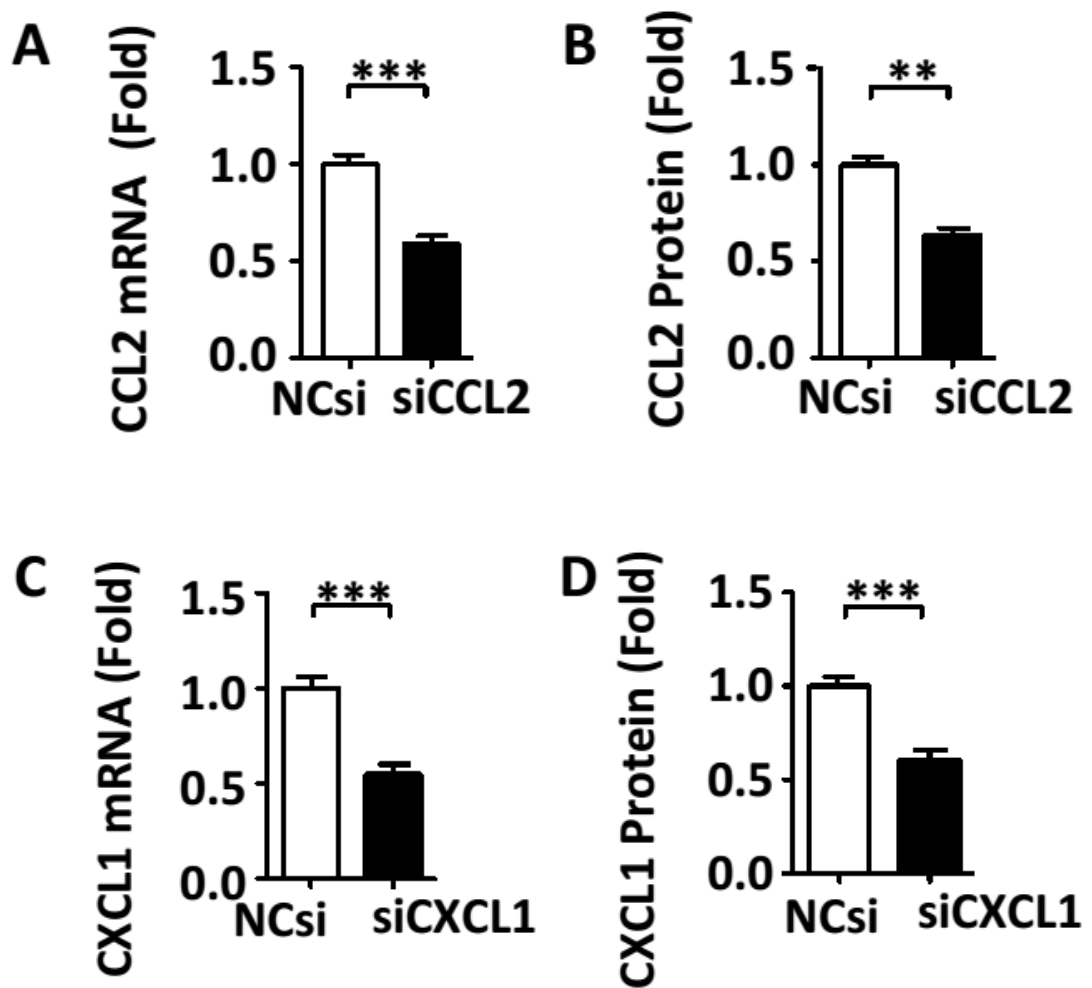


Figure 3.14 Silencing of CCL2 or CXCL1 in the SMCs using siRNAs. SMCs were transfected either with control noncoding small interfering RNA (siRNA), CCL2 siRNA (300nM) or CXCL1 siRNA (100nM) to knockdown corresponding mRNA. The real-time quantitative PCR and Quantikine ELISA kit showed a fold decrease in mRNA (**A, C**) and protein (**B, D**) levels in CCL2 or CXCL1 siRNA transfected SMCs and their conditioned medium respectively. n=3. All graphs are presented as mean \pm SEM. **p<0.01, ***p<0.001. NCsi, non-coding siRNA. siCCL2, CCL2 siRNA. siCXCL1, CXCL1 siRNA.

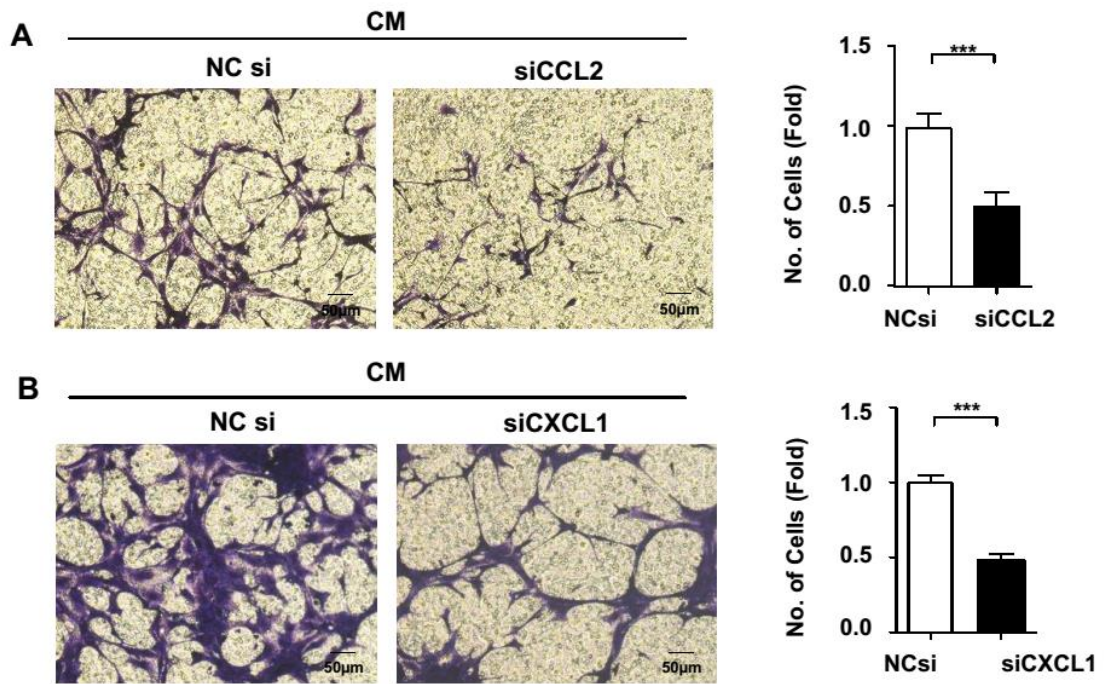


Figure 3.15 Induction of VPC migration was markedly inhibited by conditioned medium derived from siRNA knocked down SMC. Transwell assay was performed on vascular progenitor cells migrating towards SMC (transfected either with noncoding siRNA, CCL2 siRNA or CXCL1 siRNA) conditioned medium. n=5. All graphs are presented as mean \pm SEM. ***p<0.001. NCsi, noncoding siRNA. siCCL2, CCL2 siRNA. siCXCL1, CXCL1 siRNA. CM, SMC conditioned medium.

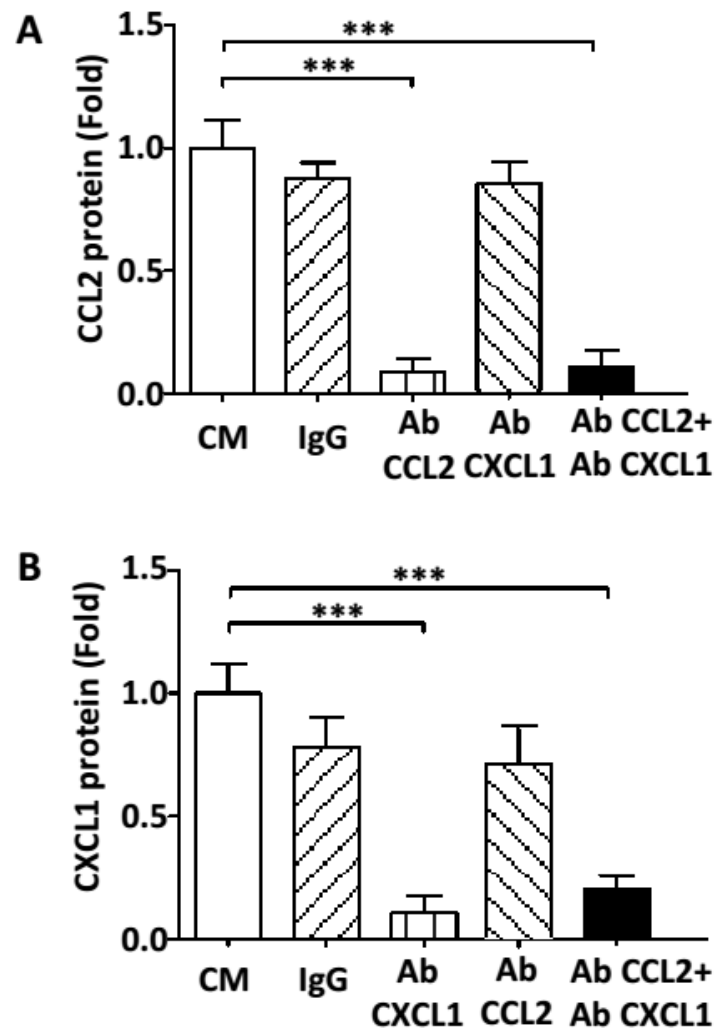


Figure 3.16 The neutralizing antibodies are effective and selective for CXCL1 and CCL2 respectively. The SMC conditioned medium was treated with either neutralising antibodies for CCL2 and/or CXCL1 or the relevant control IgG for 1 hour at 37°C, before pull-down of chemokines combined with antibodies using protein G beads. The supernatant was collected and used to measure the concentrations of CCL2 (A) and CXCL1 (B) using corresponding ELISA kits. n=3, ***p<0.001.

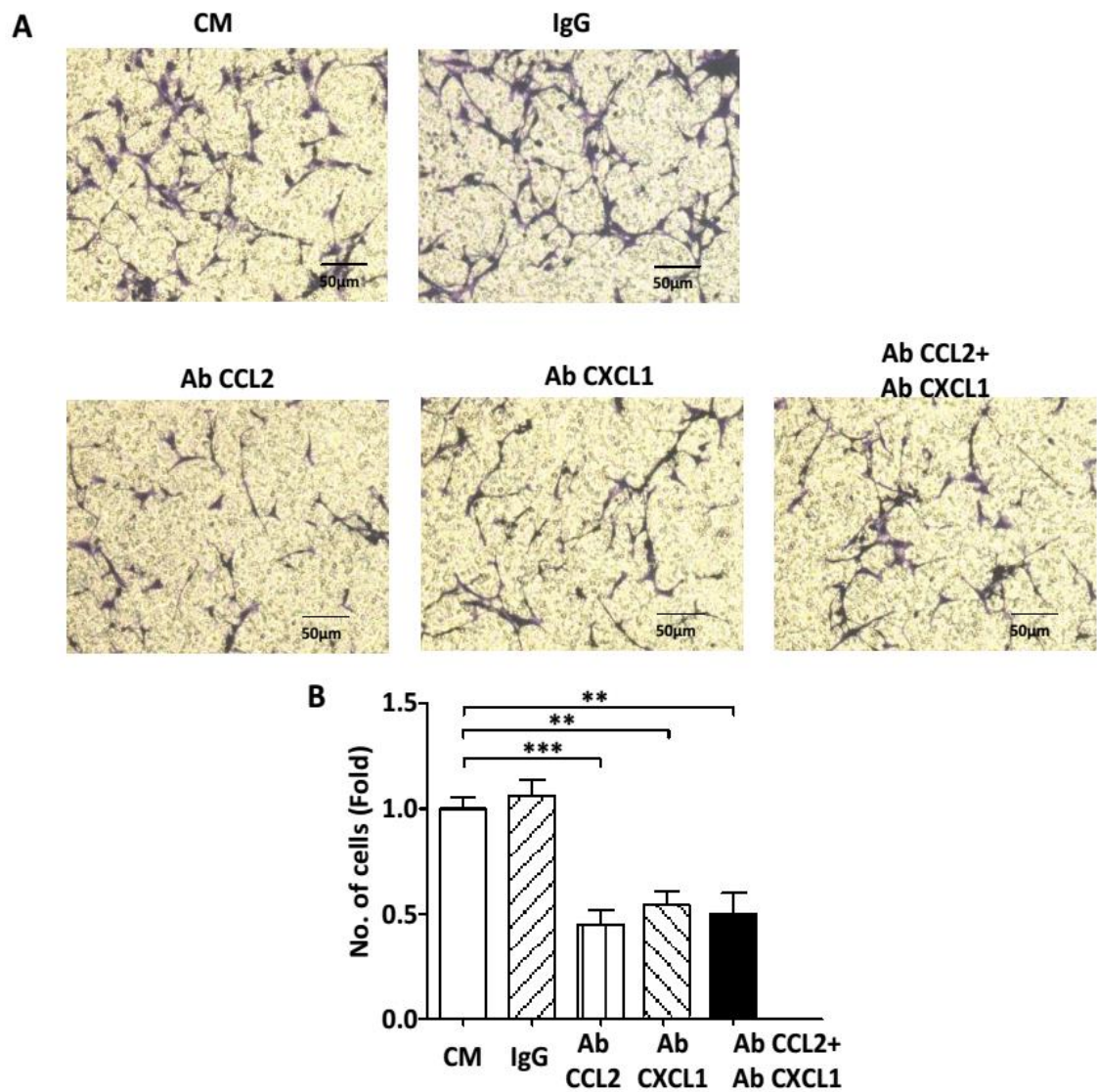


Figure 3.17 The effects of CCL2 and CXCL1 are neither cumulative nor redundant in mediating VPC migration. The transwell assay was performed on VPCs that migrated toward SMC conditioned medium (positive control) or SMC conditioned medium after depletion of chemokines with IgG, CCL2 and/or CXCL1 neutralising antibodies. $n=3$, $***p<0.001$

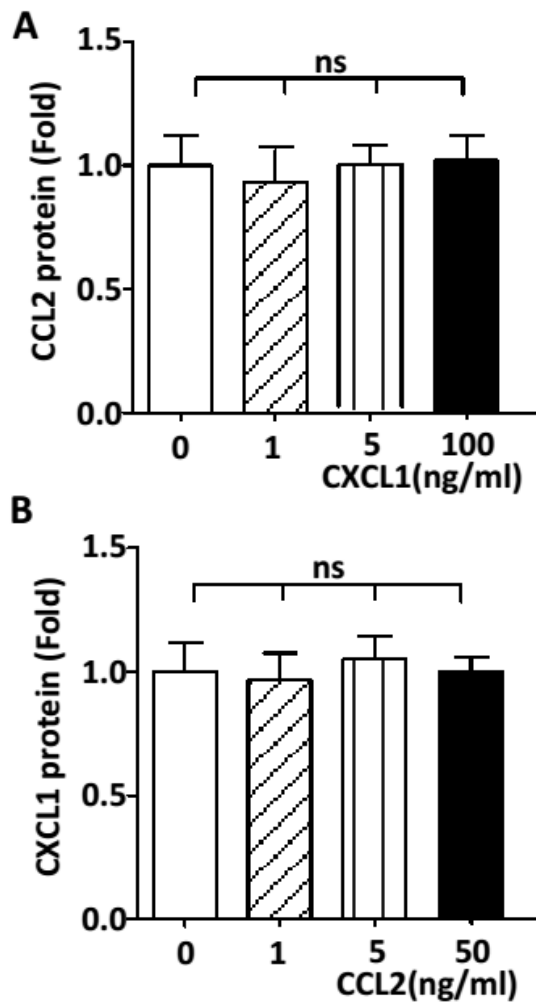


Figure 3.18 CCL2 and CXCL1 cannot stimulate VPCs to produce each other. VPCs were treated with either CXCL1 or CCL2 at indicated concentrations overnight followed by measurement of concentrations of CCL2 and CXCL1 using ELISA. The graphs are presented as mean \pm SEM of n=3, ns, $P > 0.05$.

3.4 VPC migration is mediated by CCR2 and CXCR2

To identify the panel of chemokine receptors that are expressed in VPCs, a qPCR-array of Sca-1⁺ VPCs was performed; a cloned population of VPCs and SMCs were used as controls. Data shown in Figure 3.4C and Table 3.3 indicates the presence of a variety of chemokine receptors in VPCs including CCR2 and CXCR2. Both mRNA and protein expression of these receptors were significantly upregulated after treatment with SMC-derived medium (Figure 3.19). To silence CCR2 and CXCR2 in VPCs at the gene level, VPCs were infected with CCR2 or CXCR2 lentiviral shRNA; a null shRNA was used as a control. The cells were selected for 4-5 days with neomycin and maintained in complete culture medium. Subsequent transwell migration experiments with CCR2 or CXCR2 silencing resulted in a significant decrease in VPC migration (Figure 3.20). Consistently, pre-treatment with antagonists of CCR2 or CXCR2 led to a downregulation of VPC migration in response to chemokines (Figure 3.21). These findings suggest that CCL2 and CXCL1 released from SMCs induced VPC migration via interaction with their corresponding receptors CCR2 and CXCR2.

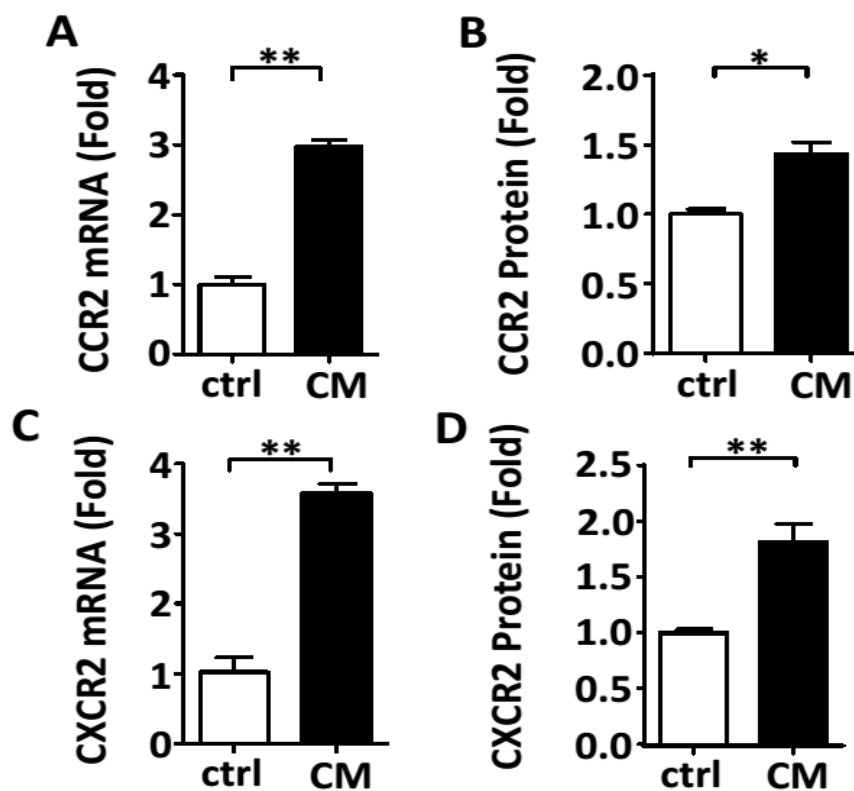


Figure 3.19 The upregulation of chemokine receptors CXCR2 and CCR2 was analysed using flow cytometry. The mRNA (A, C) and cell-surface protein (B, D) expression of CCR2 or CXCR2 was confirmed using real-time quantitative PCR and flow cytometry. n=3. ctrl, control. CM, conditioned medium.

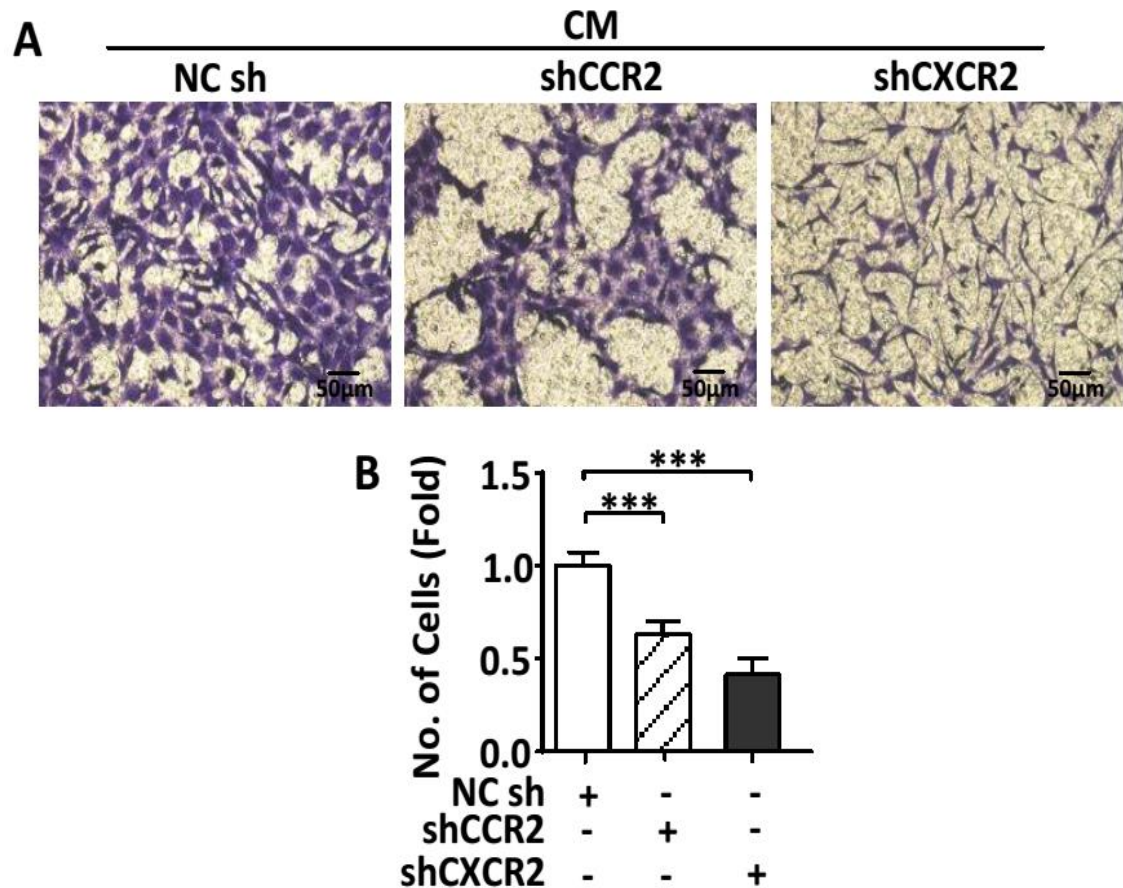


Figure 3.20 SMC-mediated migration was decreased in CCR2 or CXCR2 silenced VPCs. Vascular progenitor cells were infected with lentiviral short hairpin RNA (shRNA) (noncoding shRNA, CCR2 shRNA or CXCR2 shRNA) for ablation of CCR2 or CXCR2 before migration toward SMC conditioned medium. Noncoding shRNA served as the control. n=3. All graphs are presented as mean \pm SEM. ***p<0.001. NCsh, noncoding shRNA. shCCR2, CCR2 shRNA. shCXCR2, CXCR2 shRNA. ctrl, control. CM, SMC conditioned medium.

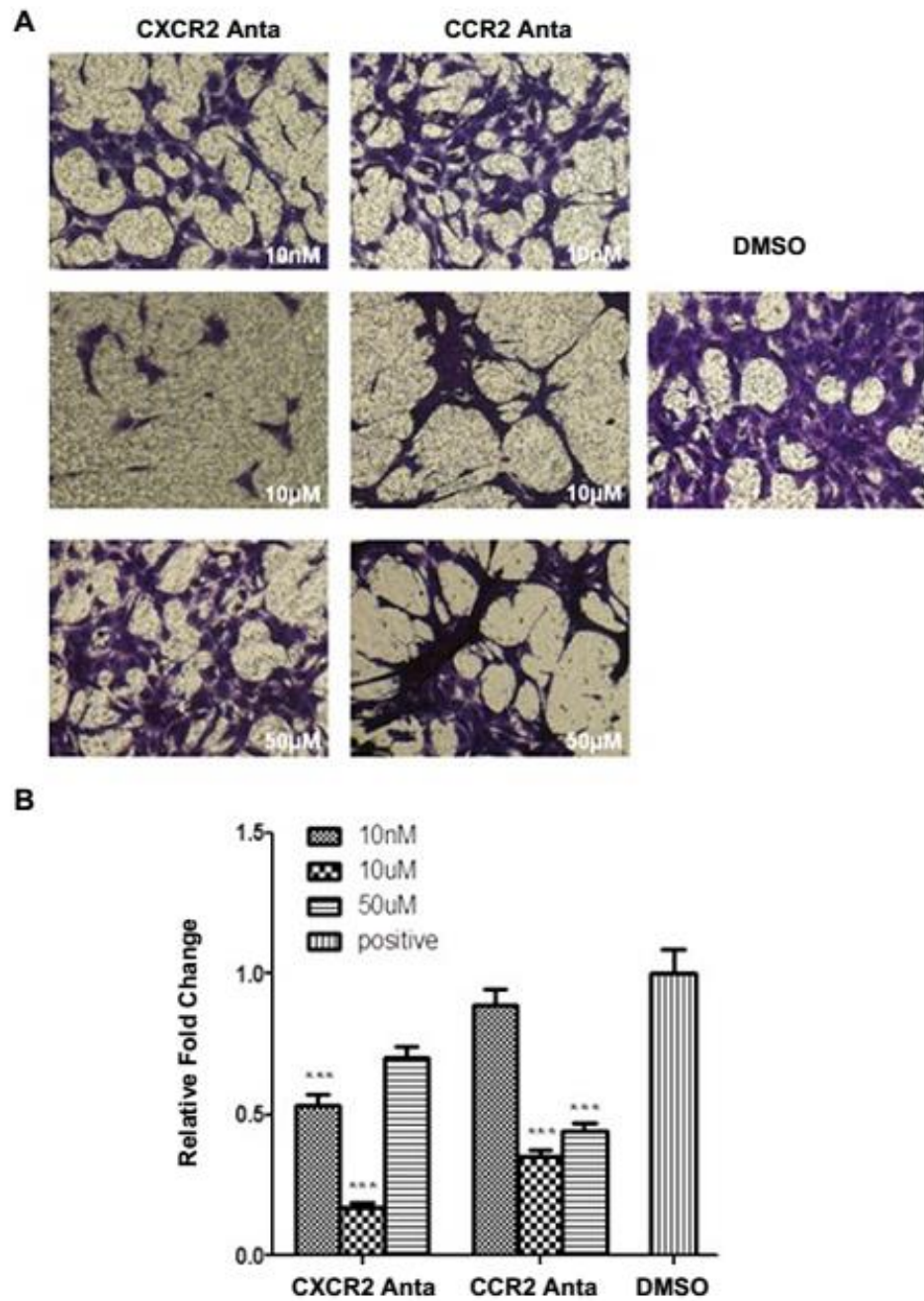


Figure 3.21 Antagonists of CCR2 or CXCR2 inhibited SMC-mediated VPC migration.

The transwell assay was performed on vascular progenitor cells that were pre-treated with a range of concentrations of either an antagonist of CXCR2 or CCR2 for 1 hour prior to the migration toward SMC conditioned medium study. **A.** Images represent each group in 20x view of fields. **B.** All graphs are presented as mean \pm SEM of $n \geq 3$. *** $p < 0.001$.

3.5 CCL2 and CXCL1 induced VPCs migration through the Rac1-p38 pathway

The Rho GTPase family members Rac1 and Cdc42 have been implicated as important regulators of cell migration (Fonseca, Freund, Bornhauser, & Corbeil, 2010). To investigate whether Cdc42 and Rac1 are involved in VPC migration, we measured the levels of GTP-Cdc42 and GTP-Rac1 using pull-down assays. Stimulation of VPCs with mouse recombinant CCL2 or CXCL1 resulted in both Cdc42 and Rac1 activation (Figure 3.22). Treatment with either ML141 (an inhibitor of Cdc42) (Figure 3.23) or NSC23766 (an inhibitor of Rac1) (Figure 3.24) resulted in a significant reduction in VPC migration induced by CXCL1 and CCL2 when compared to control.

p38 MAPK has been identified as the downstream signal transducer of active forms of Cdc42 and Rac1 and the p38 signalling pathway can induce rearrangements of the cytoskeleton that regulate cell migration (Malchinkhuu et al., 2005; S. Zhang et al., 1995). A time course, using Western blotting analysis, showed that p38 phosphorylation occurred in VPCs after treatment with SMC conditioned medium for 20 minutes (Figure 3.26 A). p38 phosphorylation was up-regulated in response to mouse recombinant CCL2 and CXCL1 treatment (Figure 3.26 B), and suppressed in response to conditioned medium derived from CCL2 siRNA or CXCL1 siRNA depleted SMCs (Figure 3.26 C). p38 phosphorylation was also suppressed in VPCs where CCR2 or CXCR2 had been knocked down using the corresponding shRNA (Figure 3.27 A). Pre-treatment of VPCs with CCR2 or CXCR2 antagonists consistently reduced p38 phosphorylation (Figure 3.27 B). Importantly, p38 phosphorylation was significantly reduced in VPCs pre-treated with NSC23766 (a Rac1 inhibitor) (Figure 3.29) but not ML141 (a Cdc42 inhibitor) (Figure 3.28). Finally, migration of VPCs was reduced in the presence of SB203580 (an inhibitor of the p38-MAPK signalling pathway) (Figure 3.31). These indicate that Rac1 regulates CCL2 and CXCL1 induced VPC migration via the p38-signalling pathway. RhoA, another important small GTPase, has also been reported to regulate the cell migration process (Fonseca et al., 2010), however, pre-treatment of VPCs with C3 transferase (a RhoA inhibitor),

did not alter VPC migration (Figure 3.25) or p38 phosphorylation (Figure 3.30). We therefore conclude that CCL2 and CXCL1 induce VPC migration by activating GTPase Rac1 and p38 signalling pathways.

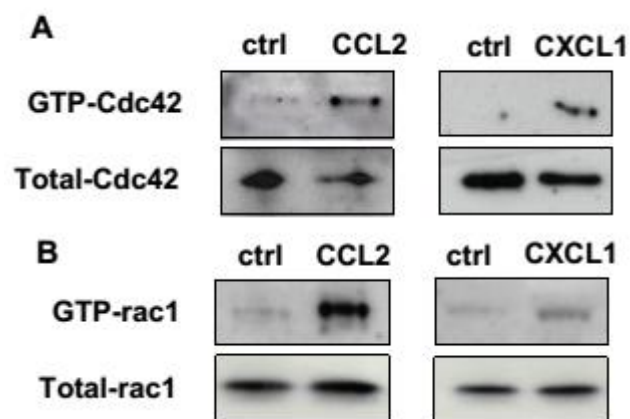


Figure 3.22 CCL2 and CXCL1 activated GTPase Cdc42 and Rac1. Vascular progenitor cells were treated with either CCL2 or CXCL1 for 5 minutes. GTP-bound Cdc42/Rac1 were pulled down by PAK-PBD beads in whole cell lysates. Cdc42 and Rac1 antibodies were applied to detect GTP-Cdc42 and GTP-Rac1 in pulled down protein lysate and total Cdc42 and total Rac1 in whole protein lysate. The representative blots are from at least 3 independent experiments.

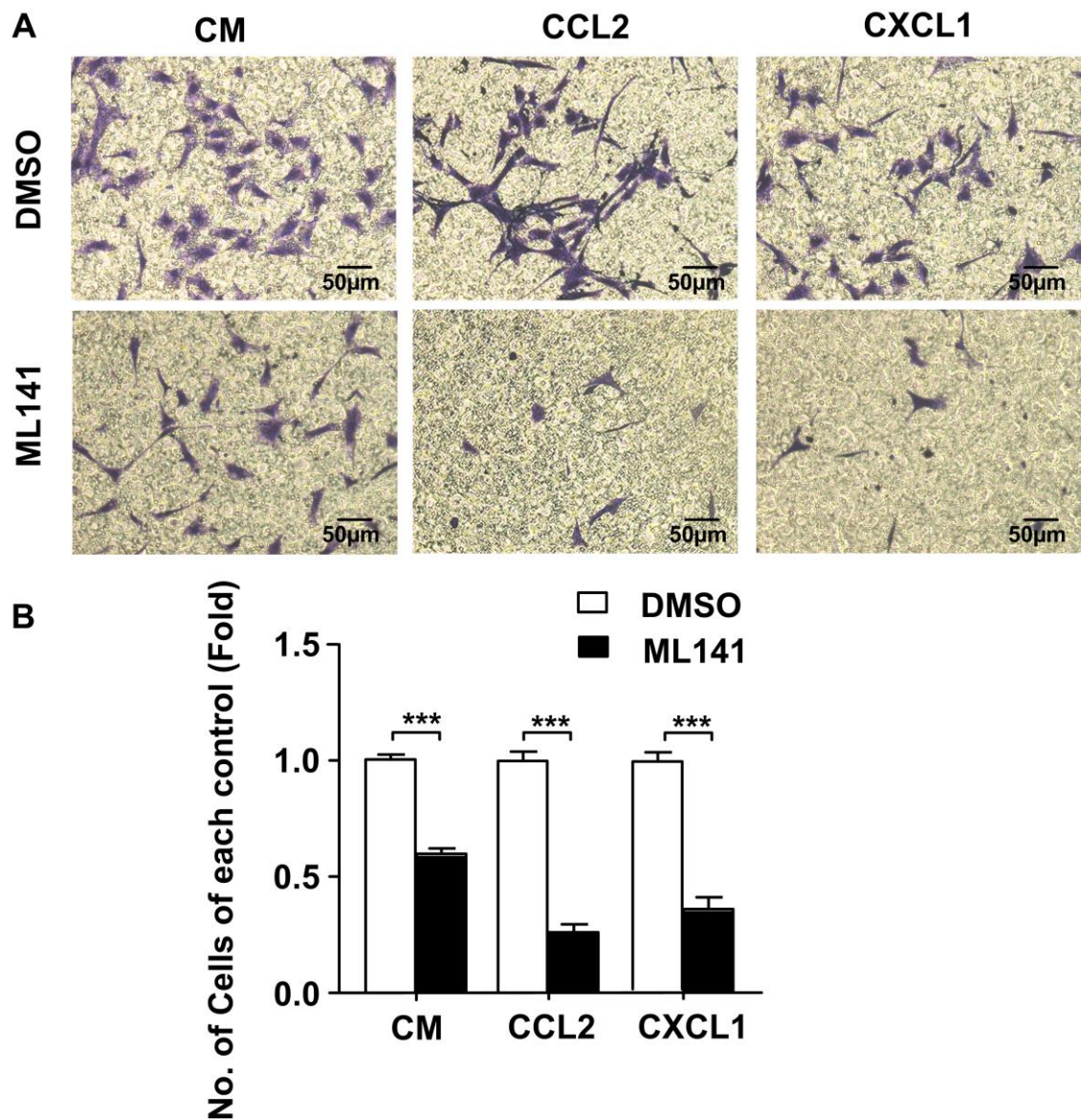


Figure 3.23 The Cdc42 signalling pathway is involved in SMC-induced VPC migration. A. The transwell assay was performed on VPCs that were pretreated with either vehicle (DMSO) or ML141 (20 μ M) for 1 hour before migration toward either SMC conditioned medium or recombinant CCL2, CXCL1. B. The quantification of the transwell assay results is presented as the fold change in cell migration towards each treatment in comparison to cells pre-treated with DMSO. n=3 ***P<0.001

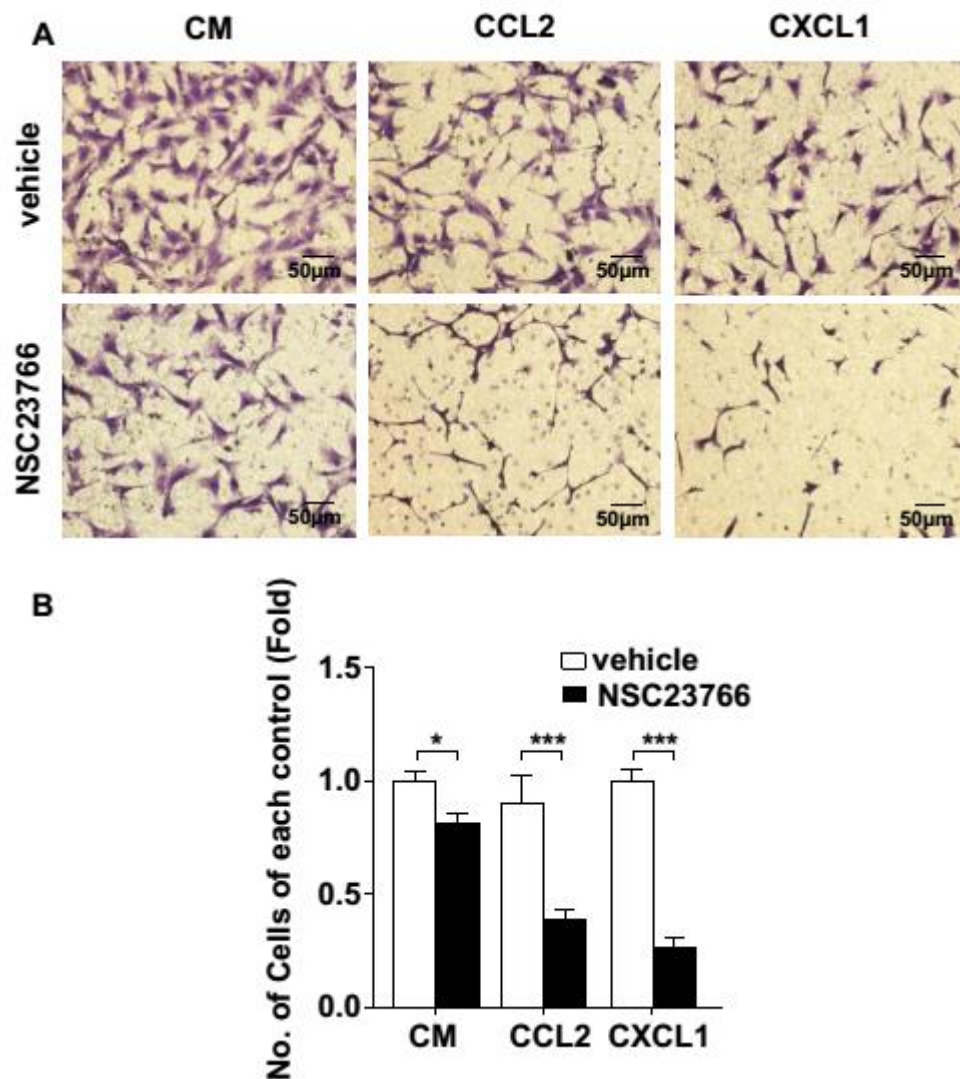


Figure 3.24 The Rac1 signalling pathway is involved in SMC-induced VPC migration.

A. The transwell assay was performed on VPCs that were pre-treated with either vehicle (control) or NSC23766 (50 μ M) for 1 hour before migration toward either SMC conditioned medium or recombinant CCL2, CXCL1. B. The quantification of the transwell assay shows the fold change between cells migrating toward each treatment and cells pre-treated with the vehicle. n=3 * P<0.05, ***P<0.001. vehicle, sterile distilled water.

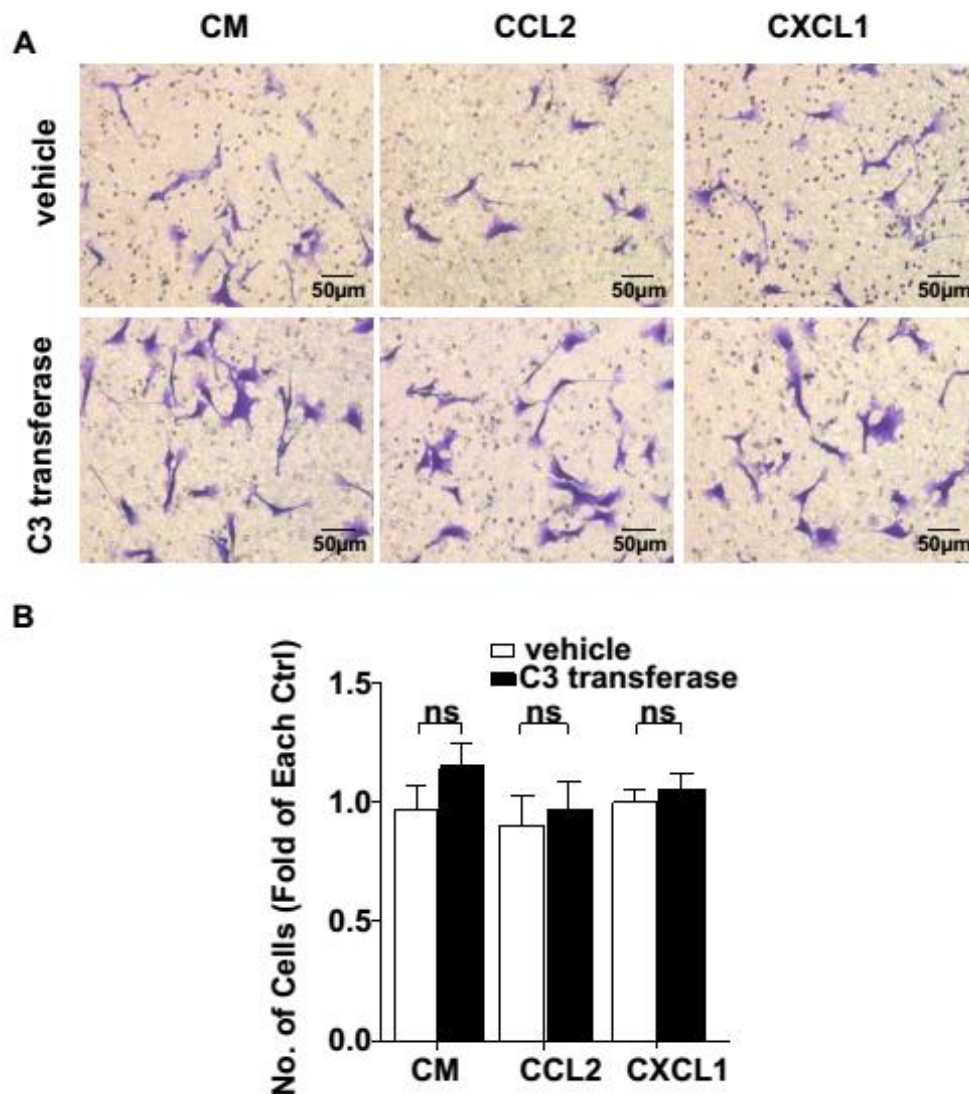


Figure 3.25 The RhoA signalling pathway is not involved in SMC-induced VPC migration. A. The transwell assay was performed on VPCs that were pre-treated with either vehicle or C3 transferase (2 µg/ml) for 2 hours before migration toward either SMC conditioned medium or recombinant CCL2, CXCL1. B. The quantification of the transwell assay is presented as the fold change in cell migration toward each treatment compared to cells pre-treated with the vehicle. n=3 ns, P>0.05. vehicle, sterile distilled water.

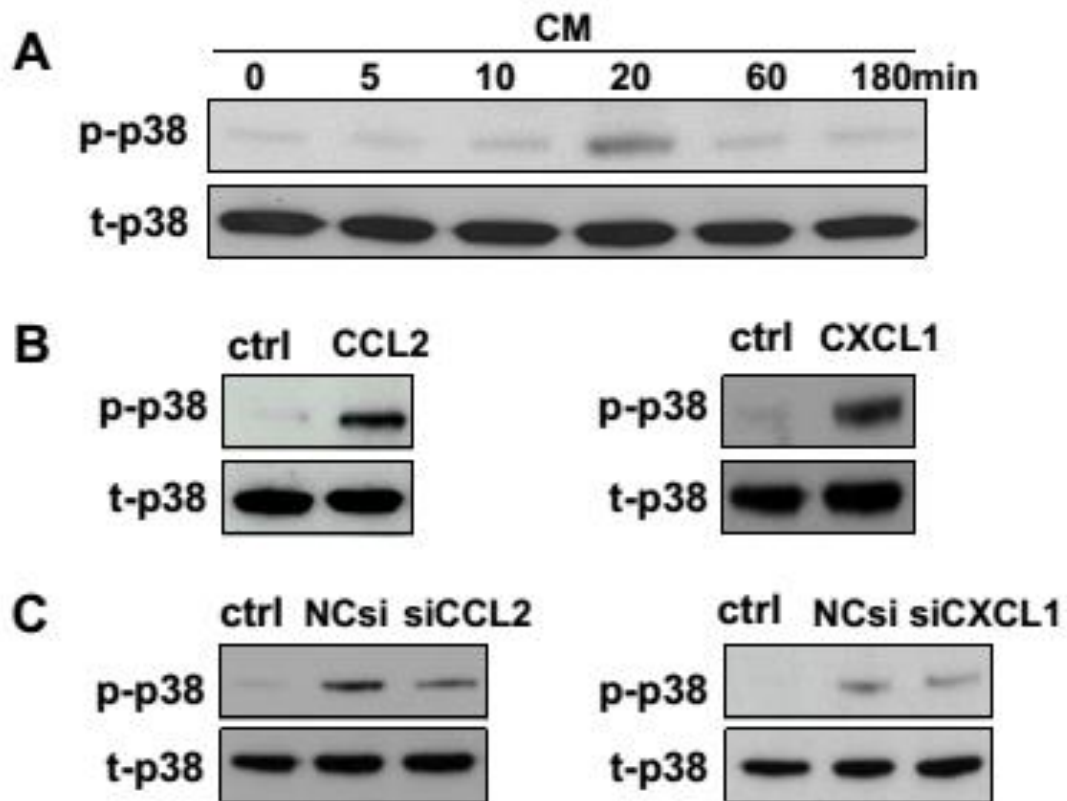


Figure 3.26 SMC conditioned medium induced p38 phosphorylation via CCL2 and CXCL1. A. Western blotting was performed on SMC conditioned medium-treated vascular progenitor cells for the detection of phosphorylated p38 (p-p38, Thr1 80/ Tyr1 82) and total p38 (t-p38) at indicated time points. B. Vascular progenitor cells were treated with recombinant CCL2 or CXCL1 respectively at 20 mins followed by Western blotting to detect p38 phosphorylation. C. The p-p38 and t-p38 on vascular progenitor cells after treatment with SMC (transfected with CCL2 siRNA or CXCL1 siRNA) conditioned medium was analysed by Western blotting. The representative blots are from at least 3 independent experiments.

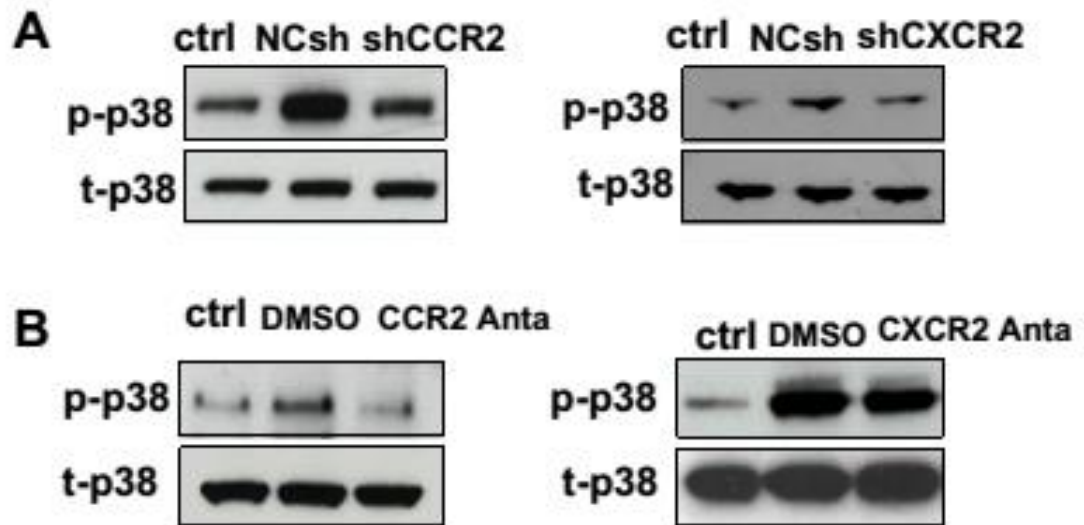


Figure 3.27 SMC-conditioned medium induced p38 phosphorylation via CCR2 and CXCR2. A. Cell lysates from CCR2 shRNA or CXCR2 shRNA transfected vascular progenitors cultured in SMC conditioned medium were harvested for the detection of p-p38 and t-p38. B. After pre-treatments with either DMSO or antagonists of CCR2 or CXCR2, p-p38 and t-p38 of vascular progenitor cells in response to SMC conditioned medium was detected using Western blotting. The representative blots are from at least 3 independent experiments.

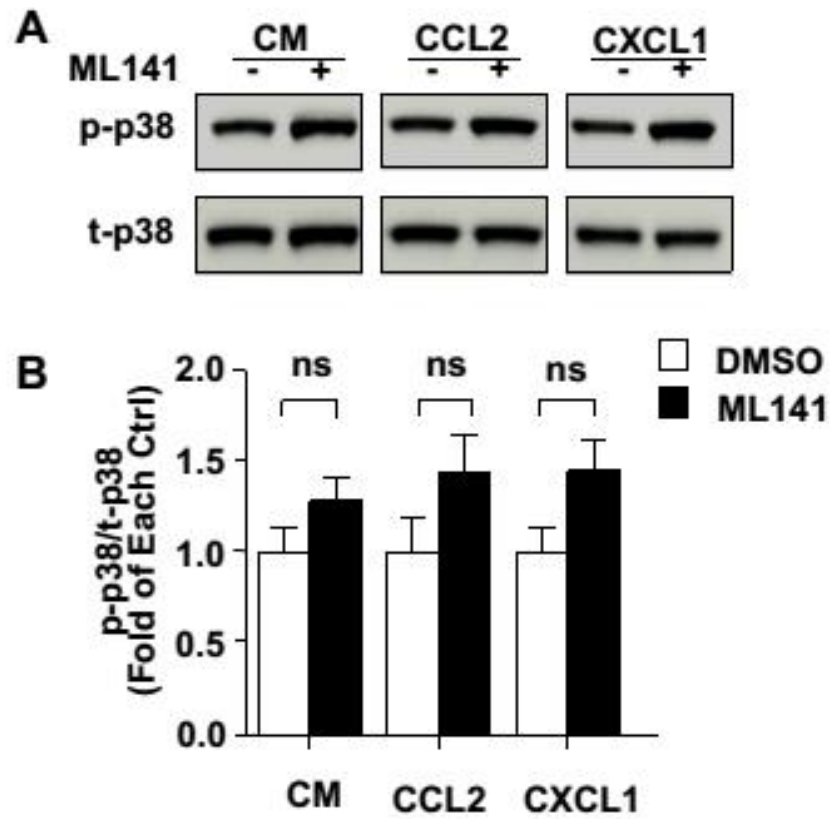


Figure 3.28 SMC conditioned medium induced p38 phosphorylation but not via the Cdc42 signalling pathway. After pre-treatment with DMSO (vehicle) or ML141 for 1 hour, VPC were stimulated with SMC conditioned medium or CCL2, CXCL1 before cell lysates were harvested for detection of p-p38 and t-p38. n=3, ns, p>0.05

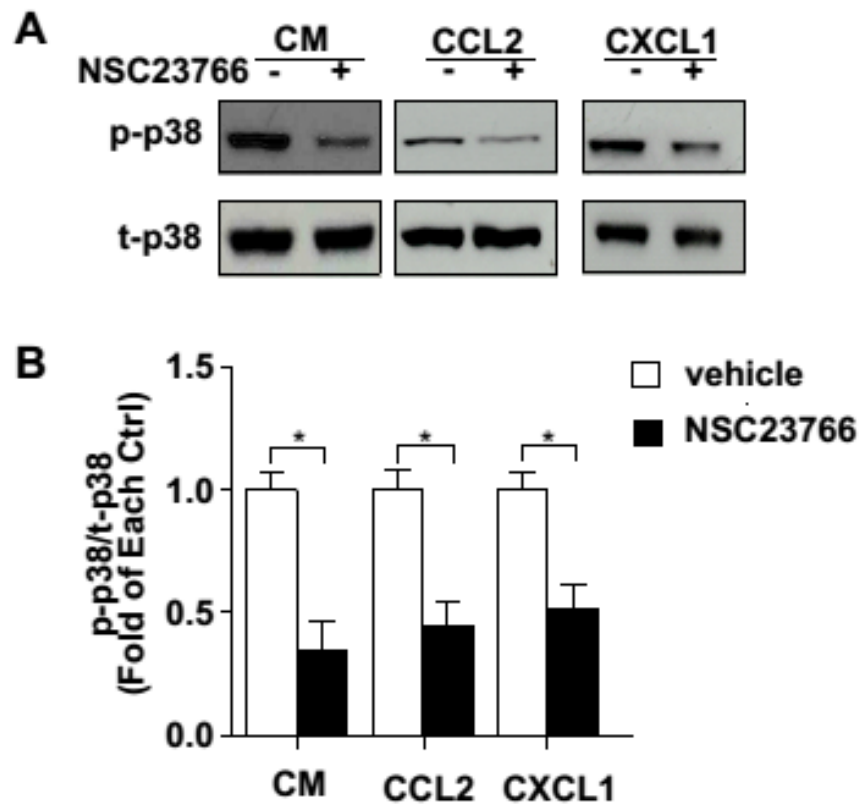


Figure 3.29 SMC conditioned medium induced p38 phosphorylation via the Rac1 signalling pathway. After pretreatment with vehicle (H₂O) or NSC23766 for 1 hour, VPCs were stimulated with SMC conditioned medium or CCL2, CXCL1 before cell lysates were harvested for detection of p-p38 and t-p38. n=3, *p<0.05. vehicle, sterile distilled water.

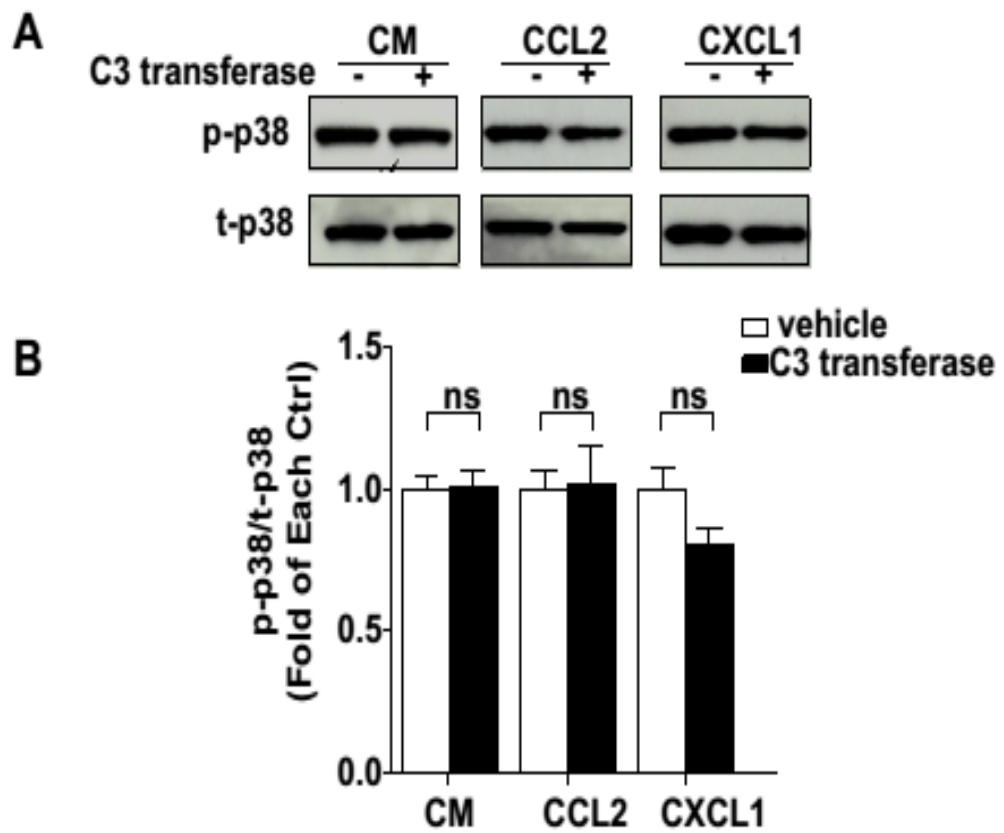


Figure 3.30 SMC conditioned medium induced p38 phosphorylation but not via the RhoA signalling pathway. After pre-treatment with vehicle or C3 transferase for 1 hour, VPCs were stimulated with SMC conditioned medium or CCL2, CXCL1 before cell lysates were harvested for detection of p-p38 and t-p38. n=3, ns, p>0.05. vehicle, sterile distilled water.

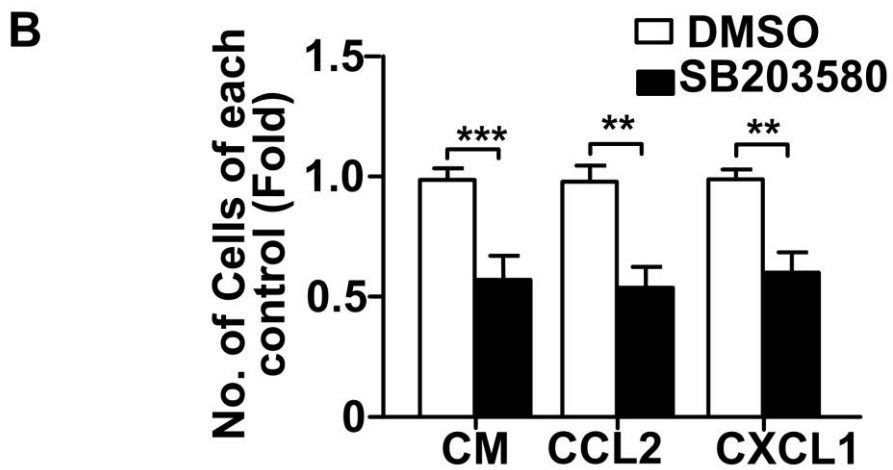
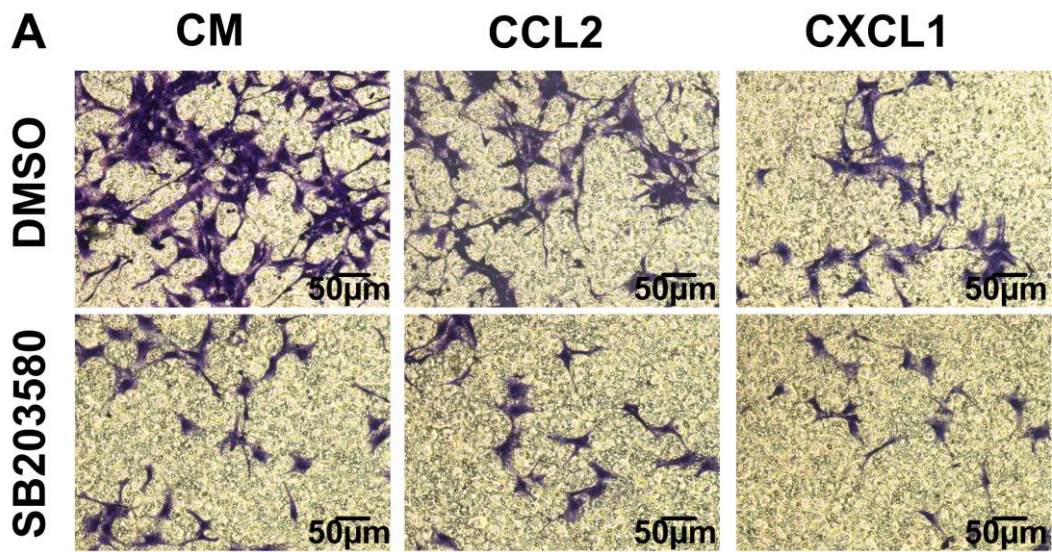


Figure 3.31 SMC conditioned medium induced VPCs migration via the p38 signalling pathway. After pre-treatment with DMSO or SB203580 for 1 hour, VPC were stimulated with SMC conditioned medium or CCL2, CXCL1 before cell lysates were harvested for detection of p-p38 and t-p38. n=3, **p<0.01, ***p<0.001

3.6 Lack of CCL2 inhibits Sca-1⁺ cell migration and neointima formation

SMCs can be described as activated and switched into the synthetic phenotype when they are cultured *in vitro* (Stegemann, Hong, & Nerem, 2005) and under these conditions they might constitutively release CCL2 and CXCL1. To study chemokine release in *in vivo* conditions, a mouse femoral artery wire injury model was used to assess whether native SMCs only produce chemokines, which attract VPC migration, after cell injury. Co-immunofluorescence staining of α -SMA and CCL2 or CXCL1 showed that in intact vessels, SMCs are quiescent and do not produce CCL2 or CXCL1 (Figure 3.32). However, once SMCs are injured they release increasing levels of CCL2 and CXCL1 (both at a short time point (6 hours) and a long time point (2 weeks) (Figure 3.32). These chemokines may play a role as potent and persistent attractants for VPC migration. To verify this, GFP-Sca-1⁺-VPCs (additionally labelled using a Qtracker[®] 655 Cell Labeling Kit) were seeded on the adventitia of vessels that were injured. *En face* confocal microscopy revealed that 72 hours after injury the number of migrated cells found on the intimal side of the vessel wall was significantly lower in CCL2^{-/-} mice when compared to WT mice (Figure 3.35). CCL2^{-/-} mice were identified by genotyping mice and measuring CCL2 levels in peripheral blood (Figure 3.34). Quantification based on either GFP-Sca-1⁺-VPCs or Qtracker showed similar results (Figure 3.35). Sca-1 immunofluorescence staining in sections of injured arteries 2 weeks post injury, showed that GFP-Sca-1⁺-VPCs remained Sca-1 positive after 2 weeks *in vivo* but that fewer cells migrated into the intimal side to contribute to neointima formation in CCL2^{-/-} mice compared to the WT mice (Figure 3.36). These results suggest a role for CCL2 in VPC migration from the adventitia to the intima, where they may contribute to neointima formation.

To further investigate the role of CCL2 in VPC neointima formation, wire-injured femoral arteries were seeded with VPCs or PBS in the adventitia. HE stained sections showed neointima formation in the vessel at 1 or 2 weeks after wire injury (Figure 3.37). Quantification of the data indicated that neointimal lesions were significantly increased in vessels seeded with VPCs. In

the 2-week post injury group, the difference in the ratio of neointima to media with VPCs or PBS was higher than that in the 1-week group. However, neointimal lesions in CCL2^{-/-} mice were decreased in vessels with either VPCs seeded in the adventitia or PBS. There is a significant reduction in neointimal lesion area of vessels with VPC seeding in CCL2^{-/-} mice when compared to the WT mice at 2 weeks.

To characterise cells contributing to neointima formation, immunofluorescence staining of α -SMA and Sca-1 was used; data showed that the cells in the neointima of PBS-treated vessels were approximately 70% α -SMA⁺ and 10% Sca-1⁺ 2 weeks after injury (Figure 3.33). Staining of α -SMA and CCR2 in Figure 3.38 revealed that the cell numbers in the neointima of VPC seeded vessels were increased, albeit fewer were α -SMA positive. These results indicate that in the PBS treated vessels, most cells contributing to neointima formation are α -SMA positive. These cells are either from media SMC migration and proliferation or from progenitor cell differentiation. When Sca-1⁺ VPC were seeded in the adventitia, the ratio of α -SMA positive cells was significantly decreased, which implies that VPCs possess the high potential to migrate and proliferate. Over a short time, most cells maintained their progenitor cell characteristics, even though several had differentiated into SMCs (Figure 3.38 A, B). CCR2 was more highly expressed on the cells in the neointima of VPC-seeded vessels (Figure 3.38 C, D). Cumulatively, these data suggest that VPCs applied to the adventitia significantly increased neointima formation after vessel injury, largely through their migration and with a lesser effect via their differentiation into SMCs. Furthermore, lack of CCL2 markedly inhibits the effect of VPCs on neointima formation.

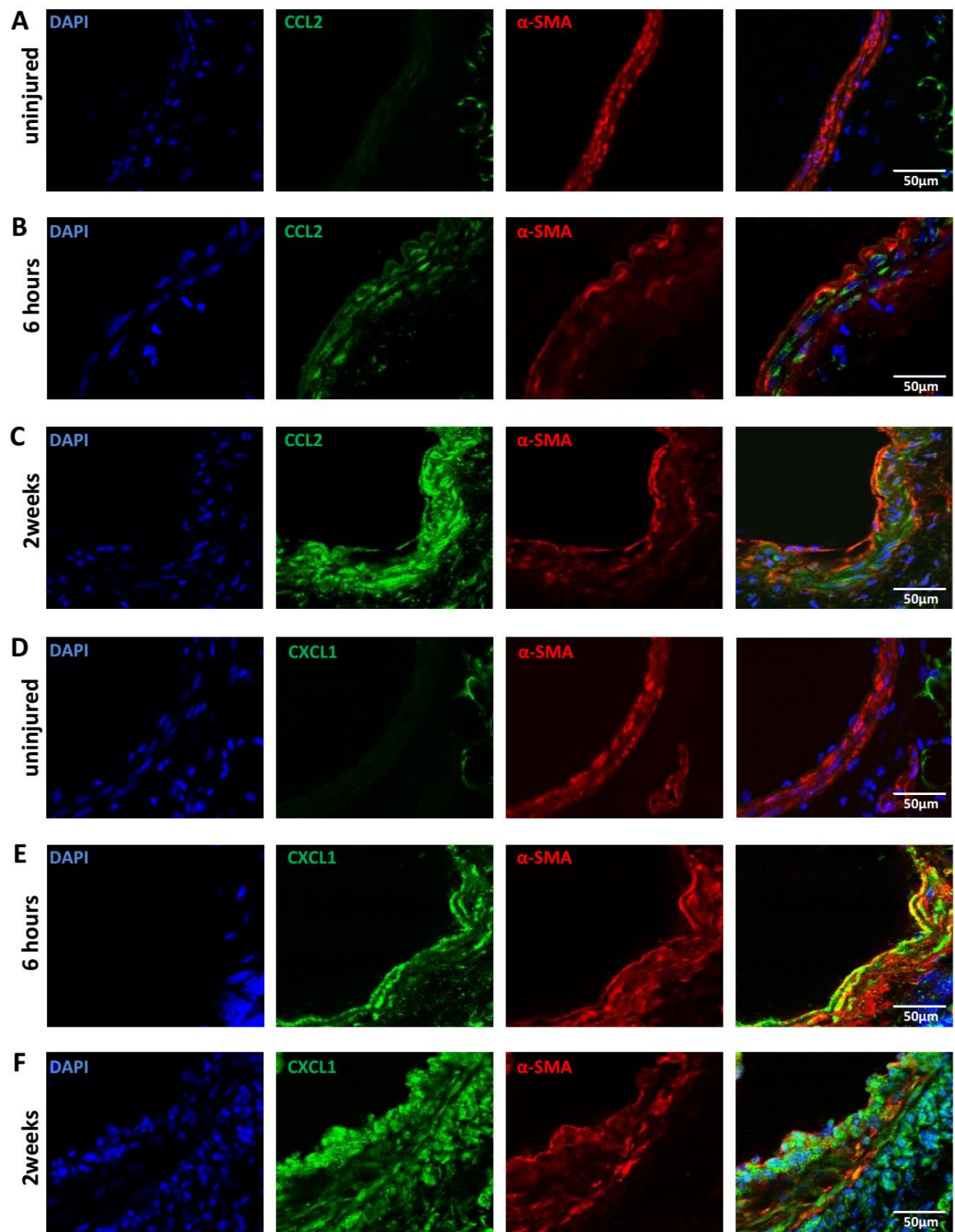


Figure 3.32 CCL2 and CXCL1 are expressed on wire-injured vessels *in vivo*. Femoral arteries of C57BL/6J mice were harvested after wire injury or from uninjured animals. Frozen sections of intact vessels or vessels 6 hours or 2 weeks after wire injury were co-stained with immunofluorescent markers either CCL2 (A, B, C) or CXCL1(D, E, F) and α -SMA. n=4 mice/group.

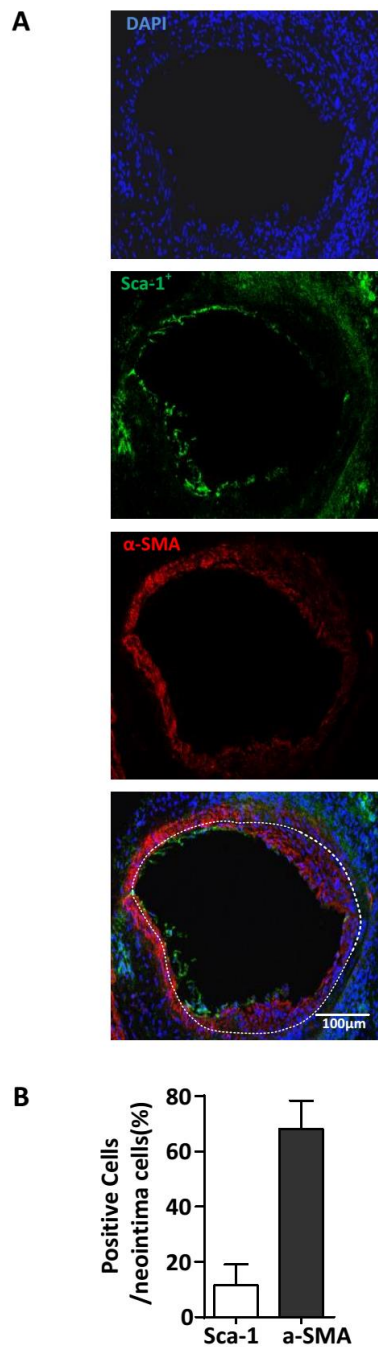


Figure 3.33 Characterisation of cells in the neointima. A. Femoral arteries that had not been seeded with VPC in the adventitia were harvested 2 weeks after wire injury from C57BL/6J mice and stained with Sca-1 and α -SMA (white dotted line indicates internal elastin, and the circled part is neointima area). B. The ratios of positively stained cells with each marker in the neointima area were quantified and are shown in the graph. Graph is presented as mean \pm SEM of n=8 mice/group

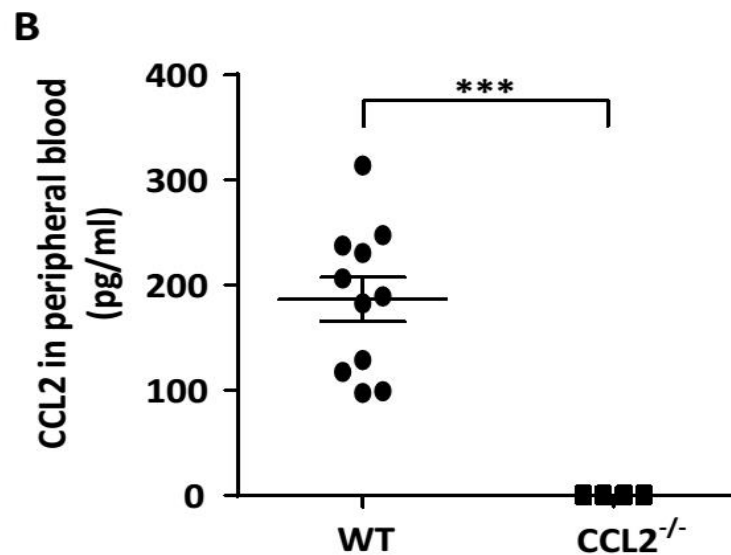
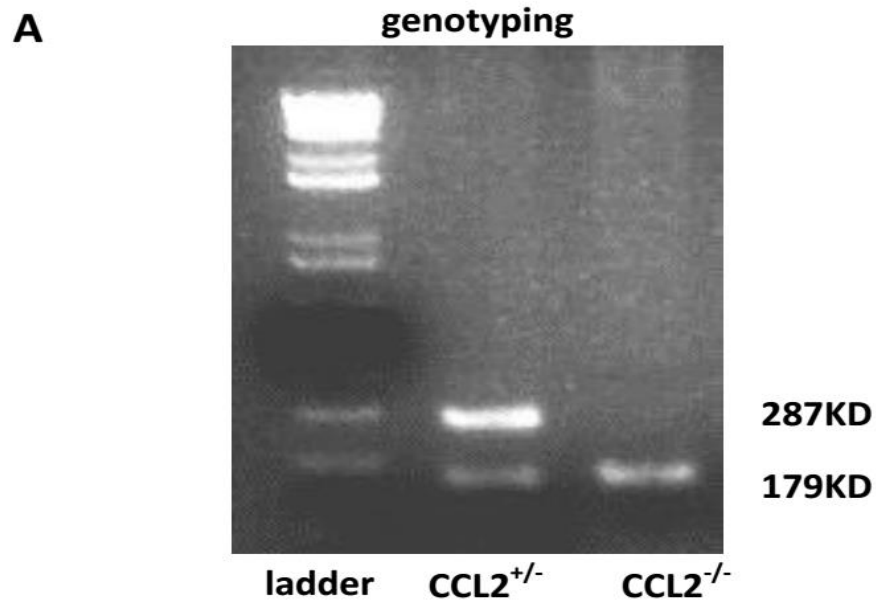


Figure 3.34 Identifying the CCL2^{-/-} mouse genotype and CCL2 levels in the peripheral blood. A. To expand CCL2^{-/-} mice number, CCL2^{-/-} mice were crossed with CCL2^{+/+} mice. A piece of ear tissue from each mouse was used to genotype offspring. The image shows heterozygote (CCL2^{+/-}) with bands at 287bp and 179bp and the mutant (CCL2^{-/-}) with only one band at 179bp. B. Peripheral blood was collected from the vena cava before euthanasia of WT or CCL2^{-/-} mice. The CCL2 level was measured in serum using a murine CCL2 Quantikine ELISA kit. The graph is presented as mean±SEM of n≥8 mice/group, ***p<0.001

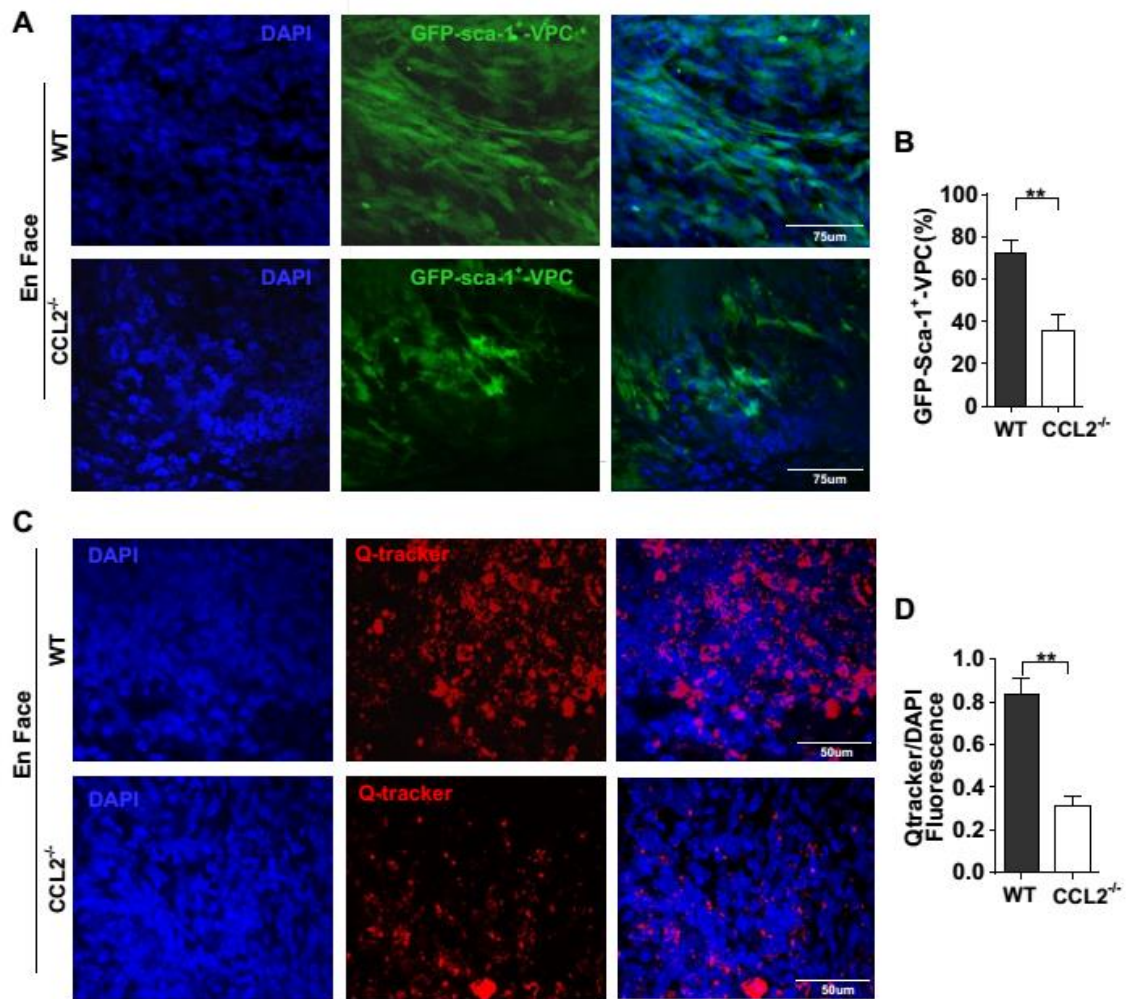


Figure 3.35 Lack of CCL2 inhibits Sca-1⁺ vascular progenitor cell migration *in vivo*. A. After using a mouse femoral artery wire injury model, GFP-Sca-1⁺ vascular progenitor cells (GFP-Sca-1⁺-VPC) (1×10^6) were seeded in the adventitia of each injured vessel. *En face* staining shows that the cells migrated to the intimal side of the vessels 72 hrs post injury of WT and CCL2^{-/-} mice. B. The percentage of GFP-Sca-1⁺-VPC within respective DAPI⁺ nucleated populations in each view was quantified. C. Sca-1⁺ vascular progenitor cells (1×10^6), which were labelled with Q-tracker to locate them as they migrated, were seeded in the adventitia of each injured femoral artery. 72 hrs post injury, the vessels were harvested. *En face* staining shows that cells migrated to the intimal side of the vessels of WT and CCL2^{-/-} mice. D. The ratio of the fluorescence intensity (Qtracker: DAPI) is represented in the graphs as mean \pm SEM of n=8 mice/group

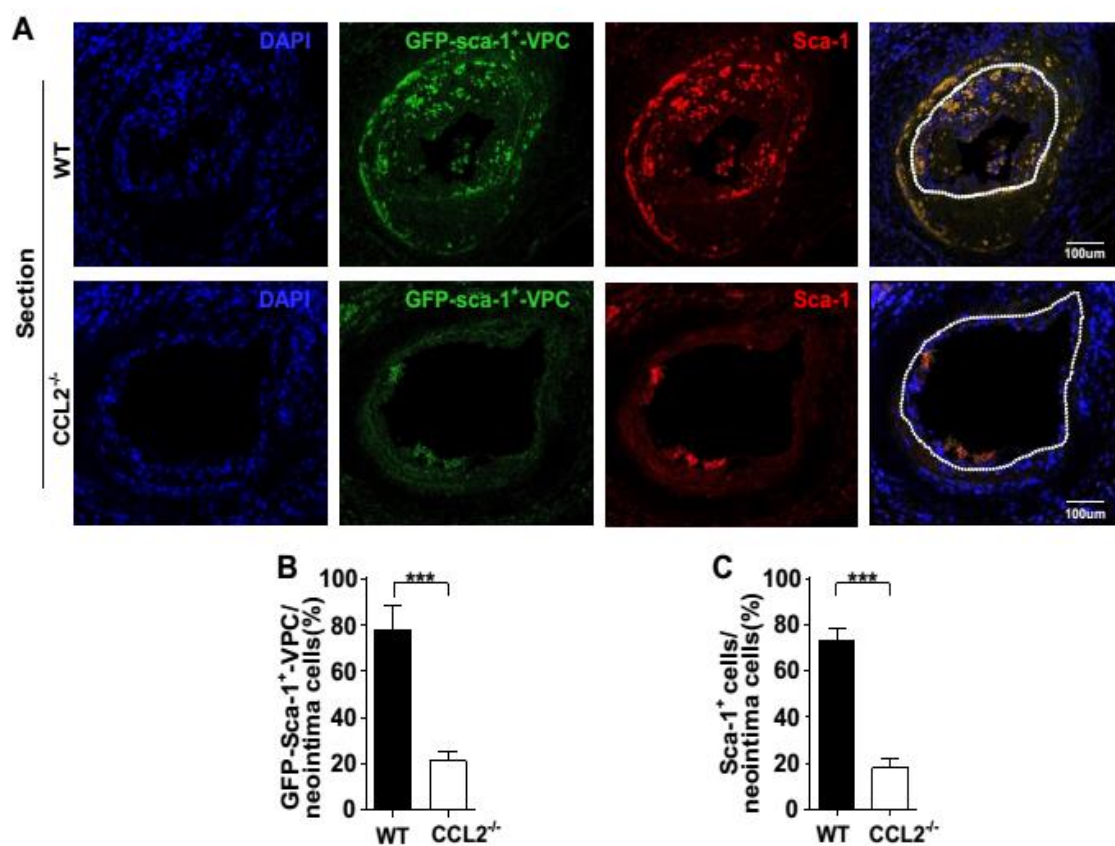


Figure 3.36 Lack of CCL2 inhibits Sca-1⁺ cell participation in neointima formation. A. The femoral artery sections from WT and CCL2^{-/-} mice 2 weeks post injury were prepared for immunofluorescent Sca-1 staining. B, C. The graphs show the percentage of GFP-Sca-1⁺-VPC or Sca-1⁺ cells within the DAPI⁺ nucleated cells in the neointima (white line indicates internal elastin, the neointima area is surrounded by the line). Representative images and graphs are presented as mean \pm SEM of n=8 mice/group. ***P<0.001. WT, wild type.

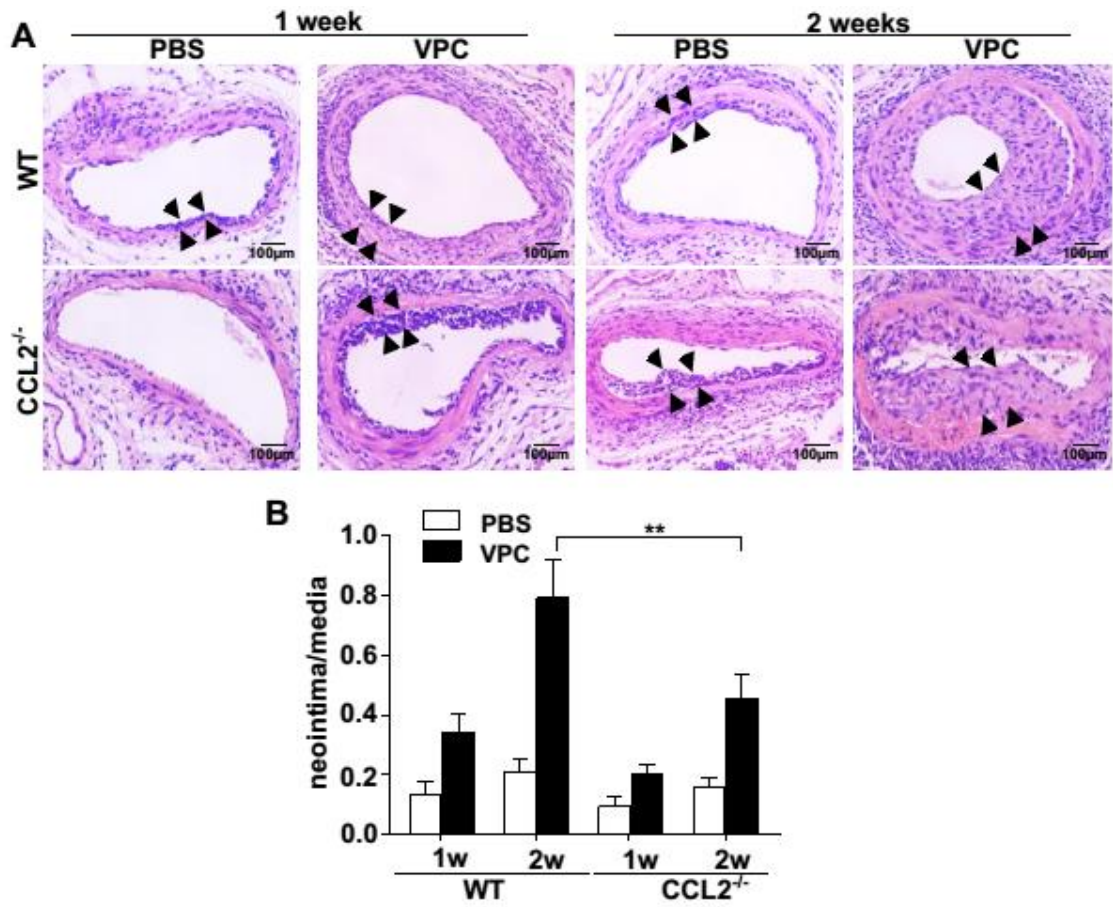


Figure 3.37 Lack of CCL2 reduces neointima formation. A. Animals were euthanized at indicated time points after injury and the femoral arteries were fixed in 4% phosphate-buffered (pH=7.2) formaldehyde, embedded in paraffin, sectioned in 5 μ m thick preparations and stained with haematoxylin–eosin. B. The ratio of neointima (the area between arrows) to media was quantified in the graph as mean \pm SEM of n=8 mice/group. 1w, 1week. 2w, 2weeks.

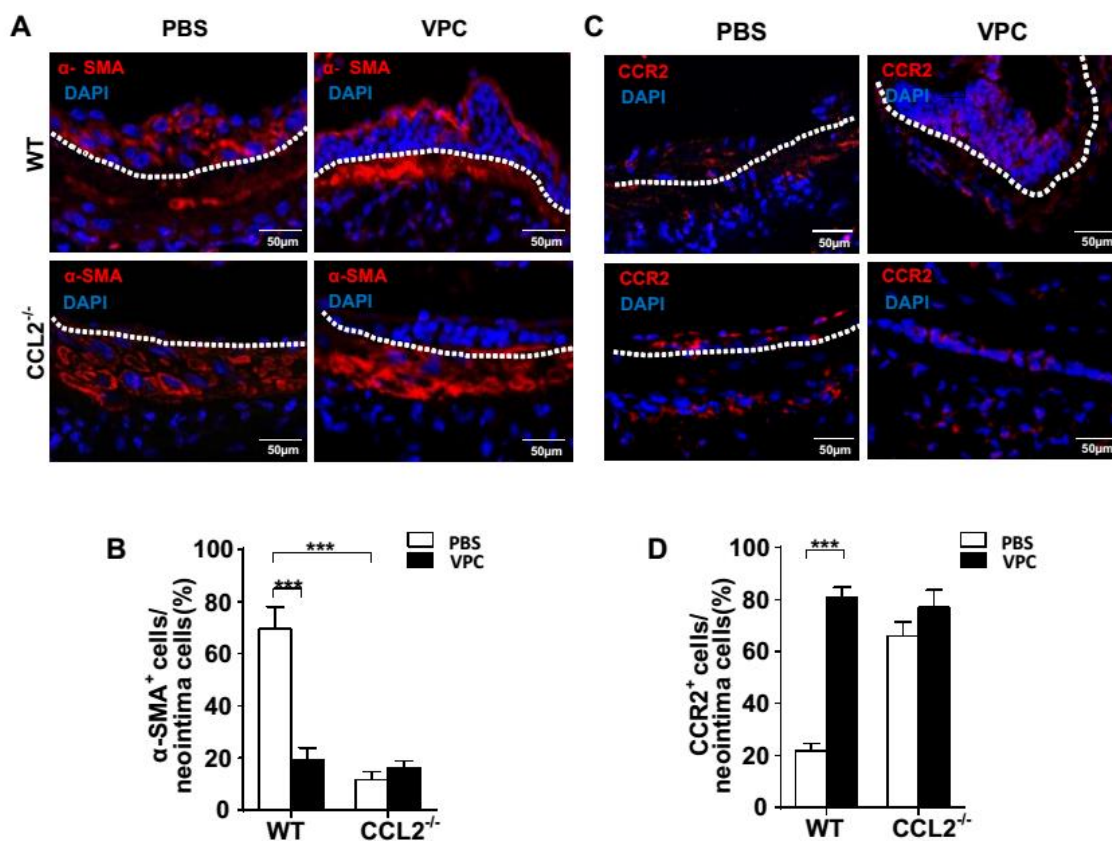


Figure 3.38 Lack of CCL2 inhibits Sca-1⁺ cells migration and differentiation into SMCs. A, C. Vessel sections were also prepared for immunofluorescent α -SMA and CCR2 staining 2 weeks post wire injury (white dotted line indicates internal elastin, and the above is neointima area). B, D. Quantification of the percentage of positively stained cells within the neointima was shown in graphs as mean \pm SEM of n=8 mice/group. ***P<0.001. WT, wild type. PBS, phosphate buffered saline. VPC, vascular progenitor cell.

3.7 Role of CCL2 released from non-bone marrow tissue

Previous studies show that SMCs, ECs and other tissues (Rollins, Yoshimura, Leonard, & Pober, 1990; X. Yu et al., 1992) are also able to secrete CCL2. To investigate which source of CCL2 plays the most important role in VPC migration-induced neointima formation, chimeric mouse models were created. Wild-type bone marrow was transplanted into an irradiated CCL2^{-/-} mouse to form a CCL2 chimera. Conversely, a wild-type chimeric mouse model was prepared by transplanting CCL2^{-/-} mouse bone marrow cells into an irradiated wild-type mouse. The levels of CCL2 in the serum of peripheral blood were measured using a murine CCL2 ELISA kit. In the wild-type chimeric mouse model, the level of CCL2 in peripheral blood was increased, despite transplantation with CCL2^{-/-} bone marrow cells, when compared to a non-transplanted wild-type mouse control. This indicated that CCL2 is mostly released from non-bone marrow tissues. In contrast, the level of CCL2 has increased in CCL2^{-/-} mice with transplanted wild-type bone marrow cells, but to a much lesser extent, thus indicating that only a small proportion of CCL2 is released from bone marrow-derived cells (Figure 3.39). Data from HE-stained sections showed that neointima lesion area correlated with the CCL2 level in the blood (Figure 3.40). These results support the observations that CCL2-induced VPC migration contributes to neointima formation.

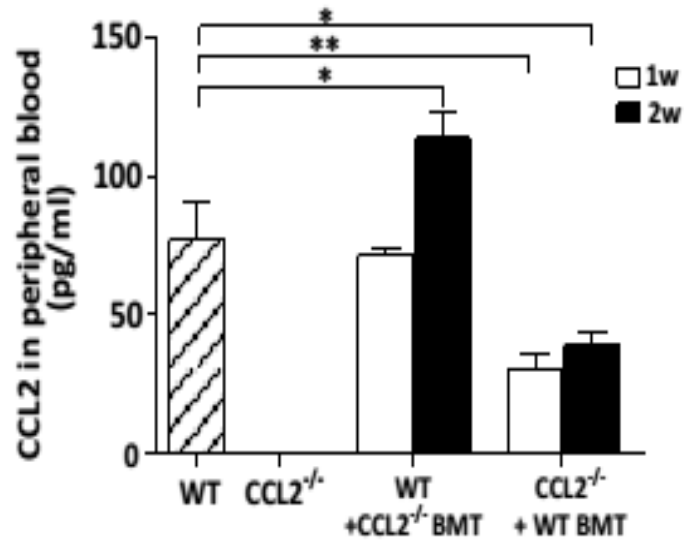


Figure 3.39 Levels of CCL2 in peripheral blood of chimeric mice. After bone marrow transplantation in chimeric mouse models, CCL2 levels in peripheral blood of indicated mice after 1w and 2w was determined using a CCL2 Quantikine ELISA kit. Data is shown as mean \pm SEM of n=5 mice/group. *p<0.05, **p<0.01, ***p<0.001. WT+CCL2^{-/-} BMT, wild type mice in which bone marrow was transplanted from CCL2^{-/-} mice. CCL2^{-/-}+WT BMT, CCL2^{-/-} mice in which bone marrow was transplanted from wild-type mice. 1w, 1week. 2w, 2weeks.

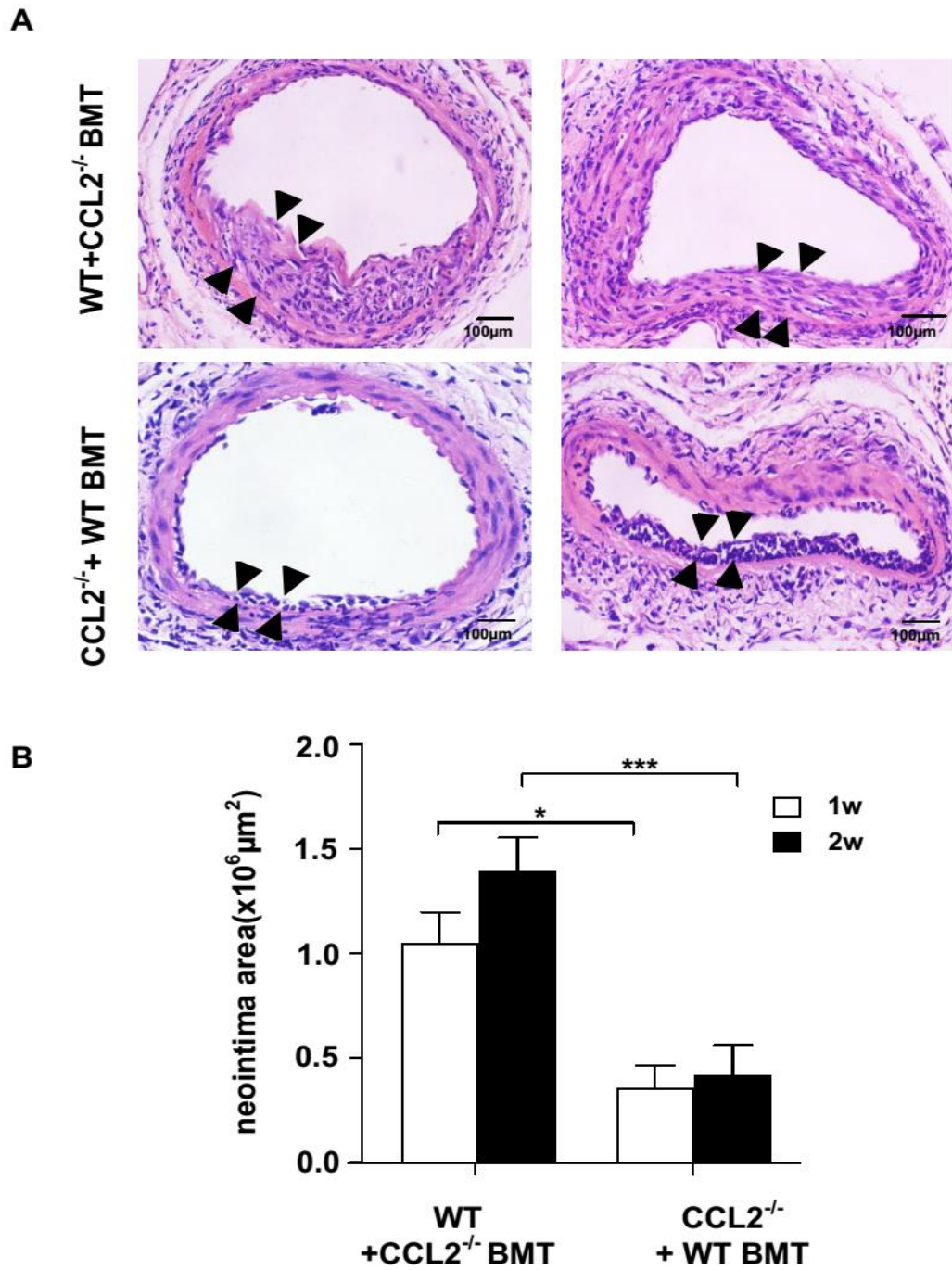


Figure 3.40 CCL2 released from peripheral tissue plays an important role in contribution to neointima formation. The vessel sections of indicated mice at various time points were stained with HE, and the neointima area (the area between arrows) is shown in the represented images (A) and quantified in the graphs (B) presented as mean \pm SEM of n=5 mice/group. *p<0.05, **p<0.01, ***p<0.001. WT+CCL2^{-/-} BMT, wild type mice in which bone marrow was transplanted from CCL2^{-/-} mice. CCL2^{-/-}+WT BMT, CCL2^{-/-} mice in which bone marrow was transplanted from wild-type mice. 1w, 1 week. 2w, 2 weeks.

3.8 CXCL1 plays a role in Sca-1 positive cell migration *in vivo*

Previous studies have demonstrated that CXCL1 can induce endothelial cell migration and tube formation *in vitro* (D. Wang et al., 2006) , and can also act as an angiogenic factor to promote tumour growth (Haghnegahdar et al., 2000). To further investigate the role of CXCL1 in the induction of VPC migration *in vivo*, VPCs were mixed with matrigel containing either PBS or mouse recombinant CXCL1 and injected subcutaneously in WT mice. After 2 weeks, the number of cells that migrated into the matrigel was found to be significantly more in the CXCL1 group than in PBS controls (Figure 3.41). Immunofluorescence staining of Sca-1, CD31 and α -SMA showed that most of the migrated cells in matrigel plugs were Sca-1 positive, whilst some were CD31 positive, and only very few were α -SMA positive (Figure 3.42). In addition, CXCL1 or control siRNA in a pluronic gel mixture was also delivered to the adventitial side of wire-injured vessels, to assess the effect of local CXCL1 knockdown on Sca-1⁺ VPC migration *in vivo*. Successful *in vivo* knockdown of CXCL1 mRNA level in injured femoral arteries was confirmed after 6 days, using real-time quantitative PCR (Figure 3.43), demonstrating a marked reduction in CXCL1 expression levels. Furthermore, *En face* confocal microscopy revealed that the number of migrated cells found on the intimal side of the vessel wall was lower in CXCL1 siRNA treated vessels compared to the control siRNA, 72 hours after seeding GFP-Sca-1⁺VPC in the adventitia (Figure 3.44). These results indicated the importance of CXCL1 in Sca-1⁺ cells migration *in vivo*.

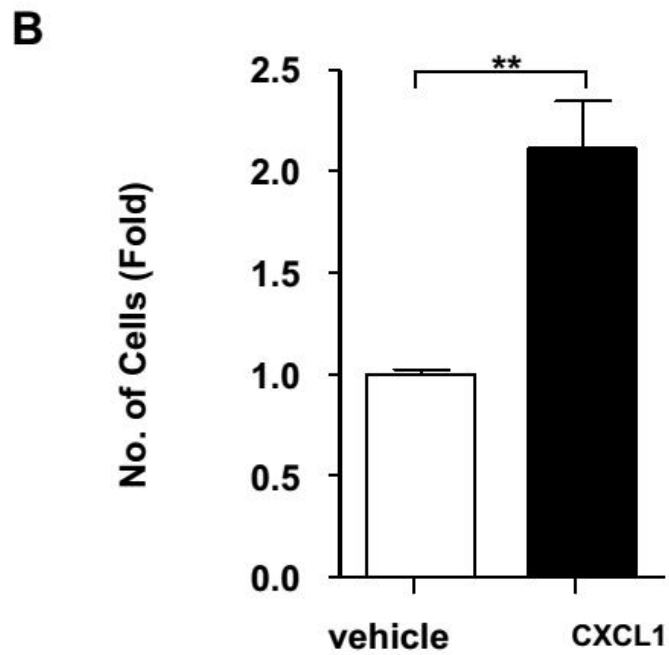
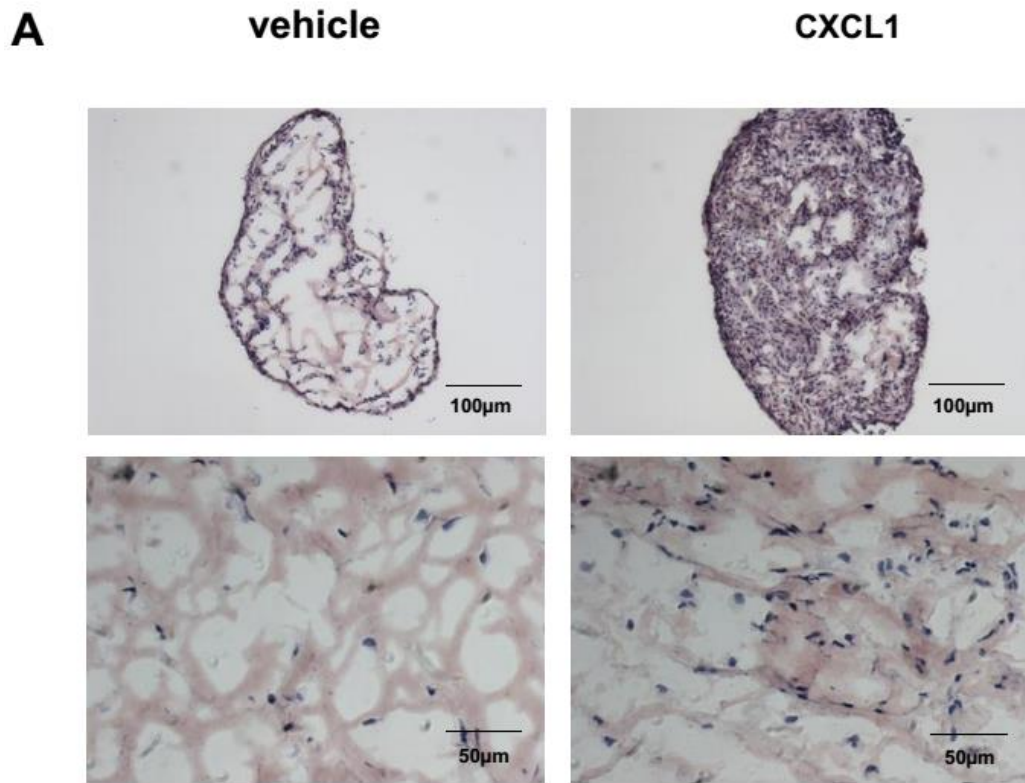


Figure 3.41 CXCL1 can induce cell migration *in vivo*. Using a matrigel plug assay, matrigel mixtures containing either vehicle (PBS) or mouse recombinant CXCL1 were injected subcutaneously into C57BL/6J mice. The matrigel plugs were harvested 2 weeks later and the frozen sections were stained with haematoxylin–eosin. Graphs are presented as mean±SEM of n=8, **P<0.01,

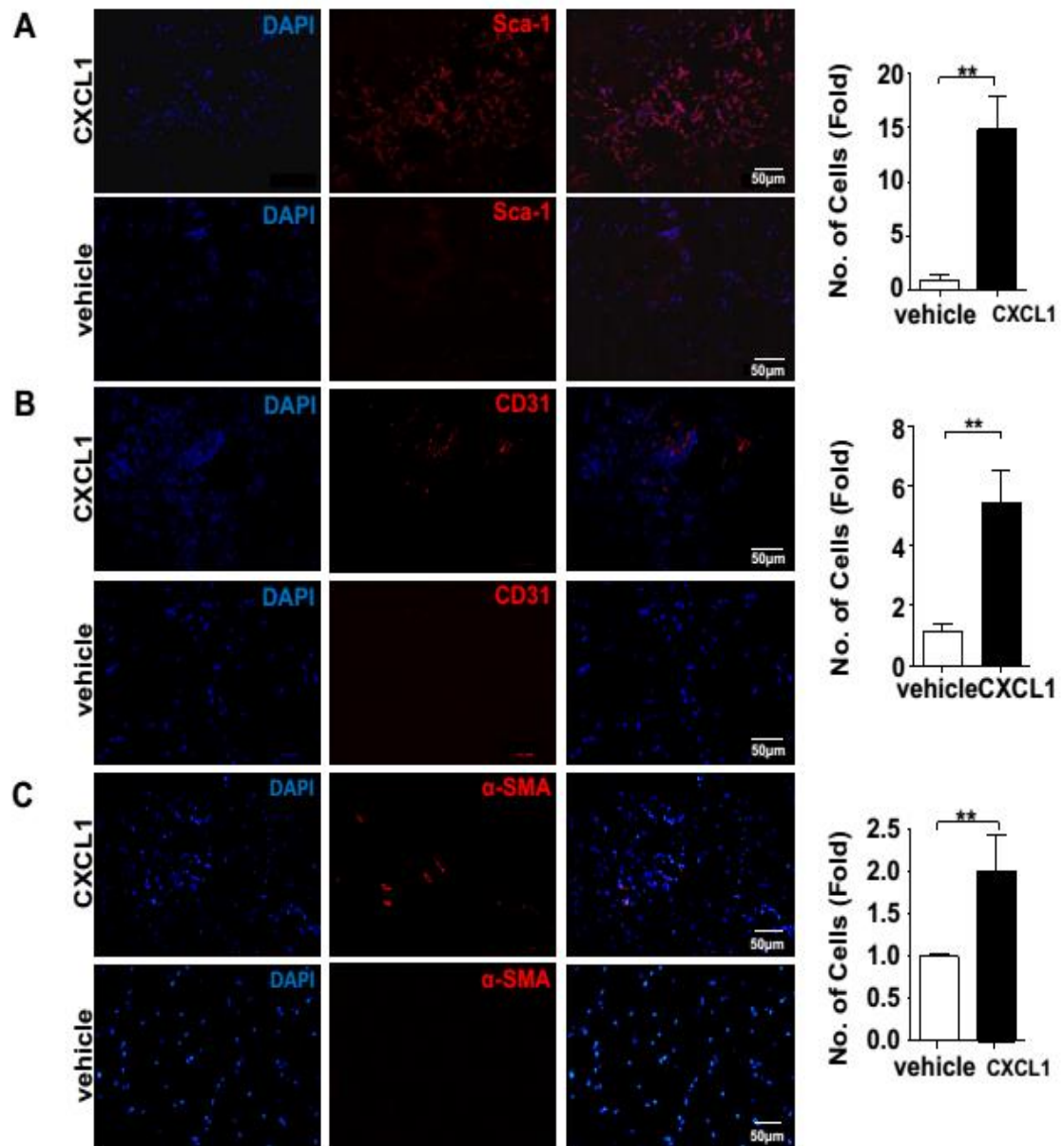


Figure 3.42 CXCL1 can induce Sca-1 positive cell migration *in vivo*. Using a matrigel plug assay, matrigel including PBS or mouse recombinant CXCL1 was injected subcutaneously into C57BL/6J mice. The matrigel plugs were harvested 2 weeks later and corresponding frozen sections were stained with immunofluorescent markers (Sca-1, CD31, α -SMA). The numbers of cells migrated into the matrigel (quantification from bottom images in A) or positively stained with each marker were quantified and shown in the graphs. Graphs are presented as mean \pm SEM of n=8, **P<0.01, ***P<0.001.

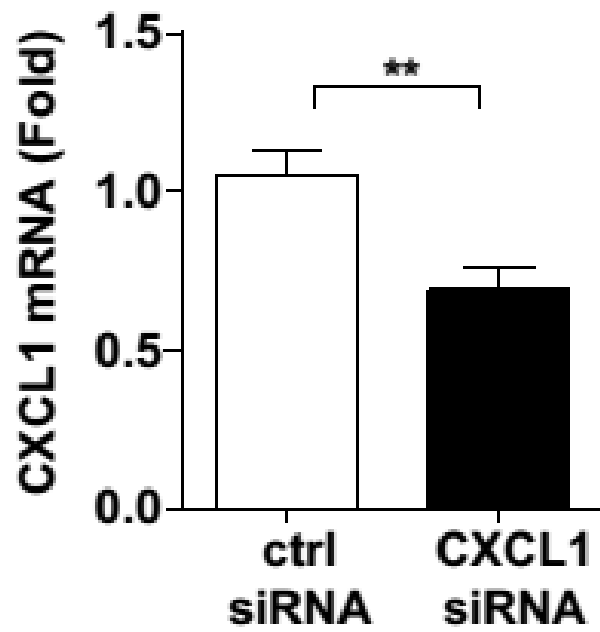


Figure 3.43 CXCL1 small interfering RNA knocked down CXCL1 mRNA level in arteries. Quantification of CXCL1 mRNA level by qPCR 6 days after perivascular application of CXCL1 or negative control siRNA dissolved in the pluronic gel. Graphs are presented as mean \pm SEM of n=4, **P<0.01

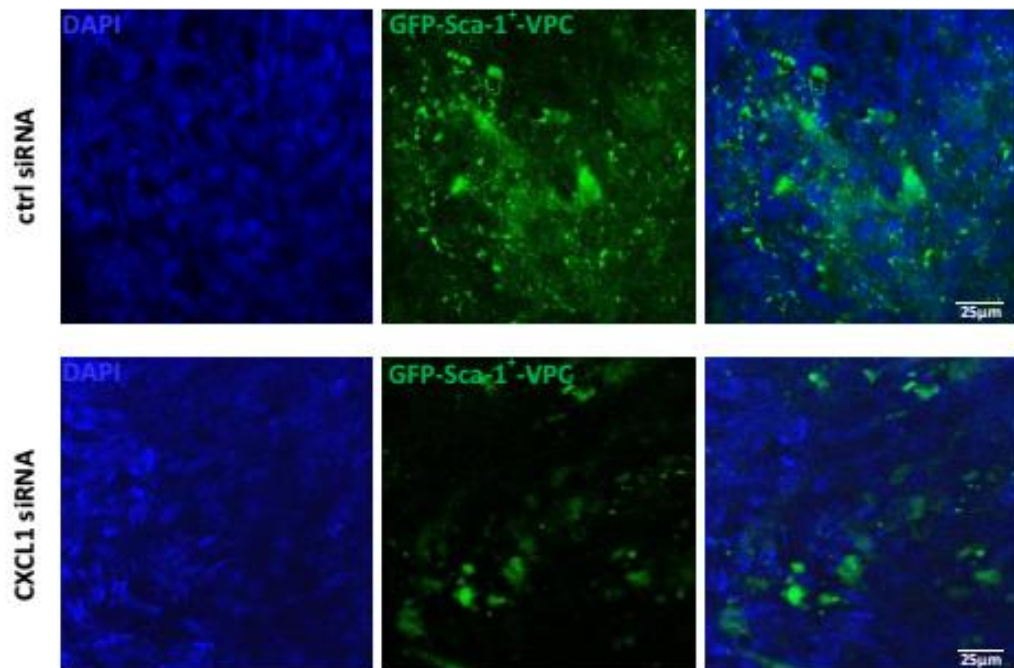


Figure 3.44 CXCL1 small interfering RNA inhibits Sca-1⁺ cells migration *in vivo*. Representative images of *En face* staining show that cells migrated to the intimal side of the vessels 72hrs post seeding GFP-Sca-1⁺-VPC (1×10^6) in the adventitia of femoral arteries treated with CXCL1 or control siRNA.

Chapter 4

Discussion

Overview

Atherosclerosis, as a main underlying pathogenesis of cardiovascular disease, is one of the leading causes of morbidity and mortality in the world. Currently, percutaneous coronary intervention (PCI) is the most effective treatment for obstructive coronary atherosclerosis. However, post-angioplasty restenosis is still the main complication that exacerbates the outcome of coronary artery disease treatment (Hoffmann et al., 1996; Keeley, Boura, & Grines, 2003; Serruys et al., 1988).

For many years, researchers have been working to elucidate the mechanisms behind post-angioplasty restenosis. Though neointimal hyperplasia and restenosis development involves a complicated series of cellular and molecular responses in the injured vessel, SMC proliferation and migration have putatively been viewed as central factors in these processes (Christopoulos & Havlir, 2013). However, in recent decades, adult stem/progenitor cells, which reside in the vascular wall, have been found to be activated and mobilised to participate in vascular remodelling after vessel injury (Hu et al., 2004). The exact mechanisms behind stem cells contribution to post-angioplasty restenosis are still uncertain.

In an attempt to explore the underlying cause of VPCs activation and mobilisation, a co-culture system of vascular SMCs and progenitor cells was developed and revealed that SMCs can release molecules into the supernatant, which stimulate progenitor cells migration towards them. To further investigate which molecules produce this effect, a multiple chemokine ELISA array was performed that showed the levels of CCL2 and CXCL1 were markedly increased in SMC conditioned medium when compared to controls. Treating progenitor cells with recombinant proteins CCL2 or CXCL1 significantly increased VPC migration. Furthermore, we found that the relationship between CCL2 and CXCL1 in progenitor cells migration is neither cumulative nor redundant.

Next, the mechanism of CCL2 and CXCL1 promotion of vascular progenitor cell migration was studied, by identification of their corresponding receptors CCR2 and CXCL2, which were upregulated on progenitor cells after stimulation with SMC conditioned medium. Blockage of

either chemokine in SMC conditioned medium or inhibition of either receptor on progenitor cells can abolish SMC-induced progenitor cell migration. Rho GTPase family members are important molecular switches which orchestrate rearrangement of the cytoskeleton to control cell migration, so, for this reason a GTPase Rac1/Cdc42 activation assay was carried out on progenitor cells treated with SMC conditioned medium. Both Rac1 and Cdc42 were activated into their GTP-bound forms. Antagonists of either Rac1 or Cdc42 can significantly reduce the migratory ability of progenitor cells. p38 phosphorylation was upregulated in SMC treated progenitor cells and inhibition of the p38 MAPK signalling pathway markedly abrogated progenitor cells migration. Furthermore, inhibition of Rac1 but not Cdc42 significantly diminished p38 phosphorylation, indicating that SMC-induced progenitor cell migration is via CCL2 or CXCL1 binding to their corresponding receptors and activating the Rac1/p38 signalling pathway. Additionally, the expression of cytoskeleton-related proteins, such as paxillin, vinculin and phosphorylated FAK were upregulated in SMC-treated progenitor cells.

Following *in vitro* mechanistic studies, animal models were utilised to further confirm CCL2 and CXCL1 roles in progenitor cell migration *in vivo*. In a femoral artery wire injury model, CCL2^{-/-} mice showed reduced progenitor cell migration from the adventitia to the intima in 3 days, and less neointima area after 2 weeks in comparison with WT mice. The comparison of the neointimal area in two types of chimeric mice (CCL2^{-/-} (BMT WT), WT (BMT CCL2^{-/-})) further indicates that the effect of CCL2 on progenitor cell migration is mostly dependent on peripheral-derived CCL2. Additionally, *in vivo* injection of CXCL1 within matrigel significantly attracted Sca-1⁺ cells migration. Furthermore, local application of CXCL1 siRNA may inhibit progenitor cells inward migration from the adventitia to the intima.

With these findings, we present the first evidence that vascular smooth muscle cell produced chemokines CCL2 and CXCL1 play a key role in mediating vascular progenitor cell migration from the adventitia to intima, where these cells contribute to neointimal formation in a mouse femoral artery wire injury model. This provides key evidence as to why restenosis and delayed re-endothelialisation persist after angioplasty and stenting and this may be fundamental for developing new drugs that can improve the long-term outcome of patients.

4.1 Characterization of vascular progenitor cells (VPCs)

In recent years, more and more studies from different laboratories have identified the presence of stem/progenitor cells that reside in the adventitia of vessel walls. They are thought to maintain the homeostasis of the vessel wall during physiological conditions but can be activated and participate in repair of the injured vessel under pathological conditions. They can differentiate into various cell types such as ECs or SMCs to participate in vascular repair or neointima formation, depending upon the variety of stimulations under the specific circumstance. Nevertheless, a conundrum remains that the identification and characterisation of adventitia progenitor cells still lack consistency between different research groups (Table 4.1). To date, there remains a lack of specific progenitor cell markers that can exclusively characterise and distinguish these progenitor cells from other cell types. Furthermore, several important questions still remain to be addressed: 1) What are the differentiation capacities of the vascular progenitor cells into ECs or SMCs? 2) What are their roles in vascular repair, re-endothelialisation and neointima formation? 3) Do their responses to stimuli differ while in the same microenvironment? 4) Why are the conclusions from different research groups so varied? Thus, there is still much progress to be made in the field of progenitor cell research. Although some progenitor cells are identified by specific markers using *in situ* immune-staining in the vessel wall or antibody selection from isolated cells, it is still uncertain whether they are activated after vascular injury and differentiate to play a role in vascular events. The current models of differentiation *in vitro* are unable to replicate the dynamic and complicated circumstances progenitor cells exist in *in vivo*.

Table 4.1 Vascular stem/progenitor cells in adventitia

Year	2004	2006	2007	2008
Reference	(Hu et al., 2004)	(Zengin et al., 2006)	(Pasquinelli et al., 2007)	(Passman et al., 2008)
Designation	Vascular progenitor cells	Vascular wall resident EPCs	Angiogenic MSCs	Smooth muscle progenitor cells
Source	ApoE ^{-/-} mouse	Human internal thoracic artery	Human thoracic aorta	Mouse embryonic/adult arteries
Cell markers	Sca-1 ⁺	CD34 ⁺ VEGFR2 ⁺ Tie2 ⁺ CD105 ⁻ CD144 ⁻	CD34 ⁺ C-kit ⁺	Sca-1 ⁺
Differentiation potential and contribution	SMC, atherosclerosis hyperplasia	EC, Hematopoietic cells	EC	SMC

year	2010	2013	2014	2016
Reference	(Pasquinelli et al., 2010)	(Tigges, Komatsu, & Stallcup, 2013)	(Psaltis et al., 2014)	(Sitnik et al., 2016)
Designation	Mesenchymal vascular cells	Adventitial pericyte-like cells or MSCs	Adventitial macrophage progenitor cells	Adult adventitial progenitors
Source	Human arteries	Mouse injured femoral artery	Mouse aortic adventitia	Human lymphoid adventitia
Cell markers	Oct-4 ⁺ Stro-1 ⁺ Sca-1 ⁺ Notch ⁺	NG2 ⁺ CD146 ⁺ PDGFR ⁺	Sca-1 ⁺ CD45 ⁺	CD34 ⁺
Differentiation potential and contribution	Adipogenic Chondrogenic Osteogenic	SMC, post-injury restenosis	Macrophages	Pericytes, Reticular cells, Dendritic cells

In 2004, Hu et al. were the first to report that a population of Sca-1⁺, c-kit⁺, CD34⁺, Flk-1⁺ progenitor cells residing in the adventitia of ApoE^{-/-} mice aortic roots contributed to hyperplasia in atherosclerosis. They found that these Sca-1⁺ adventitia progenitor cells can differentiate into SMCs in response to PDGF-BB *in vitro*. Furthermore, a chimeric mouse model was generated to demonstrate that Sca-1⁺ cells carrying the Lac Z gene can migrate from the adventitia to

contribute to neointima lesions in a vein graft model and simultaneously differentiated into LacZ-SMCs *in vivo*. This study postulates that adventitia Sca-1⁺ cells have the capacity to migrate across vessel walls and subsequently differentiate into SMCs to contribute to neointima formation. Subsequently, our laboratory also identified another population of progenitor cells derived from the adventitia of vein grafts, which are Sca-1⁺ and are able to differentiate into SMCs in response to Collagen IV. An *ex vivo* model additionally revealed increased migratory abilities of these progenitor cells in response to SDF-1 and in a carotid artery vein graft model of ApoE^{-/-} mice, the arteries enveloped with these progenitor cells showed significant exacerbation of atherosclerotic lesions.

In our study, we chose to utilise vein graft-derived adventitia Sca-1⁺ progenitor cells because these cells are derived from the pathological adventitia of an injured vessel wall, which is more comparable to the disease microenvironment. Application of these Sca-1⁺ vein graft progenitor cells to the wire-injured artery model appears a reasonable representation of the disease setting. Several seminal works on Sca-1⁺ progenitor cell differentiation have been published by our lab (Y. Chen et al., 2013; Hu et al., 2004; Wong et al., 2013), but work is lacking regarding their migratory behaviour and their role in contribution to neointima formation. We chose to study mouse due to easy access to specific gene knockout models and their fast breeding in comparison to animals with a circulatory system more similar to humans. We think our findings will be informative for future study of human disease.

The aim of the present study was to further examine the underlying mechanisms of the contribution of these pathological adventitia progenitor cells to neointima formation in injured vessels. Our data demonstrate that chemokines and their corresponding receptors play a key role in SMCs-induced Sca-1⁺ progenitor cell migration. Whilst there exists an abundance of studies that demonstrate chemokine receptor expression on various cell types such as granulocytes, monocytes, mast cells, T cells, and endothelial cells (de Nigris, Schiano, Infante, & Napoli, 2012; Katsumoto & Kume, 2013), little is known about the presence/absence of chemokine receptors on vascular progenitor cells. We further characterised these vein graft adventitia-derived Sca-1⁺ progenitor cells by screening their gene expression levels of 84 different

chemokines and chemokine receptors. Unstimulated VPCs were found to express several chemokines and a number of chemokine receptors, however at markedly lower levels in comparison to SMCs (the main cell type in the vessel wall) and macrophages (a positive control). These data provide the first evidence of the presence of chemokines and their receptors in VPCs and suggest chemokines have a potential role in mediating VPC migration.

4.2 SMCs induce VPC migration via CCL2 and CXCL1

SMCs are the main type of cell in the vascular wall and play a crucial role in both physiology and pathology of vessel events. It has been well established that SMCs change their phenotypes from contractile to synthetic after vessel injury and that their proliferation and migration have a major effect on neointima formation (Y. Cheng et al., 2009; Rudijanto, 2007; Yoshida, Kaestner, & Owens, 2008). Furthermore, activated SMCs can also release inflammatory cytokines and growth factors, resulting in an inflammatory microenvironment in the vascular wall after vessel injury (Fabunmi, Baker, Murray, Booth, & Newby, 1996; Rakesh & Agrawal, 2005). Many studies have shown that cytokines and growth factors are potent molecules that can mobilise circulating progenitor cells (Sprague & Khalil, 2009). However, only a few studies are focused on whether these local inflammatory factors can also activate resident progenitor cells in the vascular wall. As they are known to be located within close proximity to SMCs within the vessel wall, it is tempting to postulate that adventitia progenitor cells can also be activated by inflammatory molecules existing in the same microenvironment.

In our study, we demonstrated that proliferating SMCs can significantly induce vascular progenitor cell migration using *in vitro* migration assays and single cell tracking experiments in real time, indicating that the cells are migrating directionally and efficiently but not randomly. *In vivo*, GFP-Sca-1⁺ cells were applied to the adventitial side of injured femoral arteries, after 72 hours GFP⁺ cells can be easily detected on the intimal side by *en face* staining, while, 2 weeks later, neointima formation was significantly increased in these treated vessels compared to injured vessels where no cells had been applied. These data indicate that either when cultured *in vitro* or when injured *in vivo*, mouse SMCs are transformed into a proliferative phenotype, and in this condition, they are a potent attractant for VPC migration both *in vitro* and *in vivo*.

After the endothelial injury, an inflammatory response occurs in the vessel wall and chemokines can be released from both mononuclear cells and SMCs and participate in vascular events (Saederup, Chan, Lira, & Charo, 2008; Veillard et al., 2005). In our study, we utilised a wire injury artery model, which caused endothelium denudation. We assumed that after this injury,

the major cell type remaining in the vessel wall would be activated SMCs which, would transmit an inflammatory response to neighbouring adventitia progenitor cells through chemokines. A multiple chemokine ELISA on supernatant from cultured activated SMCs demonstrated that several chemokines were up-regulated. Amongst them, CXCL1, CCL2 and CCL5 show the highest level of secretion. It is also noteworthy that in a qPCR array, the gene expression levels of both CCL2 and CXCL1 were much higher in SMCs when compared to other cells, which is consistent with our finding at the protein level. Furthermore, *in vivo*, immunofluorescence staining of α -SMA, CCL2 and CXCL1 on femoral arteries revealed that few chemokines are expressed on the intact vessel wall, however after injury, there is a time-dependent increase in CCL2 and CXCL1, much of which co-localises with SMC markers. This demonstrates that once activated, SMCs can release large amounts of CCL2 and CXCL1.

We found that stimulation of VPCs with recombinant CXCL1 and CCL2 stimulated VPC migration, however, stimulation with CCL5 did not.

CXCL1 is a chemokine which has been studied in cardiovascular disease by a number of research groups in recent years. Oxidized LDL has been reported to trigger CXCL1 release from endothelial cells, which recruit atherogenic monocytes to promote atherosclerosis (Zhou et al., 2011). Additionally, CXCL1 is expressed on human endothelial cells and induces angiogenesis through enhancement of endothelial cell migration and proliferation (Miyake, Goodison, Urquidi, Gomes Giacoia, & Rosser, 2013). Microarray data revealed that the basal level of CXCL1 mRNA in untreated mouse aorta SMCs is low, but that it is significantly upregulated after TNF or/and α -LT β R treatment (Lotzer et al., 2010). The CXCL1 expressed on human arterial SMCs mediates endothelial progenitor cell adhesion in a flow chamber (Hristov et al., 2007). In rat aortic SMC, the mRNA level of CXCL1 was markedly enhanced by depletion of PTEN (Furgeson et al., 2010).

In contrast, CCL2 involvement in atherosclerosis and neointima formation is well established. In the vessel wall, CCL2 has been shown to be released by both endothelial cells (Loyer et al., 2014; Rollins et al., 1990; Takahara, Kashiwagi, Maegawa, & Shigeta, 1996) and smooth

muscle cells (X. L. Chen, Tummala, Olbrych, Alexander, & Medford, 1998; Cushing et al., 1990; Meng, Yan, Deng, Gao, & Niu, 2013), and to facilitate inflammation and induce SMC proliferation and migration.

We also identified CCL5 expression in the multi-analytic ELISA. The CCL5 expression has been identified in endothelial cells of transplant-associated accelerated atherosclerotic arteries (Pattison, Nelson, Huie, Sibley, & Krensky, 1996). It is produced by endothelial cells in response to TNF- α and IFN- γ , which regulates macrophage and T memory lymphocyte accumulation (Marfaing-Koka et al., 1995). Following artery injury, CCL5 has also been shown to be released by vascular smooth muscle cells and to induce an acute vascular inflammatory response by recruitment of inflammatory cells (Kovacic et al., 2010). Previous studies have reported that CCL5 mediates trafficking and homing of T cells, monocytes, basophils and eosinophils (Alam et al., 1993; Bischoff et al., 1993; Schall, Bacon, Toy, & Goeddel, 1990; Sun, Wang, Zhang, Zeng, & Wang, 2005). However, in our system, the migration of progenitors after stimulation with CCL5 did not change significantly when compared to the control. This result may indicate that CCL5, as a versatile chemokine, is upregulated in SMCs but does not play a functional role in cell migration, suggesting that vascular stem cells may selectively or specifically respond to certain chemokines.

To further confirm that the induction of VPC migration by SMC-conditioned medium was mediated by these chemokines in our study, we stimulated stem cells with exogenous mouse recombinant proteins CCL2, CXCL1 and CCL5 individually, and found that CCL2 and CXCL1 can significantly induce vascular progenitor cell migration. More importantly, the concentrations of each chemokine which displayed the optimum migration results were in the same range as the concentrations measured in SMC conditioned medium. In contrast following knockdown of CCL2 or CXCL1 in SMCs by their respective siRNAs, there was a significant decrease in VPC migration towards conditioned medium. Meanwhile, a BrdU assay revealed that during VPC migration, neither SMC conditioned medium nor CCL2 or CXCL1 affected VPC proliferation, which ruled out any proliferative effects on analysis of cell migratory ability. As progenitor cells possess multipotent differentiation ability, they may spontaneously

differentiate into mature vascular cells without LIF in their culture medium, so we tested SMCs and endothelial cells markers in the VPCs which had migrated. The results excluded the effects of differentiation during migration.

4.3 The mechanism underlying CCL2 and CXCL1 induced VPC migration

As the above results show, CCL2 and CXCL1 contribute to VPC migration equally, so we wanted to further investigate possible interactions between the two chemokines. At inflammatory sites, more than one chemokine takes an effect and they may cooperate with others to activate subsequent downstream signalling events. Many studies have reported synergistic effects of chemokines in leukocyte recruitment and migration, which enhance inflammation both *in vitro* and *in vivo*. For example, CXCL6 acts with CCL2 and regakine-1 acts IL-8 to induce neutrophil mobilisation synergistically (Gijssbers et al., 2005; Gouwy, Struyf, Catusse, Proost, & Van Damme, 2004); and the co-injection of CXCL10 with CCL5 significantly enhances T lymphocytes recruitment *in vivo* (Stanford & Issekutz, 2003). However, in our study, simultaneous depletion of two chemokines CCL2 and CXCL1 in SMC-conditioned medium did not further inhibit VPC migration when compared to inhibition of a single chemokine, thus indicating that there is no additive effect on VPC migration due to CCL2 acting with CXCL1.

To ensure some fundamental activities are carried out *in vivo*, redundancy is an important characteristic of many cell signalling pathways, particularly in chemokine and chemokine receptor activity (Mantovani, 1999). This redundancy is present in several forms. Chemokine receptors expressed on leukocytes usually bind to multiple chemokines to produce the same response. For example MCP-1, 2, and 3 can similarly bind to CCR2 on monocytes and activated T lymphocytes and NK cells (Campbell et al., 2001) and both IL-8 and CXCL1 bind to CXCR2 on neutrophils (White et al., 1998). This also explains why chemokine receptor knockout mice do not always have the same phenotype as mice in which one of the corresponding chemokines was knocked out (Boring et al., 1997; Gu et al., 2000). For this reason, in order to precisely study the functional roles of CCL2 and CXCL1 *in vivo*, we chose CCL2^{-/-} mice and applied CXCL1 siRNA locally rather than using CCR2^{-/-} and CXCR2^{-/-} mice.

A single cell can also produce many different chemokines in response to a single stimulus. Homocysteine induces human aortic endothelial cells to release both MCP-1 and IL-8 (Poddar, Sivasubramanian, DiBello, Robinson, & Jacobsen, 2001), and enriched monocytes stimulate HUVEC to produce both IL-8 and MCP-1 (Lukacs et al., 1995). Both MCP-1 and MIP-1 β were strongly expressed on macrophages in multiple sclerosis lesions (Simpson, Newcombe, Cuzner, & Woodroffe, 1998). As mentioned previously, we also found that several chemokines were released from proliferating SMCs, such as CCL2 and CXCL1 which confirmed this redundant chemokine production.

In addition, there is redundancy in that target cells can be activated in the same way by multiple chemokines, for example the CXCR3 ligands CXCL9 and CXCL10 play a redundant role in mediating obliterative bronchiolitis in mice (Medoff et al., 2006) and both CCR7 and CXCR4 contribute to B cell homing to lymph nodes (Okada et al., 2002). In the present study, depletion of either CCL2 or CXCL1 partially inhibits VPCs migration, but treatment with each individual chemokine fails to stimulate VPCs to release the other. Thus, we conclude that after the loss of one chemokine, the reduced induction of VPCs migration is not compensated for by the other chemokine. Simultaneous depletion of both chemokines did not further inhibit VPC migration when compared to inhibition of a single chemokine. Taken together, the effects of CCL2 and CXCL1 are neither cumulative nor redundant in the mediation of VPCs migration. There may also be additional factors in SMC conditioned medium which play a role in inducing VPC migration as the migratory ability was not completely abolished on depletion of both chemokines. Further experiments are required to determine what other chemotactic factors would additionally effect VPC migration.

As many reports have shown, CCL2/CCR2 and CXCL1/CXCR2 signal transduction mediates recruitment of neutrophils, monocytes or macrophages into inflammatory sites during progression of various diseases (Boisvert et al., 2006; Boring et al., 1998; J. Dawson, Miltz, Mir, & Wiessner, 2003; Ritzman et al., 2010; Shea-Donohue et al., 2008). Most studies report the expression of CCR2 and CXCR2 on inflammatory cells, such as monocytes/macrophages (Lei et al., 2002; C. Weber et al., 1999), neutrophils (Rose, Foley, Murphy, & Venkatesan, 2004) and

T lymphocytes (Lippert, Zachmann, Henz, & Neumann, 2004; Sebastiani et al., 2001). Several studies have also demonstrated that CCR2 is present on vascular wall cells. CCR2 was detected on human umbilical cord vein ECs and found to mediate a role for CCL2 in endothelial wound repair (K. S. Weber, Nelson, Grone, & Weber, 1999) and angiogenesis (Salcedo et al., 2000). CCR2 was also found to be activated in response to CCL2 in rat aortic smooth muscle cells and to alter SMC function in age-related arterial remodelling (Spinetti et al., 2004). CCR2 has also been identified on human saphenous vein SMCs where it mediates the induction of vein graft thickening by CCL2 (Schepers et al., 2006). In contrast, there are fewer studies into the expression of CXCR2 on vascular cells. CXCR2 has been found on human intestinal microvascular endothelial cells and umbilical vein endothelial cells and to induce angiogenic events, including endothelial cell proliferation and migration in response to CXCL8 (Heidemann et al., 2003; A. Li, Dubey, Varney, & Singh, 2002). IL-8 increased both CXCR2-expressing human and mouse endothelial monolayer permeability, to induce vascular leakage-related disease (Gavard et al., 2009). Interestingly, a study detected CXCR2 in intima/media layers of the coronary artery, which mediated IL-8-downregulation of VCAM-1 production by SMC (Hastings, Feaver, Lee, Wamhoff, & Blackman, 2009). Therefore, in this case, we are not able to exclude that other cells which express CCR2 or CXCR2 in the vessel wall, such as migrated endothelial cells. However, as most endothelial cells were detached during wire injury and the VPCs possess the higher capacity of migration into the neointima than SMCs, we can deduce that most cells expressing CCR2 and CXCR2 in our data are VPCs.

In our study, we found that basal levels of CCR2 and CXCR2 expression were much lower on VPCs in comparison to macrophages, potentially due to their quiescent status in conditions without stimuli. However, following treatment with SMC conditioned medium, both gene and protein levels of the receptors were significantly up-regulated. The ability of VPCs to migrate after CCR2 and CXCR2 genes were permanently silenced by shRNA was significantly reduced. Furthermore, the application of antagonists of each chemokine markedly abrogated induction effects of SMC on VPC migration, confirming the gene silencing results. Thus our data provide

the first evidence of expression of CCR2 and CXCR2 on vascular progenitor cells in response to SMC-derived soluble factors. Together, these results also indicate that SMC-induced VPC migration is mediated by both the CCL2/CCR2 and CXCL1/CXCR2 axis.

To elucidate the mechanism(s) of VPC migration, we studied the roles of the Rho GTPase family. Cdc42, Rac1 and RhoA are the main Rho GTPase family members that are known to regulate the formation of lamellipodia, filopodia and focal adhesions, respectively, thus orchestrating subsequent cell movement (Nobes & Hall, 1999). Some reports have demonstrated that chemokines induce cell migration via activation of Rho GTPase family members. RhoA, Rac1 and Cdc42 are all activated in the regulation of SDF-1 induced human gastric cancer cells and T cell migration (G. Chen et al., 2012; del Pozo, Vicente-Manzanares, Tejedor, Serrador, & Sanchez-Madrid, 1999). CXCL12 activates Rac1 during its regulation of lung alveolar epithelial cell migration (Ghosh, Makena, Gorantla, Sinclair, & Waters, 2012). Depletion of Cdc42 in dendritic cells resulted in a reduction of cell migratory abilities driven by CCL19, and in comparison with WT cells, their velocity decreased 20%, but directionality dropped 40% (Lammermann et al., 2009). Inhibiting RhoA activity using a dominant-negative mutant can significantly down-regulate CCL19 elicited T cell migration (Rougerie et al., 2013).

In our study, we found that both Cdc42 and Rac1 in VPCs were activated by either CCL2 or CXCL1 via their respective receptors CCR2 and CXCR2. To determine whether Cdc42 and Rac1, as other studies have shown, also play roles in CCL2 or CXCL1-induced VPC migration, we applied their specific inhibitors in migration assays. The inhibition of either Cdc42 or Rac1 impaired VPC migration in response to CCL2 or CXCL1. This indicated that both CCL2 and CXCL1 promoted VPC migration were mediated by activated Cdc42 and Rac1.

In contrast, the addition of a RhoA inhibitor did not alter VPC migration driven by SMC conditioned medium or CCL2, CXCL1, which revealed that the RhoA signalling pathway is not involved in VPC migration.

Additionally, p38 MAPK, as a stress-activated protein kinase, has been widely reported to be associated with cell migration in response to extracellular stresses. Aberrant shear stress induces

human mesenchymal stem cell migration to wounded areas through the CXCL12/CXCR4/p38 MAPK signalling pathway (Yuan, Sakamoto, Song, & Sato, 2013). p38 phosphorylation is also involved in CXCL1-induced leukocyte migration in mice (Cara, Kaur, Forster, McCafferty, & Kubes, 2001). Cytokines such as IL-4 and TNF- α can activate stress-activated pathways leading to phosphorylation of p38 MAPK, which is dependent on Rac1 and Cdc42 (Wery-Zennaro, Zugaza, Letourneur, Bertoglio, & Pierre, 2000). PAK1, as an established effector of Rac1 and Cdc42, mediates tracheal smooth muscle cell migration by activation of the p38 MAPK pathway (Dechert et al., 2001). In our study, p38 phosphorylation was up-regulated in response to mouse recombinant CCL2 or CXCL1 and was suppressed by CCL2/CXCL1 knockdown or CCR2/CXCR2 inhibition, which indicates that CCL2 and CXCL1 activate p38 phosphorylation via CCR2 or CXCR2. These results suggest that progenitor cell migration is induced by CCL2 or CXCL1 through the p38 MAPK signalling pathway. We also found that p38 phosphorylation was markedly down-regulated by Rac1 inhibition but not inhibition of Cdc42 or RhoA, suggesting that progenitor migration is induced via a Rac1/p38 signalling pathway. Although the migration assay showed the participation of Cdc42 in SMC-induced progenitor migration, it may act through an as yet unidentified signalling pathway, for example, CCL19 activates ERK1/2 and JNK, but not p38 phosphorylation in migration of cells in squamous cell carcinoma of the head and neck via the PI3K/Cdc42 signalling pathway (Liu et al., 2014; Zhao et al., 2011).

In addition, FAK, paxillin and vinculin are critical in responding to various extracellular stimuli (e.g. chemokines) to regulate cell adhesion molecule structures and the cytoskeleton and to process the signalling pathways for cell mobility. It has been reported that CXCL12 can induce pro-adhesion of progenitor B cells mediated by FAK phosphorylation (Glodek et al., 2007; Le, Honczarenko, Glodek, Ho, & Silberstein, 2005). Suppress of cytokine signalling 3 regulates Pro-B cells development through the CXCL12/CXCR4/FAK signalling pathway (Le et al., 2007). In another study, CXCL8-induced FAK phosphorylation is adhesion-dependent in CXCR2-expressing cells but adhesion-independent in CXCR1-expressing cells (Cohen-Hillel et al., 2006), and phosphorylated FAK can subsequently activate paxillin which plays an important role in leukocyte migration (Cohen-Hillel, Mintz, Meshel, Garty, & Ben-Baruch, 2009).

Furthermore, CCL5 was reported to activate T lymphocytes via FAK, paxillin and ZAP-70 compound (Bacon, Szabo, Yssel, Bolen, & Schall, 1996). MCP-1-induced lymphocyte adhesion to endothelial cells regulates vinculin and paxillin expression in lymphocytes (Mullaly, Moyse, Nelson, & Murray, 2002). In our study, immunofluorescence staining revealed that SMC-conditioned medium induced FAK phosphorylation, paxillin and vinculin-regulated VPC migration. Further studies could be focused on the interactions between CCR2/CXCR2 and cytoskeleton proteins and Rho GTPase in the signalling pathways.

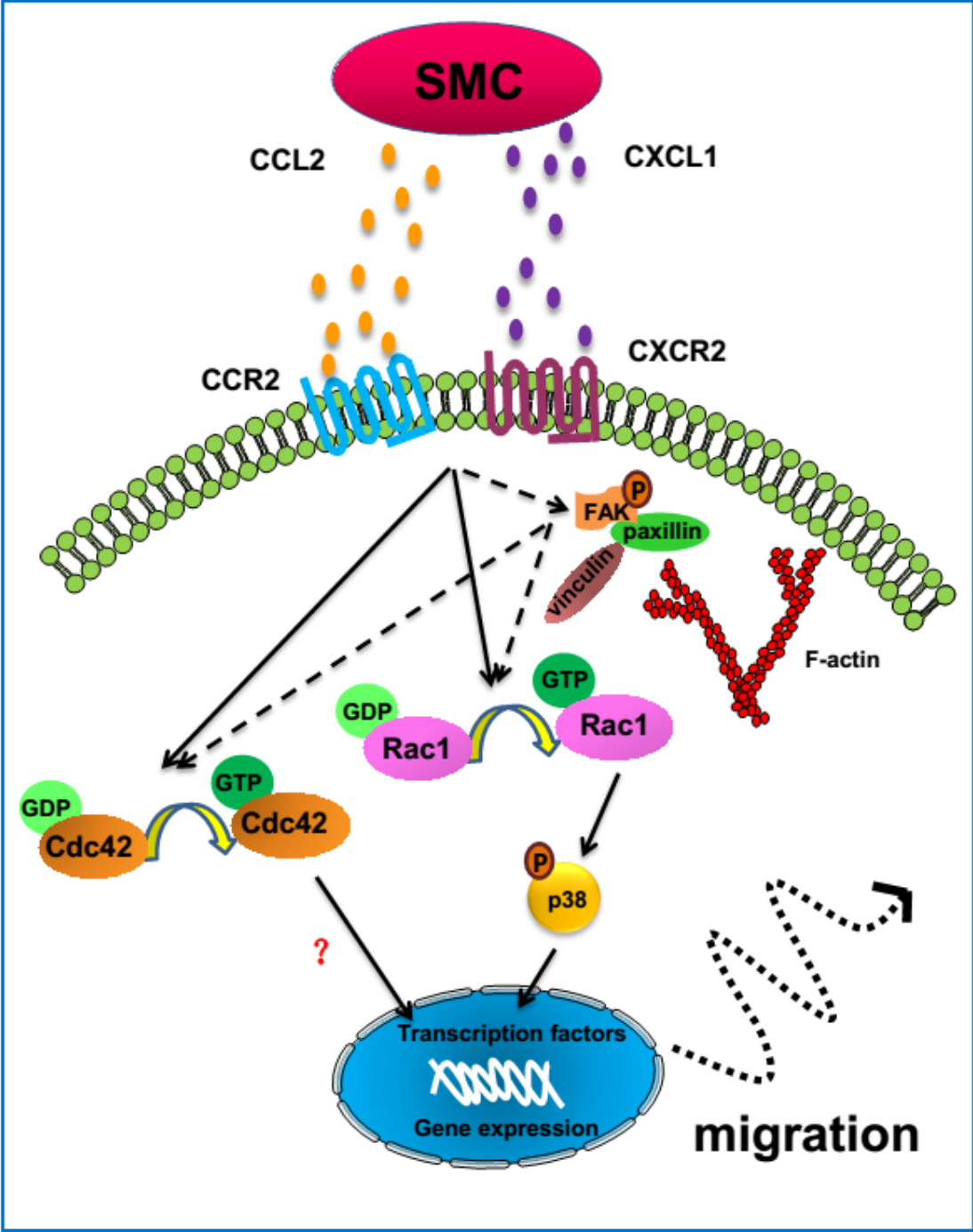


Figure 4.1 Schematic illustration of the roles of CCL2 and CXCL1 released from SMCs in enhancing VPCs chemotaxis. SMCs release CCL2 and CXCL1 into the medium. When VPCs are treated with this SMC-CM, these chemokines bind to their corresponding receptors CCR2 and CXCR2 on the VPCs. The GTPases Rac1 and Cdc42 become activated and then p38 is phosphorylated via Rac1, finally leading to increased VPC migration. SMC-CM also induces expression of cytoskeleton-related proteins paxillin, vinculin and phosphorylated FAK, which may also activate the Rac1 or Cdc42 signalling pathways.

4.4 The role of CCL2 and CXCL1 in VPC migration *in vivo*

Reports using different animal models have shown that CCL2 plays an important role in neointimal hyperplasia (Egashira et al., 2002; Tanaka, Sata, Hirata, & Nagai, 2003; Usui et al., 2002). Most studies suggest that CCL2 recruits monocyte/macrophages. In ApoE^{-/-} mice, after wire injury, CCL2 was immediately released by SMCs in the vessel wall and this was associated with platelets adherence to the denuded endothelium and subsequent mediation of monocyte recruitment at an early stage to induce neointima formation (Schober et al., 2004). In another femoral artery ligation model, local infusion of CCL2 significantly increased collateral hindlimb flow, which was accompanied by increased infiltration of monocytes, both in the perivascular and whole blood system. (van Royen et al., 2003). Furthermore, intramuscular transfection of a mutant CCL2 gene in hypercholesterolemic rabbits using a balloon injury model, confirmed the facilitative role of CCL2 in monocyte infiltration and restenosis (Mori et al., 2002). Other studies have demonstrated that CCL2 enhances SMC migration, proliferation and invasion to remodel vessels. Blockage of CCR2 using 7ND-CCL2 gene transfer in ApoE^{-/-} mice diminished vein graft-induced neointima hyperplasia through a direct inhibitory effect on SMCs proliferation (Schepers et al., 2006). Increased expression of CCL2 and CCR2 in aged rat aorta enhanced migration and invasion of SMCs (Spinetti et al., 2004). All these studies indicate that CCL2 is a crucial factor in the development of neointima formation by association with different essential factors.

It is noteworthy that CCL2-mediated neointimal hyperplasia not only formed in hyperlipidemic animals (e.g. ApoE^{-/-} mice), but has also been found in animals with normal blood lipid (e.g. C57BL/6J mice, Wistar-Kyoto rats, Sprague-Dawley rats and cynomolgus monkeys) (Egashira et al., 2002; Furukawa et al., 1999; Usui et al., 2002). For this reason, as well as due to the time-consuming nature of creating CCL2^{-/-}ApoE^{-/-} mice, in our study we chose C57BL/6J mice as the background of the control and used CCL2^{-/-} mice to validate the role of CCL2 in vascular progenitor cell driven neointima formation.

Vascular progenitor cells have previously been identified as playing a role in neointima formation (Y. Chen et al., 2013; Hu et al., 2004), in the present study, the immunofluorescence staining of wire injury-induced neointima confirmed that after 2 weeks approximately 70% of α -SMA⁺ cells and about 10% cells in the neointima were Sca-1⁺ cells. We then applied Sca-1⁺ progenitor cells to the adventitia of wire-injured femoral arteries to confirm the role of CCL2 in Sca-1⁺ progenitor cells-induced neointima formation. We found that CCL2 not only enhanced VPC migration from the adventitia to the intima in a short time period (3 days) but also accelerated neointima formation (in 1-2 weeks) by inducing VPC migration. We demonstrated that migrated progenitor cells are an important cellular component of CCL2 mediated neointima formation because CCL2 knockout resulted in a lower number of progenitor cells and reduced lesion formation. We also identified that the majority of cells contributing to neointima formation in our system were not α -SMA⁺ but express a stem cell marker (Sca-1), which indicates that though some VPCs participating in neointima formation differentiate into SMCs, most of them retain progenitor cell characteristics as they migrate. This effect was diminished by knockout of CCL2 in mice. From the above data, we confirmed other studies' viewpoints on the role of CCL2 in SMCs-contributed neointima formation. Additionally we showed that the ratio of α -SMA⁺ cells to other cells in the neointima is much lower in VPC seeded arteries than control arteries, which indicates that no matter whether these α -SMA⁺ cells are media-derived SMCs or VPC differentiated SMCs, VPC may inhibit SMCs contribution to neointima formation by their higher capacity of migration towards the intima and their maintenance of stem cell properties. A possible explanation for the higher migratory ability of the explanted/cultured VPCs compared to vascular resident VPCs might be due to the difference between culture conditions their original niche and the microenvironment in the vessel wall. To maintain local microenvironment homeostasis in the vessel wall, some inhibitory factors for stem cell migration may exist preventing resident VPC from showing as high motility as cultured VPCs. Furthermore, based on our experience of culturing VPCs *in vitro*, they display a higher proliferation and migration property only when they are seeded at a high cell density, so *in vivo*, we seeded 1×10^6 cells outside each injured vessel to maintain their higher migratory capacity. We provide the first evidence that CCL2 is crucial for vascular progenitor migration

from the adventitia to the intima where they contribute to lesion formation, but more work needs to be done to observe native adventitial cells.

In addition, as CCL2 takes its effect via CCL2/CCR2 axis, the significantly enhanced expression of CCR2 in neointimal cells in VPC seeding group suggests that the participation of VPCs in neointima formation may be dependent on CCL2-driven recruitment. However, according to previous published studies, monocytes (Han et al., 2000), peripheral EPCs (Spring, Schuler, Arnold, Hammerling, & Ganss, 2005) and activated endothelial cells (Stamatovic, Keep, Kunkel, & Andjelkovic, 2003; K. S. Weber, P. J. Nelson, et al., 1999) can also express CCR2. That is why in our study, after wire injury, 20% of cells were CCR2 positive in both the lumen face and the vessel wall of the injured arteries treated with PBS. A possible explanation is that injured endothelial cells are activated and peripheral EPCs and monocytes can be recruited to the lumen face, additionally, some monocytes also transmigrate into the vessel wall which express CCR2.

Many studies have demonstrated that bone marrow stromal cells are the main source of CCL2, which plays important roles in myeloma cell homing (Vande Broek et al., 2003), monocyte mobilization (Jung, Mithal, Park, & Miller, 2015), macrophage bone marrow homing (Y. Li et al., 2015) and arteriogenesis (Kinnaird et al., 2004). On the other hand, other studies proved that CCL2 was released from the vessel wall, by cells such as endothelial cells (Shyy, Hsieh, Usami, & Chien, 1994), smooth muscle cells (X. L. Chen et al., 1998) and adventitial macrophages. Thus, the cellular source of CCL2 that can contribute to VPC migration *in vivo* needs to be further investigated.

In our study, through the use of chimeric mouse models, we found that after bone marrow transplantation, CCL2 can be detected in the peripheral blood of a CCL2^{-/-} chimeric mouse (bone marrow was from WT mouse), where it was originally undetectable. This indicates that CCL2 in the blood is released from transplanted bone marrow cells, which confirms the notion that CCL2 is bone marrow-derived. However, in a WT chimeric mouse (bone marrow was from CCL2^{-/-} mouse), we also found that the levels of CCL2 were markedly upregulated and showed

an even higher increase compared to the CCL2^{-/-} chimeric mouse. This indicates that CCL2 from non-bone marrow tissues (e.g. SMC) contributes more to systemic CCL2 levels in the peripheral blood than in bone marrow. The quantitative data from neointimal lesions of each type of chimeric mice showed a consistent increase in neointima area with higher CCL2 levels in the blood, which further confirmed that the CCL2 from non-bone marrow tissues-induced progenitor cell migration and contribution to neointima formation. Taken together, in the present study, we confirm that CCL2 is produced from both bone marrow cells and peripheral tissues. Furthermore, we also demonstrate that CCL2 released from non-bone marrow tissues (e.g. SMCs) plays a more crucial role in accelerating neointima formation through induction of progenitor cell migration from the adventitia to the neointima.

CXCL1 has been well established as a potent chemoattractant for leukocyte arrest and migration, which contributes to several inflammatory diseases (Boisvert et al., 2006; Reutershan et al., 2006; Zhou et al., 2011). In cardiovascular disease, human endothelial cell-released CXCL1 induces angiogenesis and blockage of CXCL1, resulting in inhibition of endothelial cell migration and proliferation, which further abrogates angiogenesis *in vivo* (Lo, Lai, Li, & Wu, 2014; Miyake et al., 2013). The roles CXCL1 plays in neointima formation are distinct in different studies. CXCL1 released from activated SMCs or endothelial cells promotes neointima formation or atherosclerosis (Furgeson et al., 2010; Zhou et al., 2011). Furthermore, CXCL1 is also associated with vascular inflammation, matrix degradation and lipid deposition that leads to unstable atherosclerotic lesions (Breland et al., 2008). According to other studies, CXCL1 can also mediate circulating endothelial progenitor cells which contributes to re-endothelialisation after arterial injury (Hristov et al., 2007). Blockage of CXCL1 delayed endothelium recovery and increased neointimal area (Liehn et al., 2004). The underlying mechanism of CXCL1-related neointima formation is still poorly understood.

In the present study, the quantification of cells with different markers attracted by local administration of CXCL1 *in vivo* revealed that CXCL1 had a robust chemotactic effect on Sca-1⁺ cells migration but not on endothelial cells and SMCs. We next attempted to confirm that CXCL1-mediated VPC migration contributes to neointimal formation. Unfortunately, as a

CXCL1^{-/-} mouse is not commercially available, we could not perform the neointima formation model in them. We therefore applied CXCL1 siRNA to the adventitia of injured femoral arteries to create a local silencing of CXCL1 to investigate its effect on VPC migration into arteries. As siRNA silencing is transient, we evaluated Sca-1⁺ progenitor cells migratory ability from adventitia to intima over a short time point. Data from these experiments suggest that knockdown of CXCL1 with siRNA may result in a reduction in VPC migration, which indicates that CXCL1 may accelerate neointimal development through induction of VPC migration. To obtain more robust evidence for the role of CXCL1 in VPC contribution to neointima formation this study will be repeated in CXCL1^{-/-} mice in the future.

4.5 Future work

4.5.1 The role of chemokines derived from various cell types in VPC migration

In the present study, we demonstrated that once activated by vascular injury, SMCs can release chemokines to induce vascular progenitor cell migration and subsequent contribution to neointima formation. The scope of our study only enabled us to examine the chemokines which were markedly increased in SMC-conditioned medium, though other chemokines may have a potent effect on VPCs, despite their expression levels not being significantly up-regulated. However, in addition to chemokines, cytokines and growth factors other chemoattractants may also participate in VPC migration, thus a proteomic study or ELISA array could be performed on SMC conditioned medium to determine other important factors released by SMCs. Aside from SMCs, endothelial cells in the intima and other cell types residing in the adventitia can also release chemokines after stimulation by a number of factors, which may also become important chemoattractants for vascular progenitor cell mobilisation. In this case, it would be of great interest to study the effects of other cell types (e.g. endothelial cells) and their released chemokines on VPC mobilisation.

4.5.2 Potential interaction between CCR2 and CXCR2

As previously discussed, CCL2 and CXCL1 in our study showed neither a cumulative nor redundant relationship in the mediation of VPCs migration. What is the potential relationship between both chemokines and their receptors? Previous studies have found that a heterodimer of PF4 and CCL5 augmented CCL5-mediated monocyte adhesion on the endothelium (von Hundelshausen et al., 2005) . Simultaneous treatment with CCL2 and CCL5 also induced CCR2-CCR5 heterodimer formation on the cell membrane, which significantly increased the sensitivity of cells response to each chemokine alone (Mellado et al., 2001). In contrast, another study demonstrated that the specific ligands of either CCR5 or CCR2 have negative cooperation on binding to CCR5-CCR2b heterodimer expressing cells, which indicates that this heterodimer

receptor can only interact with a single chemokine at a time (El-Asmar et al., 2005). In fact, heterodimerization is a general interaction between distinct chemokines or chemokine receptors, and may generate various cascades of events that are antagonistic, synergistic or have new unknown effects. These phenomena then increase the complexity of cross talk among different signalling pathways. Thus, further studies should be carried out to explore the potential heterophilic interactions between CCL2 and CXCL1 or CCR2 and CXCR2, which would be an ideal model for understanding the underlying mechanisms of CCL2 and CXCL1's effects on SMC-induced VPC migration.

4.5.3 Underlying signalling pathways *in vivo*

In our study, we elucidated that CCL2 and CXCL1 induce VPC migration via CCR2 and CXCR2 and subsequently activate Rac1 and the p38 MAPK signalling pathway. However, we have not yet confirmed this result in wire injury-induced VPC migration *in vivo*. To address this, VPCs could be modified in three ways and then seeded in the adventitia of wire-injured arteries in WT mice to evaluate their migratory ability: 1) Knockdown of either CCR2 or CXCR2 on the VPCs to see whether the migration would be downregulated in comparison with the WT VPCs. 2) Overexpress dominant-active or -negative mutant of Rac1 in VPCs respectively, to compare whether the migration of VPCs with constitutively active Rac1 would show a significant increase. 3) Transfect VPCs with constructs encoding dominant-active or -negative mutants of p38 and test whether overexpression of dominant-active p38 would enhance VPC migration. It would be interesting to verify the roles of CCL2/CCR2/Rac1/p38 and CXCL1/CXCL2/Rac1/p38 signalling pathways in VPC migration *in vivo*.

4.5.4 Generation of SMC-specific conditional CCL2/CXCL1 knockout mice

In the present study, we explicitly demonstrated that SMCs induced VPC migration via the release of CCL2 and CXCL1 *in vitro*. Furthermore, we showed a significant decrease of VPC contribution to neointima formation in CCL2 knockout mice, then further demonstrated that this effect is dependent on peripheral tissue released CCL2. However, as previous studies report

that peripheral tissues such as endothelial cells in vessel wall can also release CCL2, data from the present study do not totally exclude other sources of CCL2. To conclusively assess the role of SMC-derived CCL2 in VPC migration and neointima formation, it would be interesting to generate a SMC-specific CCL2 conditional knockout mouse by crossing a Myh11-CreERT2 mouse with a Floxed CCL2 mouse, which are both commercially available. However, as there is no commercial Floxed CXCL1 mouse, it would be challenging but more innovative to create a Floxed CXCL1 mouse followed by generation of an SMC-specific CXCL1 mouse.

4.5.5 Application of lineage tracing of Sca-1 vascular progenitor cells *in vivo*

In our study, we utilised an accelerated model for analysing neointima formation by wrapping injured arteries with GFP-labelled VPC. There are two reasons why we chose this model: 1) to study the role of VPC migration in the early stages of neointima formation; 2) to trace the GFP-labelled VPC *in vivo*. However, as this model still lacks direct evidence for native vascular progenitor cells contribution to neointima formation, it would be better to trace the native vascular progenitor cells *in vivo*. Furthermore, as previous studies from our group showed vascular progenitor cells have high potential to differentiate into SMCs, it is a controversial issue as to whether SMCs present in the neointima are VPC-derived or medial SMC-derived and the percentage each source contributes to the neointima. It would be more convincing to obtain quantitative data on how many cells in neointimal lesions are derived from VPC. For this reason, a lineage tracing for progenitor cells in an animal model is essential. Specifically, we could use Sca-1-GFP transgenic mice as a control and further create SMC-specific CCL2/CXCL1 knockout mice to study the native vascular progenitor cell in SMC-derived CCL2/CXCL1 induced neointima formation.

4.5.6 Design of a CCL2, CXCL1 related drug-coated stent

As mentioned previously, CCL2 can both induce SMC proliferation and VPC migration to facilitate neointima formation. In the future, we could design new drugs to inhibit CCL2 effects on angioplasty restenosis. CXCL1 is reported to induce endothelial cells proliferation and accelerate re-endothelialisation. From our data, CXCL1 can also induce VPC migration towards impaired sites of the vessel wall. Thus, we could incorporate CXCL1 with factors for VPC EC-differentiation in new drugs, which will both further enhance endothelium repair by induction of both VPC migration, differentiation and EC proliferation.

4.6 Summary

It was believed that neointimal formation in response to endothelial injury was predominantly caused by the accumulation of inflammatory cells via their recruitment into the microenvironment (Galkina & Ley, 2007; Christian Weber, Fraemohs, & Dejana, 2007) and also from medial-derived SMCs (Yunhui Cheng et al., 2009; X. Yang et al., 2006). In this study, we utilised an accelerated model of *in vivo* neointima formation by inducing endothelial injury. From data generated in non-VPC seeded groups, we found that during the short time period of 1-2 weeks, very few cells contributed to the spontaneous development of neointimal lesions. In contrast, an obvious neointima was found in VPC seeded groups, which suggests that neointima formation is largely generated by VPC migration in the early stage of the model. The objective of this model was to show that after wire injury SMCs of the vessel wall are activated and can release chemokines able to induce VPC migration from the adventitia to the luminal side of the vessel.

These findings have two implications. The cells accumulating in neointimal lesions may be derived from migration/proliferation of vascular stem cells, which can differentiate into SMC-like cells, although mononuclear cells and medial SMCs contribute to this process. To obtain quantitative data on how many cells in neointimal lesions are derived from progenitor cells, lineage tracing for progenitor cells in animal models would be essential. Secondly, a new treatment strategy for restenosis could be considered. Current treatments used in the clinic are based on inhibition of cell proliferation (Hong et al., 2003; Sousa, Serruys, & Costa, 2003) using drugs such as sirolimus. A recent report from our group suggests that sirolimus can enhance vascular stem/progenitor cell differentiation into SMCs, but inhibits endothelial differentiation (Wong et al., 2013). Our current findings that large numbers of stem/progenitor cells are recruited to the intima in response to CCL2 and CXCL1 provide potential to direct cell differentiation into the endothelial lineage. In other words, a high number of stem cells exist during neointima formation, which may differentiate into endothelial cells if a new drug-coated stent could harness their potential and direct them specifically.

Chapter 5

Publications

5.1 Journal Articles

5.1.1 Published

1) Yu B, Wong MM, Potter CMF, Simpson RML, Karamariti E, Zhang Z, Zeng L, Warren D, Hu Y, Wang W, Xu Q. **Vascular Stem/Progenitor Cell Migration Induced by SMC-derived CCL2 and CXCL1 Contributes to Neointima Formation.** *Stem Cells*. 2016 Jun 14. doi: 10.1002/stem.2410.

2) Wong MM, Yin X, Potter CMF, Yu B, Cai H, Di Bernardini E, Xu Q. **Overexpression of HSP47 augments mouse embryonic stem cell smooth muscle differentiation and chemotaxis.** *PLoS One*. 2014 Jan 16;9(1):e86118.

3) Wong MM, Winkler B, Karamariti E, Wang X, Yu B, Simpson R, Chen T, Margariti A, Xu Q. **Sirolimus stimulates vascular stem/progenitor cell migration and differentiation into smooth muscle cells via epidermal growth factor receptor/extracellular signal-regulated kinase/ β -catenin signaling pathway.** *Arterioscler Thromb Vasc Biol*. 2013 Oct;33(10):2397-406.

5.1.2 In preparation

1) Karamariti E, Zhai C, Yu B, et al. **DKK3 stabilizes atherosclerotic plaque via promoting vascular progenitor and fibroblast differentiation to smooth muscle cells.** (submitted manuscript)

2) Le Bras A, Yu B, Issa Bhaloo S, Hong X, Hu Y, Xu Q. **Directed differentiation of stem cells into endothelial cells through epigenetic modulation. The implication for vascular regeneration.** (manuscript in preparation)

5.2 Meeting Abstracts

- 1) **Yu B, Wong MM, Potter C, Hu Y, Wang W, Xu Q. Smooth Muscle Cells Are Crucial for Vascular Stem Cells Migration and Vasculogenesis via Keratinocyte Cell-derived Chemokine.** Arteriosclerosis, Thrombosis, and Vascular Biology Peripheral Vascular Disease (ATVB | PVD) 2015 Scientific Sessions May 7–9, 2015 , San Francisco, Calif.(Oral Presentation)
- 2) **Yu B, Wong MM, Wang W, Hu Y, Xu Q. Vascular Stem Cell Migration in Response to Monocyte Chemoattractant Protein-1 Promotes Neointima Lesion Formation.** Arteriosclerosis, Thrombosis, and Vascular Biology Peripheral Vascular Disease (ATVB | PVD) 2015 Scientific Sessions May 7–9, 2015, San Francisco, California. (Poster Presentation)
- 3) **Yu B, Wong MM, Potter C, Hu Y, Wang W, Xu Q. Smooth Muscle Cells Are Crucial for Vascular Stem Cells Migration and Vasculogenesis via Keratinocyte Cell-derived Chemokine.** American Heart Association Scientific Sessions Best of AHA Specialty Conference, Nov. 7-11, 2015, Orlando, Florida (Invited Poster Presentation)
- 4) **Yu B, Wong MM, Potter C, Hu Y, Wang W, Xu Q. Smooth Muscle Cells Are Crucial for Vascular Stem Cells Migration and Vasculogenesis via Keratinocyte Cell-derived Chemokine.** 2015 BHF Centre Postgraduate Symposium 2015, King’s college London (Oral Presentation)
- 5) **Yu B, Wong MM, Hu Y, Wang W, Xu Q. Smooth Muscle Cells Induce Vascular Progenitor Cell Migration via Chemokine MCP-1.** 2013 SEMS Postgraduate Symposium 2013, Queen Mary, University of London (Poster Presentation)

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