Genetically Modified Mesenchymal Stem Cells Overexpressing CCR2 for Cell Therapy in Critical Limb Ischemia

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Declaration

Genetically Modified Mesenchymal Stem Cells Overexpressing CCR2 for Cell Therapy in Critical Limb Ischemia

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The work in this thesis is based on the research carried out at Regenerative Medicine Institute, National University of Ireland Galway. I, Arun Thirumaran, hereby certify that this thesis has been written by me as the record of work carried out by me. I also certify that this thesis, in part or as a whole, has not been submitted in any previous application for a degree or qualification.

Arun Thirumaran

Abbreviations

ABI	Ankle Brachial Index
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of variance
ATMP	Advanced Therapeutic Medicinal Products
BASIL	Bypass versus angioplasty in severe ischaemia
BM	Bone marrow
BMCs	Bone marrow cells
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CLI	Critical Limb Ischaemia
CMV	Cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagles Medium
EDTA	Ethylene Diamine Tetra Acetic Acid
EF-1	Eukaryotic translation elongation factor -1
EGF	Epithelial Growth Factor
EPCs	Endothelial Progenitor Cells
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FIV	Feline Immunodeficiency Virus
GAG	Group specific antigen
GFP	Green Fluorescence Protein
GSL-1-B4	*Griffonia Simplicifolia* lectin
H&E	Hematoxylin and Eosin
HEK293	Human Embryonic Kidney 293 cells
HGF	Hepatocyte Growth Factor
HIV	Human IMMUNODEFICIENCY VIRUS

HLA	Human Leukocyte Antigens
HLI	Hind Limb Ischaemia
hMSC	Human Mesenchymal Stem Cell
HPRA	Health Products Regulatory Authority
ICAM-1	Intercellular Adhesion Molecule 1
IFN-y	Interferon gamma
IGF	Insulin like Growth Factor
IGFBP	Insulin like Growth Factor Binding Protein
IL	Interleukin
IMS	Industrial Methylated Spirit
ISCT	International Society for Cellular Therapy
IVIS	*In vivo* Imaging System
LDI	Laser Doppler Imaging
LTR	Long Terminal Reports
MCP-1	Monocyte Chemotactic Protein 1
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MMPs	Matrix Metalloproteinase
mMSC	Mouse Mesenchymal Stem Cell
MNCs	Mononuclear Cells
MOI	Multiplicity of Infection
MSC	Mesenchymal Stem Cell
NK	Natural Killer Cells
NKT	Natural killer T cells
PAD	Peripheral Arterial Disease
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDX-1	Pancreatic and Duodenal Homebox-1
PIGF	Platelet Induced Growth Factor
PLGF	Placental Growth Factor

PVD	Peripheral Vascular Disease
RRE	Rev Responsive Element
RSV	Respiratory Syncytial Virus
RT	Room Temperature
SCA	Stem Cell Antigen
SD	Standard Deviation
SDF-1	Stromal Cell-Derived Factor 1
SIV	Simian Immunodeficiency Virus
STRO	Stromal Cell Antigen
TASC-1	Trans-Atlantic Inter Society Consenses-1
T_h	T-helper cells
TNF-α	Tumour Necrosis Factor-Alpha
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VNC	Viral Nucleoprotein Complex
VSV-G	*Vesicular Stomatitis* virus envelope glycoprotein-G
WNT4	Wingless related MMTV integration site 4

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Abstract

Critical Limb Ischemia (CLI) is an advanced stage of Peripheral Arterial Disease (PAD) and a global health problem. Patients with CLI may present with rest pain, gangrene and/or ulceration of the lower limb. Effective treatment options are limited and thus, the incidence of limb amputation is high with associated high morbidity and mortality rates. Mesenchymal Stem Cells (MSCs) are widely used with therapeutic efficacy in various diseases, including Critical Limb Ischemia (CLI). However, the use of MSCs is not without challenges. One of the major challenge in the use of MSCs in CLI therapy is the bio-distribution and retention of the transplanted cells in the target tissue. This reduced retention of locally transplanted cells at the target tissue is due to a variety of factors including the hostile environment where the cells are introduced to; while the reduced bio-distribution of transplanted cells is due to the clearance of these cells into organs like lungs, liver and spleen.

Here, MSCs are genetically modified to overexpress a potent chemokine receptor on their surface. The chemokine receptor overexpressed on MSCs in this study is, CC Chemokine Receptor type-2 (CCR2), which is known to bind with high affinity to Monocyte Chemoattractant Protein-1 (MCP-1), a chemokine secreted in high levels by ischemic and injured tissue. CCR2 is normally expressed on immune cells like monocytes that enable their migration towards injured tissue.

The hypothesis is; transplantation of CCR2 overexpressing MSCs will assist their increased persistence in ischemic tissue, enabling prolonged secretion of paracrine factors leading to therapeutic angiogenesis.

In Chapter 2, a lentiviral vector carrying CCR2 gene was designed and validated for its efficiency and CCR2 integration. The results showed, stable CCR2 integration and efficient transduction of these viruses without affecting the viability of cells.

In Chapter 3, Mouse MSCs (mMSCs) were transduced with lentiviral vector carrying CCR2, Transduced cells were selected, culture expanded and the stable overexpression of CCR2 on these mMSCs were confirmed at the transcript level and at the protein level.

Characterisation and functional assessment of CCR2 overexpressing mMSCs are detailed in chapter 4. CCR2 overexpressing mMSCs were analysed for their stem cell properties such as expression of mMSC specific surface markers and differentiation potential. The functional properties of these genetically modified cells were assessed by their ability to migrate to MCP-1 *in vitro*. CCR2 overexpressing mMSCs were found to be positively expressing CD90, CD105 and SCA-1, while negative for the expression of CD11b, CD34 and CD45. CCR2 overexpressing mMSCs were found to be migrating at significantly higher levels towards MCP-1.

Therapeutic efficacy of CCR2 overexpressing mMSCs were assessed in a mouse model of hind limb ischemia. Their ability to retain in the ischemic tissue after intramuscular injection were assessed using IVIS fluorescence imaging. The bio-distribution of these cells in various organs in a hind limb ischemia mouse model was also assed after 14 days of cell delivery.

CCR2 overexpressing mMSCs were found to improve blood flow in ischemic limbs when compared to animals that received injection of cells expressing a fluorescent protein DsRed and animals that received saline injections.

Histological analysis of samples showed there were increase in the number of endothelial cells forming capillaries in animals that received CCR2 overexpressing mMSCs compared to controls. There was improved skeletal muscle regeneration and increased immune cell infiltration in these tissues proving improvement in tissue regeneration.

IVIS imaging of tissue samples showed increased retention of transplanted cells in ischemic limb of animals that received CCR2 overexpressing MSCs when compared to animals that received control transduced cells. Moreover, analysis of bio-distribution of transplanted cells showed there were no significant clearing of these transplanted cells into any organs of these animals.

Chapter 1

Introduction

CHAPTER 1

1. Introduction

1.1. Peripheral Artery Disease

Cardiovascular disease is the leading cause of death and disability in the world[1]. Peripheral artery disease is one manifestation of this disease and contributes significantly to mortality throughout the world[2]. The prevalence of peripheral artery disease (PAD) has increased by 78% in the global population between 1990 and 2013 and it affects 17% of the general population[3]. The prevalence of PAD increases significantly with age. PAD shares many risk factors with coronary artery disease and a substantial number of patients who present with PAD die from coronary disease. There is a twofold risk for patients with PAD to develop major cardiovascular events in 6 months as compared to non-PAD patients[4]. Elderly patients with PAD are at the highest risk of developing coronary artery disease (68%) and stroke(42%)[5].

1.1.1. Symptoms and Risk Factors

Patients with PAD may present with intermittent claudication or a more severe manifestation of Critical Limb Ischemia (CLI). PAD can be assessed by measurement of the ankle-brachial Index (ABI) which can also give information on disease severity. ABI is calculated by dividing the blood pressure at the ankle by the highest brachial blood pressure. ABI of <0.9 is considered abnormal and is an indication of PAD. ABI of 0.7-0.9 is classified as mild, 0.5-0.69 is classified as moderate and ABI less than 0.5 is considered as severe. Patients with PAD may be asymptomatic in the initial stages followed by intermittent claudication and subsequently CLI which presents with rest pain, gangrene or ulceration. CLI may result in amputation in patients who have no revascularization option. Risk factors of PAD include diabetes, smoking, hypertension, obesity, dyslipidemia[6]. The pandemic of diabetes mellitus is a major risk factor leading to the increased prevalence of PAD. Asymptomatic patients and those with intermittent claudication are treated by aggressive risk factor modification and exercise. Treatment for those with CLI is challenging; while some have,

revascularization options those without this option are at high risk for amputation and indeed death. This thesis will deal with development of new therapeutic approaches to CLI as there are currently no suitable treatment alternatives for patients with so-called no option CLI.

1.1.2. Classification

The most commonly used classification of CLI is the Rutherford classification. Progression of CLI occurs in a stepwise manner with increase in occlusion in each progressive step. The Rutherford classification is similar to the Fontaine classification and it has six stages. Stage 0 is the asymptomatic stage where the disease is diagnosed by changes in the ABI. Stage 1 represents mild claudication, stage 2 represents moderate claudication, stage 3 represents severe claudication, stage 4 is accompanied by rest pain, stage 5 is characterised by minor tissue loss and ischemic ulceration, and finally, stage 6 is characterised by severe tissue loss or gangrene[7,8].

1.1.3. Critical Limb Ischemia

As described above, CLI is an advanced stage of PAD. Patients with PAD may present with intermittent claudication or CLI. CLI occurs when the blood flow to the lower extremities of the limbs are impaired and the nutritional and oxygen supply to the tissue becomes restricted resulting in a hypoxic environment which leads to tissue damage or gangrene[2]. The gold standard for the treatment of CLI is revascularisation procedures aiming to recover blood flow to the affected limbs [8]. Without revascularisation, patients with CLI are at the risk of limb loss and progression of gangrene that could lead to sepsis. Balloon angioplasty, endovascular revascularisation procedures and surgical bypass are the most common revascularisation procedures. Amputation is the only option for patients who have failed or not suitable for surgical bypass or endovascular treatments and are considered as no option patients[7]. Patients with CLI have a 1-year amputation rate of 30% and a mortality rate of 25%.

New approaches to this condition are urgently required and this is particularly the case for patients with diabetes mellitus. Patients with diabetes mellitus are at 2-3-fold increased risk of developing PAD. New treatment options include gene therapy and stem cell therapy or combinations using genetically modified stem cells. MSCs are proven to be efficacious in promoting therapeutic angiogenesis in animal models of cardiovascular disease[9–11].

1.1.4. Pathophysiology

The main cause of CLI in the western world is obstructive atherosclerotic disease. CLI can also be caused by vascular embolic disease, vasculitis, and in Asia can be caused by thromboangiitis obliterans. Pathophysiology of CLI affects both the macro and micro vascular system and the surrounding tissues. The initial response to the onset of ischemia is arteriogenesis or angiogenesis to enhance the blood flow to the ischemic tissue. These responses often fail to provide recovery in blood flow resulting in vasodilation often referred as vasomotor paralysis[12,13] . Ischemia results in the alterations of the function and structure of endothelial cells. Changes to endothelial cell function leads to activation of platelets and also leads to the production of free radicals and leukocyte adhesion which eventually leads to microthrombi[7].

1.1.5. Current Standard of Care for Critical Limb Ischemia

An international guideline, the Trans-Atlantic Inter Society Consenses-1 (TASC-1), was published in 2000, providing standardised recommendations for treatment and care of patients with CLI based on clinical symptoms and the level of occlusions in their lower extremities. The guideline recommended patients to undergo endovascular or surgical revascularisation based on their disease stage. Since the publication of the TASC guideline in 2000, the endovascular and surgical techniques have improved and several other international guidelines have been published for the treatment and management of CLI. These guidelines focus on the secondary prevention of PAD by cessation of smoking, lowering lipid levels, management of diabetes and hypertension and anti-platelet therapies [2,7,14,15].

Revascularisation strategies have evolved over the last years and there are several new devices in the market (drug eluting stents and balloons) and new approaches aimed at achieving revascularisation at much enhanced rates than possible a decade ago. However, a randomised clinical trial comparing endovascular techniques with bypass surgery, the BASIL trial, showed no significant differences between the groups at 1-3 year follow up. This study showed there were long term benefits to undergoing bypass surgery at an earlier stage. Patients who underwent endovascular procedures had to undergo bypass surgeries at later stages of disease progression and performed worse in comparison to the ones which received a vein graft[16,17]. Increases in a first endovascular strategy over the last two decades have shown short term favourability in patients with high risk CLI[18–20]. These comparative studies show 1 year post intervention increase in limb salvage of up to 85% in patients who have undergone endovascular treatments, but also showed there was an increase in the need for re-interventions at a later point in those CLI populations. This is reported to be due to the higher number of endovascular procedures performed in comparison to bypass surgeries. Endovascular techniques are constantly evolving as newer devices are introduced into the market and the increase in the number of endovascular interventions can also be attributed to the reduced cost factor and the improved recovery time when compared to open bypass surgeries which require longer hospital admissions. Recent studies have shown that the increase in endovascular interventions does not improve the outcomes necessarily but, the benefits are often short term, mainly due to the need for repeated interventions[21,22]. Another argument put forward for, a first endovascular intervention is that even if there is a failure in the procedure over time, there is the possibility of a bypass graft at a later stage. However, the argued advantages of open surgery to endovascular intervention is considered irrelevant due to the high mortality rates associated with CLI patients. According to a UK study, there are differences in opinions about the merits of angioplasty vs surgery as the first line of intervention among surgeons and radiologists; bypass graft or endovascular intervention.[23]. This points to the lack of randomised control trial data to help optimise patient outcomes. Based on the available clinical data it is impossible to defend endovascular or surgical bypass as the first intervention for patients with CLI[24]. The choice is often made on patient specific factors such as age and severity of the disease and availability of a suitable graft.

Emergence of advanced endovascular techniques and devices will be critical to the success of endovascular techniques while the future of bypass surgery will possibly be dominated by polymer/ biomaterial and stem cell based developments to enable competent grafts that could promote clinical recovery in patients with PAD[25].

1.1.6. Cell and Gene Based Therapies

Since the 1990s several studies have demonstrated the beneficial effects of gene and cell based therapies for patients with PAD[26–28]. Numerous studies using gene and cell therapy to promote therapeutic angiogenesis show promising results in CLI[29–31]. Results of clinical trials involving gene and cell therapies for CLI patients with no option for revascularisation procedures are starting to emerge. Most of the clinical trials are gene therapies for CLI focused on different genes and different modes of gene delivery to improve therapeutic angiogenesis and limb salvage. Multiple cell based therapies involving MSCs are showing promise in pre-clinical research in various animal models. While the safety and efficacy of cell based therapy is widely recognized, a large number of those studies are pilot studies have small sample size, and are non-controlled and non-blinded. Clinical efficacy in human trials are not conclusive in terms of amputation free survival and reduction in amputation rates[32,33]. Larger placebo controlled randomised clinical trials show no improvement in critical parameters that can attribute to long term limb salvage in patients with PVD[32]. Gene therapy based treatments of PAD have had negative results in phase 3 trials. Stem cell therapy has been suggested as a superior approach. A recent meta-analysis has shown that cell based therapy appears beneficial in uncontrolled but not controlled trials. Thus, either approach alone may not be successful and approaches using genetically modified cells may be necessary[34].

1.1.7. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adult stromal cells which are multipotent and have the ability to differentiate into multiple cell types including bone, cartilage, tendon, muscle, fat and stromal tissue[35]. Bone marrow cells that could differentiate

into mesenchymal cells were first reported in 1970 by Friedenstein[36]. He reported colony forming cells from monolayer cultures of bone marrow and spleen cultured from guinea pigs showed osteogenic differentiation *in vitro*. The term 'MSCs' were coined by Caplan who pioneered research into identifying, characterising and optimising the *in vitro* culture of bone marrow derived stromal cells[37,38]. The term MSCs were given because of their ability to differentiate into mesenchymal type cells. They are now also referred to as mesenchymal stromal cells because of their origin; from the stroma of marrow. MSCs are hence non-hematopoietic cells that are found in the bone marrow or other organs that can be culture expanded *in vitro* and are plastic adherent[39]. MSCs are also isolated from adipose tissue, umbilical cord blood, liver and spleen, and are self-renewing[40], with a capacity to differentiate into multiple cell lineages that can form adipocytes, chondrocytes, myotubes, osteoblasts, tenocytes, neural-cells, and hematopoietic supporting stroma[35,41–43].

In addition to their multi-lineage differentiation potential, MSCs secrete a variety of cytokines and growth factors in a paracrine and autocrine manner. The effects of these secreted factors are commonly referred to as trophic effects. Trophic effects of MSCs enable them to suppress the local immune system, enhance angiogenesis and differentiation of intrinsic stem cell populations within tissues promoting wound healing, and inhibit fibrosis and apoptosis. [44,45]. MSCs also are known to migrate towards sites of injury[46] due to the expression of chemokine receptors[39,47,48]. Human MSCs are known to express major histocompatibility complex class 1 (MHC-1), but they do not express human leukocyte antigen class II (HLA)[49]. MSCs from different sources vary in the expression levels of surface molecules and adhesion factors. Typical human MSCs express several markers which are used for their selection and isolation. Adult human MSCs do not express haematopoietic markers CD45, CD34, CD14 or CD11 or co-stimulatory molecules such as CD80, CD86, CD40 or adhesion molecule CD31, CD18, and CD56, but they express CD105, CD73, CD44, CD90. CD71 and Stro-1. They express adhesion molecules such as CD106, CD166 and CD29[50–53]. MSCs isolated from other species do not always express the same level of markers as human MSCs. MSCs isolated from murine bone marrow typically expresses CD90, CD105, SCA-1 ,CD140a, CD140b and do not express CD34, CD45 and CD11b[54,43].

Ryan *et al* (2005) reported the immunomodulatory mechanism by which MSCs avoid allogenic rejection in both humans and animals, is due to the lack of expression of MHC-II on MSCs. MSCs disrupt functions of NK cells and T cells directly and indirectly through modulation of dendritic cells and induce a suppressive local micro environment through production of prostaglandins and interleukin-10 (IL-10)[45].

The ability of MSCs to differentiate into different lineages of cells, to secrete various chemokines and other factors along with their immune modulatory and immune suppressive characteristics make them an ideal candidate for various therapeutic applications in the field of regenerative medicine. Cellular therapies provide a new hope for patients suffering from various diseases involving tissue damage and loss. MSCs are being studied as therapeutic agents for a wide range of conditions including diabetes mellitus[55–58], cardiovascular [59–63], liver [64–68], kidney [69–71], bone diseases[72–74] and several autoimmune diseases [75–79].

1.1.8. MSC Therapy for Critical Limb Ischemia

MSCs, due to their immunological features, potential for homing and the paracrine and autocrine factor secretion makes them an ideal candidate for cell therapy in wide range of diseases including in critical limb ischemia. MSCs as explained before offer number of advantages. They are easy to isolate and expand, can be delivered locally or systemically and they engraft into tissue and secrete paracrine factors promoting angiogenesis, arteriogenesis, have immune modulatory properties, secrete chemokines and express receptors for chemokines enabling them to migrate towards site of ischemia/ injury[39,80]. The therapeutic potential of MSCs are widely reported in pre-clinical settings and are currently being explored in clinical trials[34].

Several preclinical studies have confirmed the efficacy of MSCs in promoting therapeutic recovery in ischemia of the leg.

Comparison of therapeutic potential of MSCs and mono nuclear cells in a rat model of Hind Limb Ischemia (HLI) for 2 days showed improvement in blood flow and increase in capillary density in the animals treated with MSCs. However, the laser

Doppler blood perfusion index was significantly high in animals that received mononuclear cells. Transplanted MSCs were shown to differentiate into endothelial cells, while mononuclear cells did not[81]. This study shows transplanted MSCs can differentiate into endothelial cells and it also showed the MSCs survived better in ischemic conditions *in vitro* than mono nuclear cells (MNCs). One mechanism by which MSCs provide therapeutic efficacy is by promoting the formation of collateral vessels that help bypass the blocked blood flow to the limbs[82]. Another is by secreting paracrine factors that aid in regeneration, angiogenesis and arteriogenesis[83]. Paracrine action of MSCs is believed to be the most effective mode of action whereby angiogenesis is achieved in an *in vivo* setting[44].

MSCs can be stimulated *in vitro* by altering their culture condition, co culture with immune cells or activation factors, priming the cells with growth factors or chemokines[80].

Hypoxia pre-conditioned mMSCs injected into mice HLI model showed enhanced skeletal muscle regeneration, improved blood flow and vascular formation after 7 days. Hypoxic preconditioning primes MSCs to secrete more paracrine factors enabling them to secrete chemokines that promote angiogenesis. Hypoxia preconditioning increased the expression of Wnt4 (Wingless-related MMTV integration site4)[84].

Stimulation of MSCs can be achieved by treating them with cytokines or growth factors. Intra muscular injection of mouse bone marrow derived MSCs pre-treated with EGF into diabetic mice after induction of ischemia showed increased capillary density, muscle regeneration and enhanced perfusion. Muscle tissues showed increased EGF receptor and AKT expression. These cells were Akt negative and VCAM negative when cultured before exposure to EGF[85].

Differentiation of transplanted MSCs into endothelial cells forming collateral vessels is rarely reported. Transplantation of a million cells into the thigh muscle of BALB/c mice after induction of ischemia by ligation of femoral artery showed improved limb function, reduced amputation, and reduced muscle fibrosis. The transplanted MSCs were analysed for their secretome profile before transplantation and were shown to secrete VEG-F, FGF, PLGF, and MCP-1. The transplanted cells were shown to increase the VEG-F FGF levels in the tissues in comparison with controls. This study shows the capacity of MSCs to secrete paracrine factors *in vitro* and *in vivo* enabling capillary remodelling through tropic factors secreted[82].

Use of adipose tissue derived MSCs are also investigated in several studies for their advantages over bone marrow derived MSCs. Isolation of adipose derived MSCs less complicated than bone marrow derived MSC isolation and they are shown to have similar tri lineage differentiation potential, secretome profile and can be used in therapeutic applications to enhance angiogenesis. Adipose derived mMSCs are shown to have increased blood flow recovery in mice after intramuscular injection[86]. Human adipose derived MSCs were tested for their efficacy in mouse model of HLI. Human adipose derived MSCs cultured in endothelial differentiation media has exhibited mesenchymal stem cell type phenotype. Injection of these cells into a mouse model of HLI has shown increase in capillary density, blood flow, decreased amputation and increased myogenic differentiation in tissue sections[87]. Adipose derived MSCs injected intra muscularly in another study into immune compromised mice model of HLI showed similar results of increased perfusion, increased capillary density, decreased limb loss, increased muscle regeneration when cells were injected 7 days after induction of HLI in a mouse model[88].

Improved isolation methods of MSCs can also increase their potential to induce therapeutic angiogenesis in animal models of HLI. One such method is to utilise the enhanced angiogenic potential of cells from haematopoietic and endothelial lineages. These cells have retained their ability to differentiate into vessels. Transplantation of these cell populations with proangiogenic potential could enhance

regeneration in ischemic tissue. Bone marrow derived cells with aldehyde dehydrogenase activity (ALDH); an enzyme with high expression in primitive haematopoietic progenitors. Bone marrow derived cells that were isolated based on their high level of ALDH activity ($ALDH^{hi}$) were tested for their regenerative potential in an immune compromised mouse model for HLI with femoral artery ligation, in comparison with cells with low ALDH activity. Cells with high level of ALDH activity showed augmented ischemic recovery and increased collateral density in ischemic limbs. These cells were recruited to ischemic regions, but did not integrate into ischemic tissue[89].

MSCs are ideal candidates for autologous and allogeneic cell therapy[79,90] due to their immune-modulatory properties. Allogeneic approaches could be advantageous when autologous cell sources are dysfunctional due to disease, or they cannot be culture expanded for treatment due to the donor age[91]. Although MSCs isolated from healthy donors are perceived as higher quality cells as compared to cells from a patient, comparison of MSCs from healthy donors and from patients with CLI revealed no difference in gene expression between the cells, nor was there any difference in the migration potential, or neovascularisation potential to recover blood flow in a mouse model of CLI. However, there were differences in differentiation potential of MSCs, as the cells from CLI patients failed to differentiate into chondrocytes *in vitro*, and they showed signs of senescence and decrease in proliferation and doubling time *in vitro*[92].

In a study where the application of autologous MSCs to advanced CLI patients was examined, there was 73% limb salvage[93]. Comparison of MSCs from responders and non-responders in the study revealed significantly increased secretion of IL-4, IL-6, MIP-1b, increased expression of cell surface markers CD90, and CD44 and Snail in responders, while expression of E-Cadherin, PDX-1 genes were significantly upregulated in non-responder cells. This study proves that the quality of cell therapy product in terms of expression of cell surface markers, secreted factors and genes expressed plays a critical role in determining the therapeutic outcome[93].

A double blinded randomised placebo controlled phase I/II study to assess the safety and efficacy of bone marrow derived MSCs in no option patients concluded there was a significant improvement in ABI[94]. This study along with three other placebo controlled studies report similar outcomes in terms of improvement in ABI[26,95,96].

1.2. Limitations of MSC Therapy - Study Rationale

MSCs are proven to be efficacious in terms of promoting therapeutic angiogenesis in multiple preclinical models for CLI and clinical data also suggests benefits[97], although large controlled trials have not yet been published. There are many challenges in the use of MSCs in CLI including autologous cell dysfunction and immune mediated rejection of allogeneic approaches (although as mentioned above this may be less of a problem with MSCs). Another challenge with MSC therapy is the poor survival, engraftment, viability and retention of transplanted cells at the target tissue[98]. Long term survival of transplanted cells is essential for promoting therapeutically viable results in the clinical setting. High levels of cell death are often reported in pre-clinical studies employing transplantation of MSCs. This is particularly due to ischemic conditions to which the cells are often introduced[99]. Ischemic injury is often followed by inflammation, and ischemic tissues secrete a myriad of chemokines and cytokines triggering infiltration of leukocytes, macrophages and other immune cells[100]. Several strategies to improve the cell survival and enhance cell adhesion have been developed. Loss of viability plays a crucial role when cells are delivered locally to the site of injury. Another major hurdle in MSC therapy is the bio distribution and retention of transplanted cells. Less than 1% of systemically transplanted cells reach the target site while locally delivered cells are cleared away once they have failed to survive the ischemic conditions[101,102]. Long term survival of MSCs at the site of injury is required for the paracrine mechanisms to have therapeutic effect.

The rationale for this study is to enhance the bio-distribution and retention of MSCs at the ischemic tissue by overexpressing chemokine receptors on their surface. Ischemic muscle secretes several chemokines, of which MSCs express receptors or

gets activated in the presence of others[38]. The homing potential of MSCs can be attributed to the expression of a range of chemokine receptors and adhesion factors along with the interaction of MSCs with endothelial cells and growth factors. Various receptors expressed on MSCs like CCR1, CCR2[103–105], CCR4, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6 have all been shown to play a role in migration[106–108].

Adhesion molecules like integrins and growth factors like PDGF-AB, IGF-1, EGF also play a role in the migration of MSCs[104]. Genetically modifying MSCs to over express these chemokine receptors will enable them to be retained in the ischemic tissue for prolonged periods thereby enabling paracrine mechanisms that enhances therapeutic angiogenesis.

1.2.1. Genetic Modification of Mesenchymal Stem Cells

MSCs are multipotent adult stem cells that can be isolated from various sources like bone marrow, adipose tissue, umbilical cord and placenta. MSCs are self-renewing, and easily culture expandable. They can differentiate into multiple lineages including adipocytes, osteoblasts, chondrocytes, myocytes, neurons, and tenocytes[35,41–43]. In addition to the multi lineage potential of MSCs, they are known to secrete various paracrine and autocrine factors[83] which are known to have various roles. These tropic effects of MSCs are broad and it can be angiogenic, promoting differentiation, activating immune cells, inducing chemotaxis, anti- apoptotic, inhibiting tissue necrosis, recruiting immune cells, inhibiting fibrosis, or evading immune rejection when transplanted[44]. These secreted factors of MSCs have a role in recruiting other immune cells and in the repair of damaged tissue[109,110]. The migration potential of MSCs is primarily due to the presence of various growth factor receptors and chemokine receptors[111,112]. They also express adhesion factors on their surface[39,50,104]. MSCs express various adhesion molecules and chemokine receptors which aid in their migration potential to various sites of inflammation and injury[50,104,108,112–114].The above-mentioned properties of MSCs make them ideal for use in therapeutic applications in cellular therapy of various diseases such as use in neurological diseases, for the treatment of cancer, to treat renal failure, for liver and

lung diseases, treatment of ocular defects, treating GVHD and several other a variety of other therapeutic applications.

MSCs are also attractive targets for genetic modification. Genetic modification will provide MSCs with exciting possibilities to enhance their potential for use in regenerative therapies.

Various approaches can be employed for the genetic modification of MSCs, including viral and non-viral methods of genetic modification. An ideal genetic modification should not be toxic, should not alter the function, secretory profile, surface markers, tissue function and differentiation potential of MSCs. MSCs can be engineered in multiple ways to be used in a variety of clinical conditions to improve its survival, improve homing potential, enhance anti apoptotic properties, enhance angiogenesis, enhance anti-inflammatory potential, improve bone and cartilage formation.

1.2.2. Viral and Non-Viral Modification of Stem Cells

Vectors used in gene therapy may be classified into viral and non-viral vectors. Non-viral gene delivery consists of synthetic gene delivery methods which include naked plasmid DNA, liposome mediated gene transfer, chemical transfection agents, and DNA conjugates[115].

Non-viral gene therapy is a promising alternative to virus based gene delivery systems. Non-viral gene transfer consists of direct carrier-free delivery of transgene by physical method or should be using synthetic chemical based methods. Non-viral gene delivery is cost effective in large scale and safer in comparison with viral vectors. There is no host cell immune activation or chance of virulence caused by oncogene activation[116]. One of the drawbacks of non-viral gene delivery is that the method is transient, and there is no integration of the transgene into the target cell genome. This causes loss of transgene expression upon cell division and thus only results in

short term expression of transgene in the target cell. It is also limited by the efficiency of gene transfer, with some cell types notoriously hard to transfect[117].

Viral vectors are non-virulent modified viruses which are engineered to carry genetic material, infect host cells and help integrate the genetic material into the host chromosome resulting in stable gene transfer[118]. An ideal viral vector should have the ability to infect the target cell and deliver the desired size of transgene into the host cell. The viral vector should be safe and non-toxic to the target cells and should not induce any unwanted immune response in the host cell. They should be easy to prepare and purify[119].

Viral vectors are efficient gene carrier systems and they use the mechanisms used by wild type viruses to pass through the cell membrane and attach the genetic material to the genome of the host cell. They are engineered so that their non-essential viral genes (virulence gene) is replaced or removed to accommodate the gene of interest. The envelope proteins of the viral vector governs the tropism of the virus and viral vectors are engineered by swapping the envelope proteins of viruses so that they can be designed to gain entry into different or specific cell types enabling targeted gene delivery[120].

1.2.3. Lentiviral Vectors for Genetic Modification of Stem Cells

Lentiviruses are retroviruses distinguishable by their ability to cross the nuclear membrane through the nucleopore. Lentiviruses are derived from several wild type viruses including HIV, FIV, SIV, visna virus and equine infectious anaemia virus. The first lentiviral vectors were based on HIV-1 virus and this is one of the most common and widely used lentivirus[119].

The HIV viral genome consists of regulatory genes, (*tati* and *rev*) accessory (*vpr, vif,vpu* and *nef*), in addition to the core retroviral genes *gag, pol* and *env.* Lentiviral vectors are replication incompetent by deletion of genes responsible for replication and infectivity. These genes are replaced by gene of interest whose transcription is

driven by long terminal repeats (LTRs) or engineered promoters. Lentiviruses possess all the advantages of retrovirus, large transgene capacity, vector integration allowing long term transgene expression and low pro-inflammatory activity. Lentiviral transduction initiates minimal host immune response *in vivo* demonstrating reduced antigenicity compared to other vector types[121].

Three generations of lentiviral vectors are used for gene delivery methods thus far, from HIV-1, first generation vector to the self-inactivating third generation vector containing only the minimal elements required for the target cell integration. Lentiviral vectors are produced by transient co-transfection of packaging plasmid vectors into specially designed highly expressing cell lines such as human embryonic kidney cell lines (HEK293T) that enable the multiplication of lentiviral particles[119].

The effect of genetic modification in all cell types needs to be analysed, not just in the expression of the transgene, but also by looking at the core stem cell properties of MSCs that are being modified. Various genetic modification procedures can have adverse effects on the cell function and properties. It is imperative that the analysis of stem cell properties be carried out after genetic modification to ensure there is no change in the underlying stem cell characteristics that made the cell the optimum choice of genetic modification.

In a study conducted by researchers in our group to assess the effect of lentiviral mediated genetic modification of rat MSCs, analysis of differentiation potential and cell viability of genetically modified cells along with the expression of transgene in *in vitro* ischemic conditions were assessed. Three vector systems were tested for their efficiency in transgene expression. The most efficient vector system was found to be the one where the transgene is under the EF-1α promoter. Transgene silencing and reactivation of reporter gene was analysed and the cell viability assayed at different MOI of virus. There was no loss of viability due to genetic modification. A differentiation assay into adipocyte lineage was also successfully performed.

Subsequently, MSCs were genetically modified to express pro survival genes, catalase, HSP-27, HSP-70, SOD1 and SOD3. Cell survival and transgene integrity was assessed in ischemia and hypoxia. The results concluded there was high level of transgene expression without any negative effects on the quality and function of MSCs using lentiviral genetic modification.[122]

In another study where lentiviral modification was used to transduce MSCs with Bone Morphogenic Protein 2 (BMP2), BMP2 transduction enhanced osteogenic differentiation while reducing adipogenic differentiation. MSCs transduced with BMP2 proliferated at slightly higher levels when compared to un-transduced controls. BMP2 secretion was analysed successfully using ELISA. BMP2 transduced cells differentiated successfully into adipocytes, *in vivo* tumorigenicity assay showed no tumour formation in cells transduced with BMP2 or in control GFP transduced cells. The cells showed the same surface marker profile as the unmodified cells by flow cytometry and there were osteogenic differentiation markers present on BMP2 modified cells. This proved there were no adverse effects from the genetic modification by lentivirus successfully improved osteogenic differentiation of MSCs[115].

1.3. Ischemic Tissue and Chemokines Secreted

1.3.1. Chemokines in Ischemia and Injury

Chemokines or chemotactic cytokines are a small family of signalling peptides that regulate proliferation, differentiation, chemotaxis, arteriogenesis, angiogenesis, immune cell recruitment, and skeletal muscle regeneration[100]. Chemotactic secretary factors range from 8-10kDa which helps in the infiltration of monocytes and other immune cells to the site of injury[123]. The chemokine super-family is divided into four sub-families: CXC, CC, C and CX3C chemokines, based upon the presentation of invariant cysteine (C) residues within the mature peptides. Chemokine receptors are G protein-coupled receptors for CXC, CC, C or CX3C chemokines[124], named CXCR,CCR,CR and CX3CR receptors[125] (table 1 and table 2).

1.3.2. MCP-1/ CCR2 Ligand Receptor Interactions

Monocyte Chemoattractant Protein (MCP-1/CCL2) is a chemokine that belongs to the CC chemokine family. It belongs to a family of four chemo-attractants, namely MCP-1,2,3 and 4. MCP-1 is produced by several cell types including, astrocytic, endothelial, epithelial, fibroblasts, mesangial, microglial, monocytic and smooth muscle cells[126,127]. MCP-1 is upregulated in tissue during infection, injury and ischemia and serves in recruiting macrophages, dendritic cells and T cells to the site of injury. CCR2 is the receptor for MCP-1. CCR2 is a trans membrane G protein coupled transmembrane receptor and has two isoforms, CCR2a and CCR2b with only difference in C terminal chains[128].

CCR2/MCP-1 is one of the most extensively studied chemokines in HLI models. Monocytes and macrophages are important in angiogenesis, arteriogenesis, and muscle regeneration. CCR2 has role in regulating monocyte infiltration during inflammation. CCL2/CCR2 deficient mice failed to recruit monocytes under different inflammation conditions. Inflammatory cells are attracted to the collateral artery by CCL2 during arteriogenesis and penetrate the vessel wall releasing large amount of growth factors[129] which stimulate endothelial and smooth muscle cell proliferation, necessary for collateral growth.

Table 1:1.1: CXC, CX3C and XC families of chemokines and chemokine receptors

Receptor	Ligand	Cell type expressed
CXCR1	CXCL8, CXCL6,	Neutrophils, monocytes
CXCR2	CXCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6	Neutrophils, monocytes, microvascular endothelial cells
CXCR3-A	CXCL9, CXCL10, CXCL11	Type 1 helper cells, mast cells, mesangial cells
CXCR3-B	CXCL4, CXCL9, CXCL10, CXCL11,	Microvascular endothelial cells, neoplastic cells
CXCR4	CXCL12	Widely expressed

CXCR5	CXCL13	B cells, follicular helper T cells,
CXCR6	CXCL16	CD8 T cells, NK cells and memory CD4 T cells
CX3CR1	CX3CL1	Macrophages, endothelial cells, smooth muscle cells
XCR1	XCL1, XCL2	T cells, NK cells

Table 1:2: CC families of chemokines and chemokine receptors

Receptor	Ligand	Cell type expressed
CCR1	CCL3(MIP-1α), CCL5(RANTES), CCL7(MCP-3), CCL14(HCC1)	T cells, monocytes, eosinophils, basophils,
CCR2	CCL2(MCP-1), CCL8(MCP-2), CCL7(MCP-3), CCL13(MCP-4), CCL16(HCC4)	Monocytes, immature dendritic cells, memory T cells
CCR3	CCL11(eotaxin), CCL24(eotaxin-2), CCL7(MCP-3), CCL5(RANTES), CCL8(MCP-2), CCL13(MCP-4)	Eosinophils, basophils, mast cells, Th2, platelets
CCR4	CCL17(TARC), CCL22(MDC)	T cells(Th2), basophils, dendritic cell(mature), macrophage, platelets
CCR5	CCL3(MIP-1α), CCL4(MIP-1β), CCL5(RANTES), CCL11(eotaxin), CCL14(HCC1), CCL16(HCC4)	T cells, monocytes
CCR6	CCL20(MIP-3β), LARC	T cells (memory and regulatory), B cells, dendritic cells
CCR7	CCL19 (ELC), CCL21(SLC)	T cells, dendritic cells (mature)
CCR8	CCL1 (I309) CCR9	T cells (Th2), monocytes, dendritic cells
CCR9	CCL25 (TECK) CCR10	T cells, IgA+ plasma cells
CCR10	CCL27 (CTACK), CCL28 (MEC)	T cells

CCR2/MCP-1 interaction is extensively studied in HLI in recruiting monocytes/macrophages. MCP-1 infused into the proximal end of ligated femoral artery in rabbit hind limbs showed increased collateral formation and increased

collateral conductance by angiogram. Increased monocyte accumulation was seen in the collateral artery walls of animals that received MCP-1. Infusion of ICAM-1 antibody diminished MCP-1 induced collateral artery formation. This suggested that the mechanism of action of MCP-1 was through the recruitment of inflammatory cells[130]. HLI studies on MCP-1 defective mice showed decreased restoration of perfusion[131,132]

1.3.3. Role of MCP-1 and CCR2 in Chemotaxis

MSCs express small level of CCR2 along with other potential chemokine receptors that play a role in their migration and homing potential[103]. Migration of CCR2 expressing MSCs towards MCP-1 is mediated by an intracellular adaptor molecule FROUNT. When MCP-1 binds to CCR2, it activates a cascade (figure 1.1) consisting of phosphatidylinositol 3-OH kinase (PI3K). FROUNT binds with activated CCR2 and forms clusters at the cell front during chemotaxis resulting in polarisation of MSCs which leads to rearrangement of the cytoskeleton[105,133].

The mechanism by which bone marrow derived MSCs migrate towards MCP-1 was identified by previous researchers in our group. They concluded that the migration of bone marrow derived MSCs towards MCP-1 is through the binding of MCP-1 to CCR2 expressed on the surface of MSCs. MCP-1 induced significant migration of BMMSCs and re-localised F-actin. Chemotaxis of MSCs were initiated when MCP-1 binds to CCR2 leading to the release of Gα and Gβγ subunits from the Gαβγ complex on G-protein coupled receptors. They also demonstrated that MSC migration towards MCP-1 exhibit dose dependent inhibition, where increase in the concentration of MCP-1 is shown to inhibit migration[134].

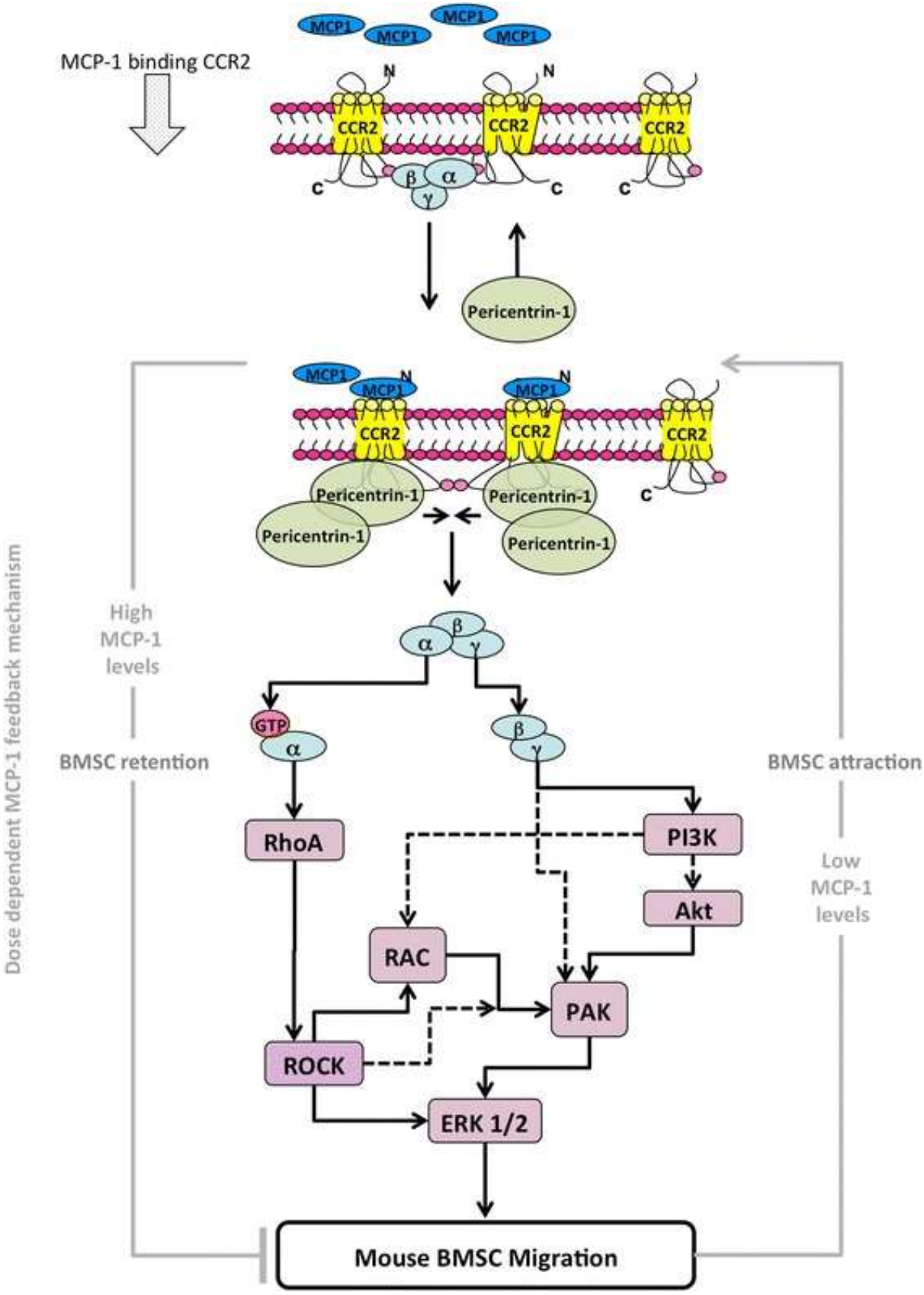

Figure 1:1: Dose-dependent MCP-1 feedback migration and activation[134]

1.4. References

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ROCK activity and the G βγ complex mediate chemotactic migration of mouse bone marrow-derived stromal cells ROCK activity and the G βγ complex mediate chemotactic migration of mouse bone marrow-derived stromal cells. *Stem Cell Res. Ther.* **6**: 136.

Chapter 2

Design Production and Optimisation of Lentiviral Vectors for Mesenchymal Stem Cell Modification

CHAPTER 2

2. Design Production and Optimisation of Lentiviral Vectors for MSC Modification

2.1 Introduction

Mesenchymal stem cells (MSCs) are fibroblast like, plastic adherent cells that are known to be multipotent, self-renewing, immunomodulatory and have a role in tissue regeneration and repair. MSCs also secrete several paracrine factors that are shown to have vital roles in chemotaxis, immune-suppression, angiogenesis, tissue response and repair. These characteristics of MSCs make them ideal candidates for therapeutic use in various diseases[1]. Genetic modification of MSCs enable researchers to fine tune the therapeutic properties of MSCs and to enhance their therapeutic potential enabling them to secrete specific factors, directing them for targeted therapies.

Genetic modification of MSCs can be achieved by viral and non-viral means. Viral vectors provide several advantages over non-viral systems of gene transfer. Several types of viral vectors such as retroviruses, adenoviruses, adeno associated viruses and herpes simplex virus are used for targeted delivery of transgenes into specific cell types. One of the major advantages of viral vectors such as retrovirus is the ability to integrate the gene of interest to the host chromosome thereby providing stable long term expression of the transgene. These viral vectors can infect dividing and non-dividing cells. Viral vectors also evade the immune system and are thus more effective in delivering transgene to the target cells. Both viral and non-viral gene delivery methods have strengths and weaknesses, but several modifications of the systems have improved the efficiency, reduced toxicity and pathogenicity and improved the integration and effective expression of the transgene[2].

Lentiviral vectors are commonly used viral vectors for gene therapy and genetic modification of different cell types. Lentivirus are a subclass of retroviruses and they belong to the class *Retroviridae*. Most of the lentivirus vectors in use today are based on the HIV-1 virus. Unlike other retroviruses, lentiviruses can infect and replicate in

both dividing and non-dividing cells[3] making them effective gene transfer vectors for a variety of cell types. They also integrate the transgene into the host chromosome providing long-term stable transgene expression[4]. Lentivirus being based on the HIV-1 shares the same genome structure as most retroviruses. The genome of HIV contains genes that code for 9 viral proteins. The structural genes, *gag, pol* and *env* encode for structural proteins. The *gag* gene codes for the viral core structural proteins, the *pol* gene codes for enzymes required for viral replication. The *env* gene codes for surface glycoprotein GP160. In addition to these structural genes and proteins for which they encode, the virus contains viral regulatory genes, *Tat* and *Rev* which code for regulatory proteins which activate viral transcription and regulate post-translational splicing and export of viral transcripts. Four accessory proteins are also coded by 4 additional genes in the viral genome. They are *Vif*, *Vpr*, *Vpu* and *Nef* and have a role in *in vivo* replication and pathogenesis of the viral particles. The viral genome is flanked by LTRs (long terminal repeats). The LTR is required for genome packaging and reverse transcription; and integration of viral particles.

Since lentiviruses in use to-date are based on the pathological HIV-1 retrovirus, several modifications have been made to increase the safety and eliminate the pathogenicity of these viruses. The components needed for the virus production are split between several plasmids (3 plasmids for the 2nd generation lentiviruses and 4 plasmids for the 3rd generation lentiviral vectors) thereby splitting the viral genome sequences needed for replication, packaging and production. All lentiviral systems in use today are replication deficient and self-inactivating after integration into the host chromosome, achieved by the deletion of the 3' LTR region.

The initial generation of lentiviral vectors were the ones that could undergo a single round of infection only. This was achieved by splitting the genome of the HIV-1 into two plasmids, one which codes for the viral DNA with *env* gene deleted, and the second plasmid carrying the *env* gene. The expression of transgene in these viruses were driven by the 5'LTR and the transgene was placed in the *env* gene[5]. Improvements in vector generation resulted in the first-generation lentiviruses

where the genome was split into three distinct plasmids. The first plasmid carried a viral packaging construct that codes for the *gag, pol* and regulatory proteins under a mammalian promoter, the second plasmid with a modified *env* gene that coded for a pseudo-typed surface receptor called VSV-G (Vesicular Stomatitis Virus envelope Glycoprotein-G) that helps the viruses to be targeted to a wide range of host cells[6]. The third plasmid was the transfer vector genome construct that contained the transgene, flanked by wild type LTRs and the RRE (Rev Responsive Element), sequence needed for packaging, reverse transcription, integration, but coded for no HIV proteins. The tat gene was deleted and an internal promoter was used for transgene expression. The second-generation lentiviral vectors had the accessory genes (*Vif*, *Vpu*, *Vpr* and *Nef*) deleted. The deletion of these genes does not affect viral replication in most of target cells. Therefore the second generation lentiviral vectors only have *gag*, *pol*, *tat* and *rev* genes remaining and the *env* is replaced by VSV-G[7]. Third generation lentiviral vectors have the *tat* gene deleted and *rev* is provided in a separate plasmid. The 5' promoter in the LTR is replaced by stronger promoters from CMV or RSV. This generation of vector has only three of the 9 genes from HIV and the packaging is through 4 plasmids. The first plasmid is the packaging construct containing *gag* and *pol* genes, the second plasmid codes for *rev*, the third plasmid codes for a VSV-G *env*, the fourth plasmid carries the transgene under a stronger promoter[4,8,9]. A number of modifications to the transfer vector such as addition of post transcriptional regulatory elements[10] and heterologous polyadenylation enhancer elements along with the use of independent and stronger internal promoters enhance the gene transfer performance and expression[9].

Lentiviral vectors for the over expression of CCR2 was designed in house. Gateway™ Cloning technology (Invitrogen, CA, USA) was used to generate lentiviral packaging vectors that can be used to produce lentiviral vectors for genetic modification of MSCs. Gateway recombination reaction enables the transfer of specific gene sequences from an entry clone (CCR2 in pDONR in this case) to a destination vector resulting in the production of an expression clone that is ready for gene expression.

Introduced by Invitrogen in the 1990s, Gateway cloning technology has emerged as a powerful tool for gene transfer between plasmids enabling researchers to move genes of interest between different vector systems. The principle of Gateway recombination is the recombination between two plasmids at a specific site termed the “att” site. This recombination is reversible. The process of production of an expression clone from an entry clone and a destination vector is termed as the LR recombination reaction. This is due to the nomenclature of the att sites on both the plasmids, attL and attR (figure 2.1).

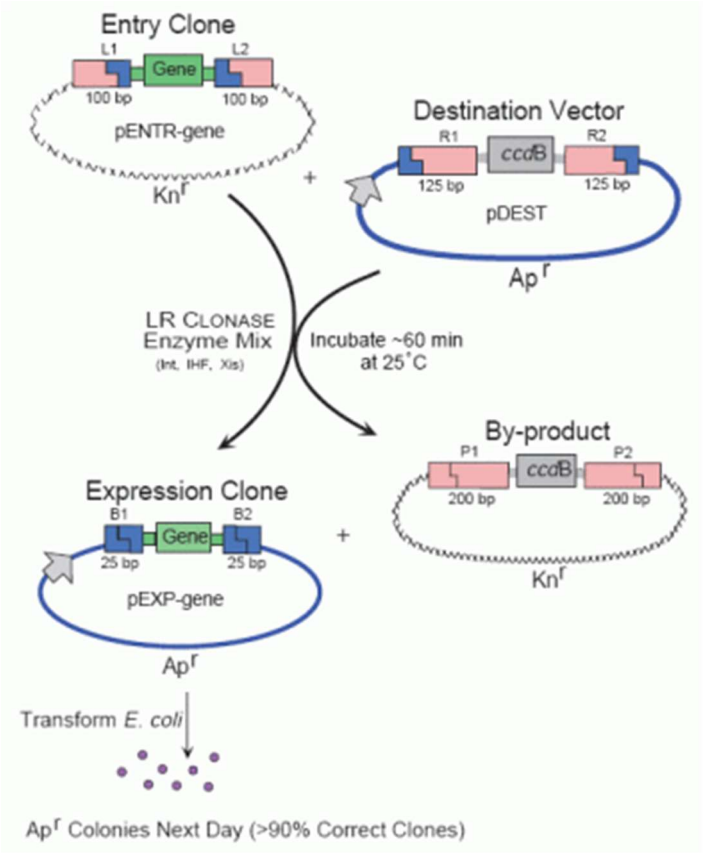

Figure 2:1 Schematic representation of a Gateway LR cloning reaction leading to the production of an expression clone

The expression clone can be selected by its antibiotic resistance (typically ampicillin resistance) once the plasmid is transformed into a suitable strain of bacteria by plating the bacterial culture on to an LB agar dish containing suitable selective antibiotics.

This chapter aims at designing an efficient lentiviral vector carrying mouseCCR2 gene, validating it for stable transgene integration and testing its efficiency in transducing cells.

2.2 Materials and Methods

2.2.1 Lentiviral Vector Design

Mouse CCR2 ORF C-DNA clones were purchased form GeneCopoeia, (Rockville MD, USA). The ORF CDNA clone was used to generate the expression clone for CCR2 under a suitable promoter to produce lentiviral particles. Several Gateway compatible lentiviral destination vectors were designed, cloned and tested for their selectable genotype, efficiency and transgene expression levels, as outlined in sections 2.2.3, table 1.1.

2.2.2 Entry Clones for CCR2 and DsRed

Gateway™-compatible ORF c-DNA entry clone for mouse CCR2 and Gateway™-compatible Ds Red entry clones were purchased from GeneCopoeia (Rockville, MD, USA) and the plasmid DNA was reconstituted in recommended volume of TE buffer. The CCR2 ORF C-DNA entry clones were in a Gateway compatible kanamycin resistant vector (figure: 2.2).

To prepare ample amount of CCR2 entry clone for the recombination reactions the entry clone was transformed into Alpha Gold competent bacterial strains (Origene) and propagated. Briefly, 50 µl of competent bacterial cells were thawed on ice and transferred to a pre-chilled sterile Eppendorf tube. Five microliters (10µg) of the respective plasmid was added to the bacterial cells and mixed gently by pipette. The tubes are incubated in ice for 30 min. The tubes were transferred to a 42^0C water bath and incubated for 30 seconds. The tubes were then transferred to ice for 2 min. One millilitre SOC medium was added and the tubes were incubated at 37^0 C for 1 hr in an incubator shaker at 225rpm. The transformed bacterial cells were centrifuged at 6000rpm for 2 min to pellet the cells. The pellet was re-suspended in 100µl of SOC medium. The cell suspension was plated on to an LB agar plate with kanamycin

25µg/ml) and incubated at 37^{0}C overnight. Single bacterial colonies that were formed overnight were picked and grown in 5ml of LB broth with Kanamycin overnight. The overnight- grown cultures of bacteria were used to isolate the plasmid DNA using Qiagen mini prep plasmid isolation kit as per the manufacturer's instructions. A glycerol stock of the bacterial culture was also made by mixing equal volumes of bacterial culture with sterile glycerol, and stored at -80^0 C for future use.

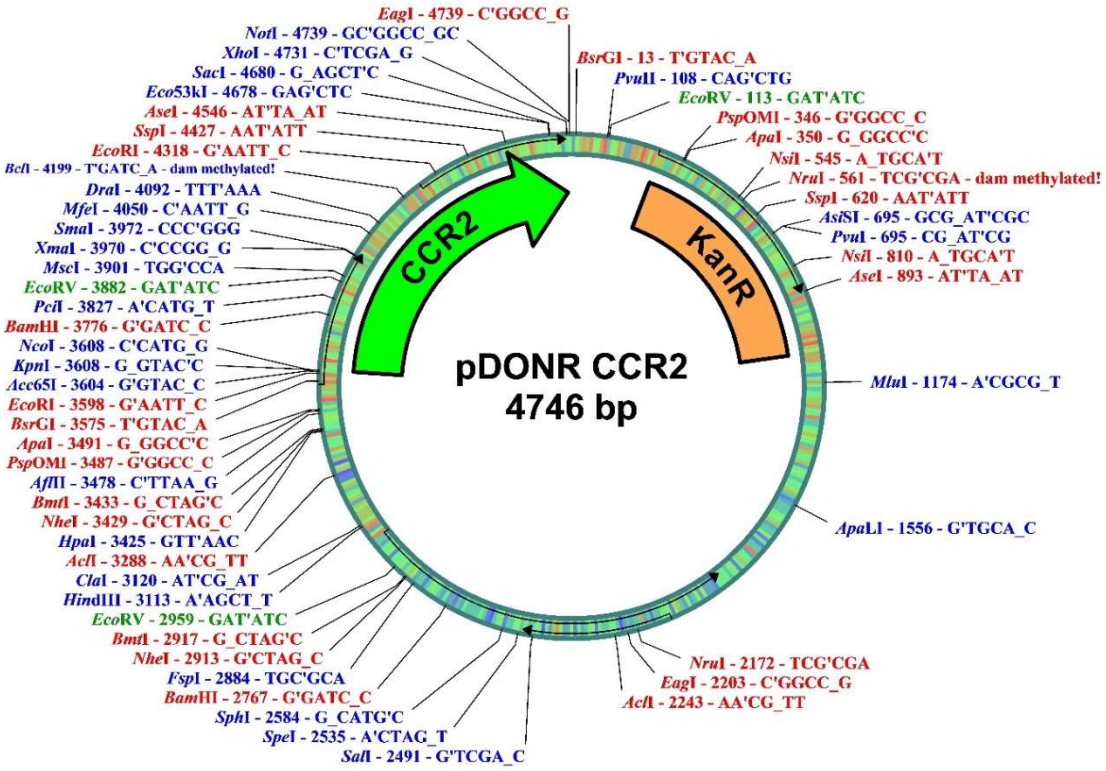

Figure 2:2 Map of the CCR2 entry clone

The Isolated plasmid DNA was quantified by Nanodrop™ and analysed by restriction digestion to confirm the plasmids isolated are the original CCR2 entry clone. All restriction enzymes and their corresponding buffers were obtained from New England Biolabs (NEB) unless specified. One microgram of plasmid DNA was used per restriction reaction. Restriction digest was performed separately with EcoR1 and *EcoR V* restriction endonuclease for 1 hr at 37^{0}C. The reaction mixture was as follows. To a 1.5 ml Eppendorf tube 1ul of restriction enzyme, 1.5 µl of the enzyme buffer, 0.5 µl of BSA (if recommended). The reaction was stopped after 1hr. The samples were mixed with 0.5 volume of loading dye and ran alongside suitable molecular weight ladder in a 1.2% agarose gel electrophoresis for 45 min at 125volts.

2.2.3 Types of Gateway Destination Vectors Tested

Various gateway compatible expression systems were tested to assess gene expression and integration of the gene of interest. Details of the destination vectors tested and their details are in the table 2.1 below.

Table 2:1 Types of lentiviral destination vectors tested

Destination vector	Promoter	Selectable marker
pLenti 6/v5/DEST	CMV promoter	Blasticidin
pWPT	EF-1 alpha	nil
pLEX_307	EF-1 alpha	Puromycin

Both CCR2 and EGFP / DsRed plasmids were cloned in the above-mentioned destination vectors and analysed for the expression of transgene in 293T cells as well as on mMSCs in chapter 3.

2.2.4 pLEX_307 Gateway Vector

The destination vector used for the gateway recombination reaction was the pLEX_307 which was a gift from David Root (Broad Institute of MIT and Harvard) (Addgene plasmid # 41392). The plasmid is an empty backbone for lentiviral expression system that contains an EF-1α promoter and a selectable marker for puromycin resistance enabling the selection of gateway expression clones. The ampicillin resistance gene enables the propagation and selection of the empty plasmid in a bacterial strain.

2.2.5 Bacterial Transformation and Plasmid Preparation

The destination vectors were propagated in top10 competent bacterial cells (Invitrogen). Transformations were performed as explained in the previous section (section 2.2.2). The transformed cell suspension was plated on to an LB agar plate with $LB_{Ampicillin}$ (100µg/ml) for destination vector and all other plasmids unless specified) and incubated overnight at 37^0C. Single bacterial colonies were picked and grown in 5ml of LB broth with ampicillin overnight. The overnight grown cultures of bacteria were used to isolate the plasmid DNA using Qiagen mini prep plasmid

isolation kit as per the manufacturer's instructions. A glycerol stock of the bacterial culture was also made and stored at -80^0 C for future use. A higher volume (500ml) of bacterial cultures were made for maxi prep of the desired plasmids from the glycerol stock when required.

2.2.6 LR Cloning for CCR2 and Ds Red plasmids

2.2.6.1 Production of Gateway CCR2 / DsRed Gateway Clones

Gateway recombination reaction was performed to produce pLEX_CCR2 and pLEX_Ds Red gateway expression clones. Gateway compatible ORF entry clone for mouse CCR2 was cloned into the Plex_307 lentiviral expression destination backbone using LR cloning (Invitrogen). Similarly, the gateway compatible Ds Red entry clone was also cloned into the Plex_307 lentiviral expression destination backbone. Briefly, the LR clonase ii enzyme was thawed on ice and mixed briefly before use. The LR cloning reaction mix consisted of the following. 1-7 µl of the entry clone CCR2 ORF or Ds Red (50-150 ng), 1 µl of destination vector Plex_307 (150ng/ml), the reaction volume was adjusted to 8 µl with sterile TE buffer. 2 µl of LR clonase II enzyme was added to the tubes. The tubes were incubated at room temperature for 18 hours. The reaction was stopped by adding 1µl of Proteinase K. The tubes were vortexed and incubated at 37^0 degrees for 10 min.

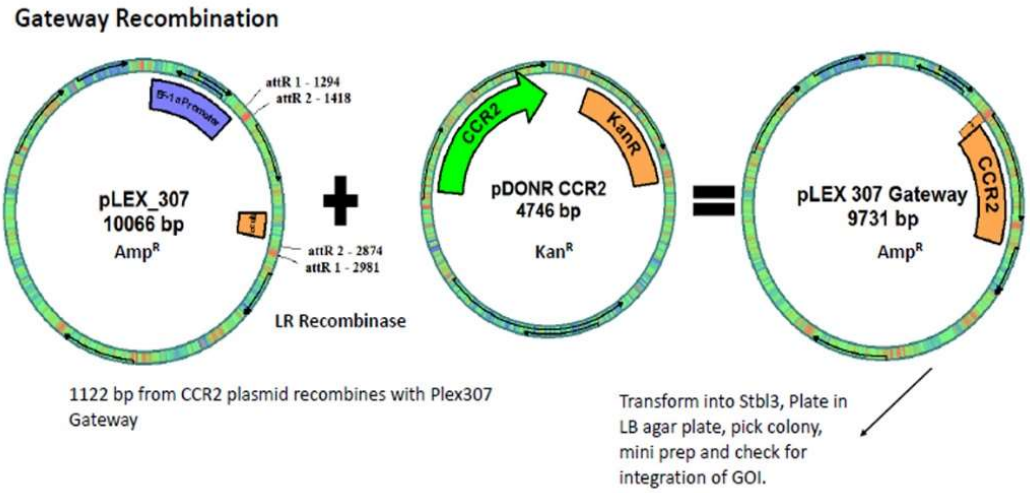

Figure 2:3 Gateway recombination: recombination between CCR2 ORF (entry vector) and pLEX 307 (destination vector) resulting in the production of pLEX 307 CCR2 (expression vector)

The resulting product containing the expression clones were transformed into top10 competent bacterial strain (Invitrogen) and propagated. A glycerol stock of the same was maintained at -80^{0}C for future use as described in section 2.2.2. Integration of gene of interest into the expression clones were analysed from a plasmid mini prep using restriction digestion. The expression clones were also sequenced to confirm the integration of the gene of interest.

2.2.6.2 Bacterial Transformation and Plasmid Isolation

The recombination reaction was transformed into top10 competent bacterial strain (Invitrogen). The bacterial transformation was carried out as explained previously (section 2.2.2). Colonies of bacteria grown were picked and grown in LB $_{amp}$ (100µg/ml) broth overnight and plasmid DNA was isolated using Qiagen mini prep plasmid isolation kit as per the manufacturer's instructions.

2.2.6.3 Analysis of Transgene Integration

The integration of CCR2 ORF into the Plex 307 lenti backbone is confirmed by restriction digestion of the resulting expression vector after LR recombination. Restriction endonucleases were selected based on their ability to cut inside the integrated CCR2 ORF sequence. All restriction enzymes and their corresponding buffers were obtained from New England Biolabs (NEB) unless specified. 1ug of plasmid DNA was used per restriction reaction. Restriction digest was performed separately with *EcoR1*, *EcoR V* and *Afl III* restriction endonuclease for 1 hr at 37^{0}C. The reaction mixture was as follows. To a 1.5 ml Eppendorf tube 1ul of restriction enzyme, 1.5 µl of the enzyme buffer, 0.5 µl of BSA (if recommended). The reaction was stopped after 1 hr. The samples were mixed with 0.5 volume of loading dye and ran alongside suitable molecular weight ladder in a 1.2% agarose gel electrophoresis for 45 min at 125volts.

2.2.7 Lentiviral Vector Production

2.2.7.1 Lentiviral Packaging Plasmids

Second generation lentiviral vectors were used in this study and the packaging plasmids were a gift from Didier Trono Laboratory (EFPL, Lausanne, Switzerland). All packaging plasmids were propagated in DH5α strain of competent bacteria and maxi prepped using Qiagen plasmid maxi prep kit using manufacturer's instructions.

The packaging plasmids used were as follows and are shown in Figure 2.1,

pPax2 (Addgene plasmid 12260): Empty backbone 2nd generation lentivirus packaging plasmid that contains a CAG promoter and encodes for gag, pol, env and tat proteins of the virus.

pMD2.G (Addgene plasmid 12259): Second generation lentiviral packaging plasmid that codes for envelope proteins. The plasmid codes for a stable pseudo typed G protein of the vesicular stomatitis virus under a CMV promoter.

pRSV Rev (Addgene plasmid: 12253): This plasmid is a third-generation lentiviral packaging plasmid that codes for post transcriptional regulators for efficient gag and pol gene expression.

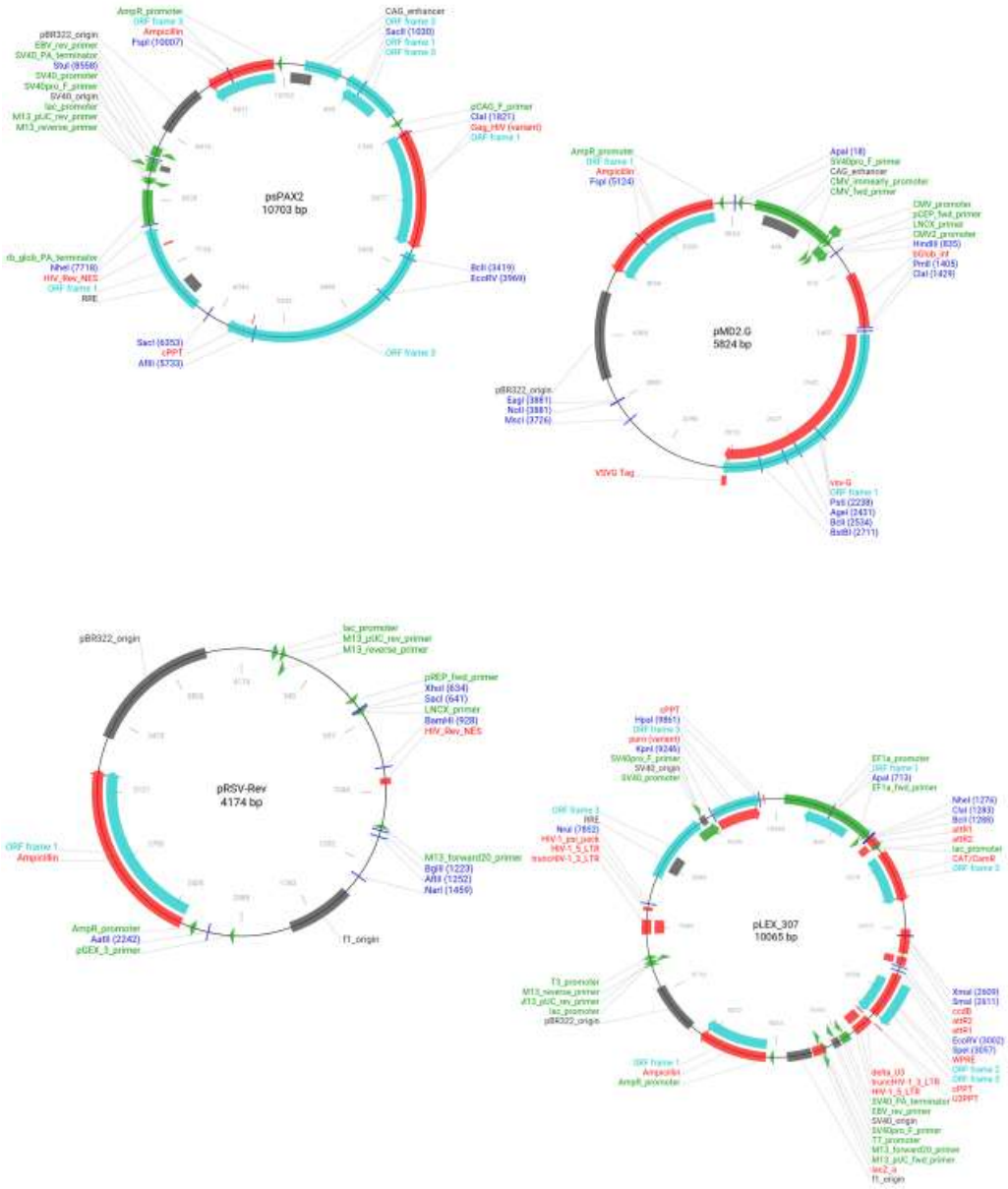

Figure 2:4 Maps of pPax2, pMD2.G pRSV Rev lentiviral packaging plasmids and pLEX_307 Gateway destination vector

2.2.8 Production of Lentiviral Particles

2.2.8.1 Tissue Culture

General considerations: All tissue culture procedures were performed under sterile conditions in a class2 biological safety cabinet. All media and supplements were filter sterilised through a 0.22µm vacuum filter unit (Sarstedt, Numbrecht, Germany). All cell types were maintained in complete medium supplemented with 10% Foetal

Bovine Serum and antibiotic mix unless specified. All the cells are grown at 37^0C, with 5% CO_2 and 95% air in a humidified CO_2 incubator.

The lentiviral particles were produced in Human Embryonic Kidney cells transformed with the SV4OT antigen (HEK 293T).

HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5g/L) medium (Sigma Aldrich, St. Louis, Missouri, USA) supplemented with 10% (v/v) FBS (Sigma- Aldrich, St. Louis, Missouri, USA), 1% Penicillin streptomycin solution (Sigma-Aldrich, St. Louis, Missouri, USA).

HEK 293 cells were thawed in a water bath, added dropwise to 10 ml DMEM high glucose medium. Roughly 30,000 cells/cm2 were seeded to a T175 flask. The cells were regularly checked under an inverted microscope to assess the growth and morphology. The growth media was replaced every 3-4 days until the cells reached 70-80% confluency. Once the flasks were70-80% confluent the media was aspirated off and the remaining media was washed off with sterile PBS, 3ml of 0.25% trypsin per T175 flask was added and incubated for 5 min at room temperature. After the cells were dissociated form the plate, 10 ml of complete media was added to neutralise the trypsin. The contents were transferred to a sterile 15 ml polystyrene tube and centrifuged at 400G for 5 min at room temperature. The pellet was re-suspended and cells were mixed with Trypan blue and using a Neubauer haemocytometer. Viability of the cells were determined by trypan blue exclusion staining during the counting process.

2.2.8.2 Passage of Cell Lines

Confluent cell lines were trypsinized routinely and passaged / cryopreserved as required. Briefly, the media was aspirated off and the cells were washed with sterile warm PBS to remove any residual media. 3 ml of 0.25% trypsin/EDTA solution was added per T175 flask and the flasks were incubated for 5 min at room temperature until the cells were dissociated. The Trypsin solution was neutralised by adding 10 ml

of complete growth medium per T175 flask. The detached cell solution was transferred to a sterile centrifuge tube and pipetted by centrifugation at 400G for 5 min at room temperature. The cell pellet was re-suspended in 10 ml of complete media. The cells were counted using a haemocytometer and re-suspended as desired. Viability of the cells were determined by trypan blue exclusion stain during the counting process. Approximately, $1x10^6$ cells were seeded per T175 flask and 25 ml of complete media was added before leaving the flasks in the incubator.

2.2.8.3 Cryopreservation and Thawing of Cell Lines

When cryopreservation of cultured cells was required, the passaging procedure explained above was performed and the cells were counted and re-suspended in freezing media comprising 10%(v/v) DMSO and 90% FBS. The cell suspension was aliquoted to sterile 1.5ml cryovials (Nunc) and placed in a -80^0C freezer overnight. The vials were placed in liquid nitrogen storage tanks for long term storage.

Thawing of cryopreserved cells were done in a 37^0C water bath. Once the contents of the cryovial is thawed, the cell suspension was transferred gently to a sterile tube containing 10ml pre-warmed complete media. The cells were counted and re-suspended in desired cell numbers and seeded on to a T75 flask and incubated overnight in a CO_2 incubator. The media was replaced next day after washing off any non-adherent cells and residual DMSO using sterile PBS.

2.2.8.4 Co-Transfection of Plasmids for Lentiviral Vector Production

Four million HEK 293T cells were cultured as described previously. When the cells were 70% confluent co-transfection was carried out using JetPEI transfection agent (Polyplus). For each 15cm plate of 293T cells a mix of 13µg of psPAX2.2, 5.6µg of Pmd2.G and 5.6µg of pRSV-Rev lentiviral packaging plasmids along with 13µg of transgene in lentiviral back bone were made up to 500µl using sterile 150mM NaCl in a 15ml polypropylene tube. The tube was vortexed and centrifuged at 400G for 1 min. In a separate polypropylene tube, 41µl of JetPEI transfection reagent was made up

to 500µl in sterile 150mM NaCl. The tube was vortexed gently. The JetPEI solution was added to the tube containing the mix of plasmids, vortexed and centrifuged at 400G for 1 min and incubated at room temperature for 30 min. The HEK293T medium was replaced by virus production medium (high glucose DMEM medium with 1x Pen Strep and 10% FBS heat inactivated (65^0C for 30 min)). The mix of JetPEI and the plasmids were added drop-wise to the HEK 293T cells. The plates were gently swirled to enable uniform mixing and incubated overnight. The medium was replaced the following day. The volume of media was maintained at 15 ml per 15cm dish.

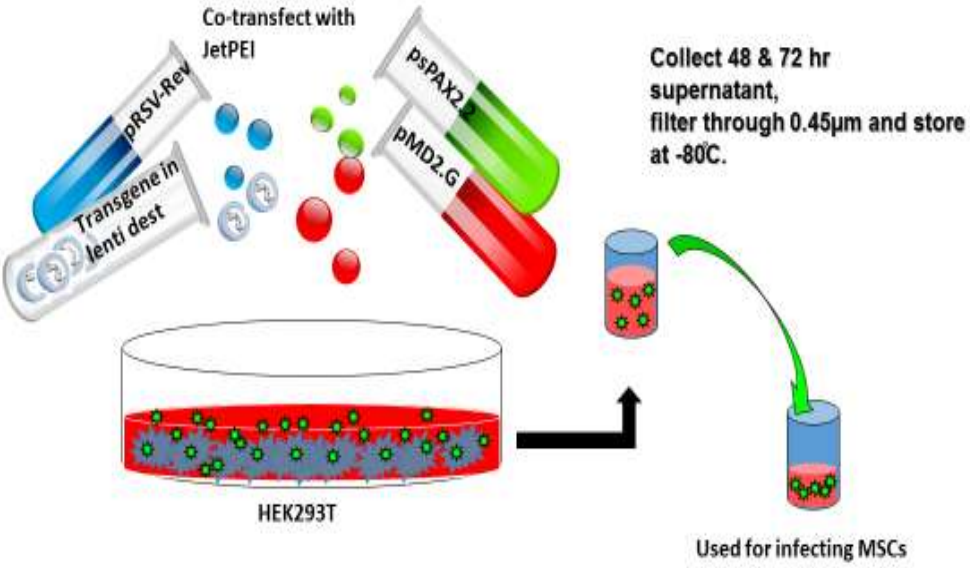

Figure 2:5 Schematic showing the production of lentiviral vector

Forty-eight hours' post transfection, viral particles released into the media was collected, filtered through a sterile 0.45µm filter and stored at 4^0C. 15ml of virus production media was added to the dishes and incubated overnight. At 72 hr post-transduction the media was collected again, filtered through a sterile 0.45µm filter and combined with the first harvest. The collected medium containing the viral particles were aliquoted, tittered and stored at -80^0C until they are used for transduction of MSCs.

2.2.9 Titration of Lentiviral particles

2.2.9.1 Transduction of HEK 293T cells

Titration of lentiviral vector was carried out in HEK 293 T cells. HEK 293 T cells were grown in high glucose DMEM, supplemented with 10% FBS, 1% PenStrep. HEK 293T cells were plated at 100,000 cells per well in a 6 well plate. Two wells served as un-transduced control while 2 wells per volume was used for 2 volumes of vector (1ml and 3ml un-concentrated vector), The vectors and controls were added to their respective wells and incubated overnight. The medium was replaced the next day. The cells were trypsinized after 48 hours and genomic DNA was isolated to analyse the GAG integration by PCR.

2.2.9.2 Determination of GAG Integration by PCR

Genomic DNA from the control and transduced cells were isolated using genomic DNA isolation kit as per the manufacturer's instructions (Qiagen). The cells were trypsinized and genomic DNA isolated and reconstituted in 50 µl TE buffer. Briefly, the DNA concentration was determined by Nanodrop™ ND-1000 Spectrophotometer (Thermo Scientific, MA, USA). PCR for GAG integration was carried out using 100ng/µl of genomic DNA sample. PCR reactions were run on a StepOne™ Plus real time PCR machine (Applied Biosystems, CA, USA) using a Qiagen Quantitect SyBrGreen PCR kit. The PCR reaction was set in a 96 well PCR plate and the reaction setup was as follows.

The reaction conditions were as follows, 95^0C for 15 min, 40 cycles of: 94^0C for 15 sec, 55^0C for 30 sec, and 72^0C for 30 sec.

The GAG primers used were of the following sequence,

GGA GCT AGA ACG ATT CGC AGT TA

GGT TGT AGC TGT CCC AGT ATT TGT C

The reaction mixture was as follows,

Mix per reaction: 10 µl Quantitect SyBr Green reaction mix

0.6 µl Forward 10 µM GAG primer

0.6 µl Reverse 10 µM GAG primer

2.8 µl water

2 µl of template Genomic DNA

Along the test samples of transduced and un-transduced genomic DNA, 2µl of gag DNA standards containing 10^3, 10^4, 10^5, 10^6,10^7 copies per µl were also included in the reaction.

2.2.9.3 Calculation of Virus Titre

Example: copy number of gag gene per 1 ml of vector = 48548.448.

The value obtained for un-transduced 293T cells were subtracted and is divided by 3500 (100ng DNA) from the standard graph. So, according to the example, 48548.448/3500=13.87

This value was multiplied by number of cells seeded, 100,000: 13.87x 100,000=1,387,000. This was divided by the volume of vector added: 1,387,000/1 (1ml vector added) = $1.3x10^6$ Tu/ml.

Titre (Tu/ml) = (number of target 293T cells) X (Copy number per cell) ÷ volume of viral vector added ml).

2.3 Results

2.3.1 Propagation and preparation of entry clones

To prepare ample amount of CCR2 entry clone for the recombination reactions the entry clone was transformed propagated in competent bacterial strain; the plasmid DNA was isolated and tested by restriction digestion. The CCR2 entry clones yielded the correct size fragments of DNA when run on an agarose gel after the restriction digest. The restriction digest of the CCR2 entry clone with EcoR1 yielded fragments of 721bp and 4026bp while the restriction digest with *EcoRV* yielded fragments of 923bp, 978bp and 2846bp (Fig 2.6) All the above-mentioned fragments were of the expected size as per the sequence which confirms the propagated plasmids were the right CCR2 entry clones.

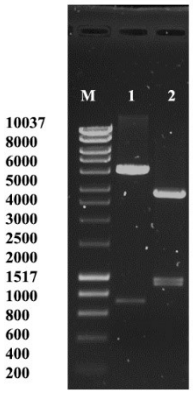

Figure 2:6 Restriction analysis of CCR2 ORF C-DNA entry clone

Lanes: 1: 1Kb Hyper Ladder Marker, 1: CCR2 Entry clone EcoR1: 721bp, 4026bp, 2: CCR2 Entry clone *EcoR* V: 923bp,978bp, 2846bp

2.3.2 Generation of expression vectors

Expression vectors were produced by LR cloning reaction. The recombination of entry clones which has the transgene (CCR2, DsRed, eGFP) with destination vector results in the production of the expression clones that has the transgene integrated at the att sites of the gateway cassette. Three different destination vectors were tested and the results are as explained below.

2.3.3 Selection of Destination vector. / types of destination vectors tested.

Three different destination vectors were used to produce expression clones and were tested for their transgene expression levels and selectable properties. The first destination vector tested, the pLenti 6/v5/DEST vector had a CMV promoter and a selectable marker for blasticidin enabling the selection of transformed cells to be selected by culturing them in blasticidin containing media.

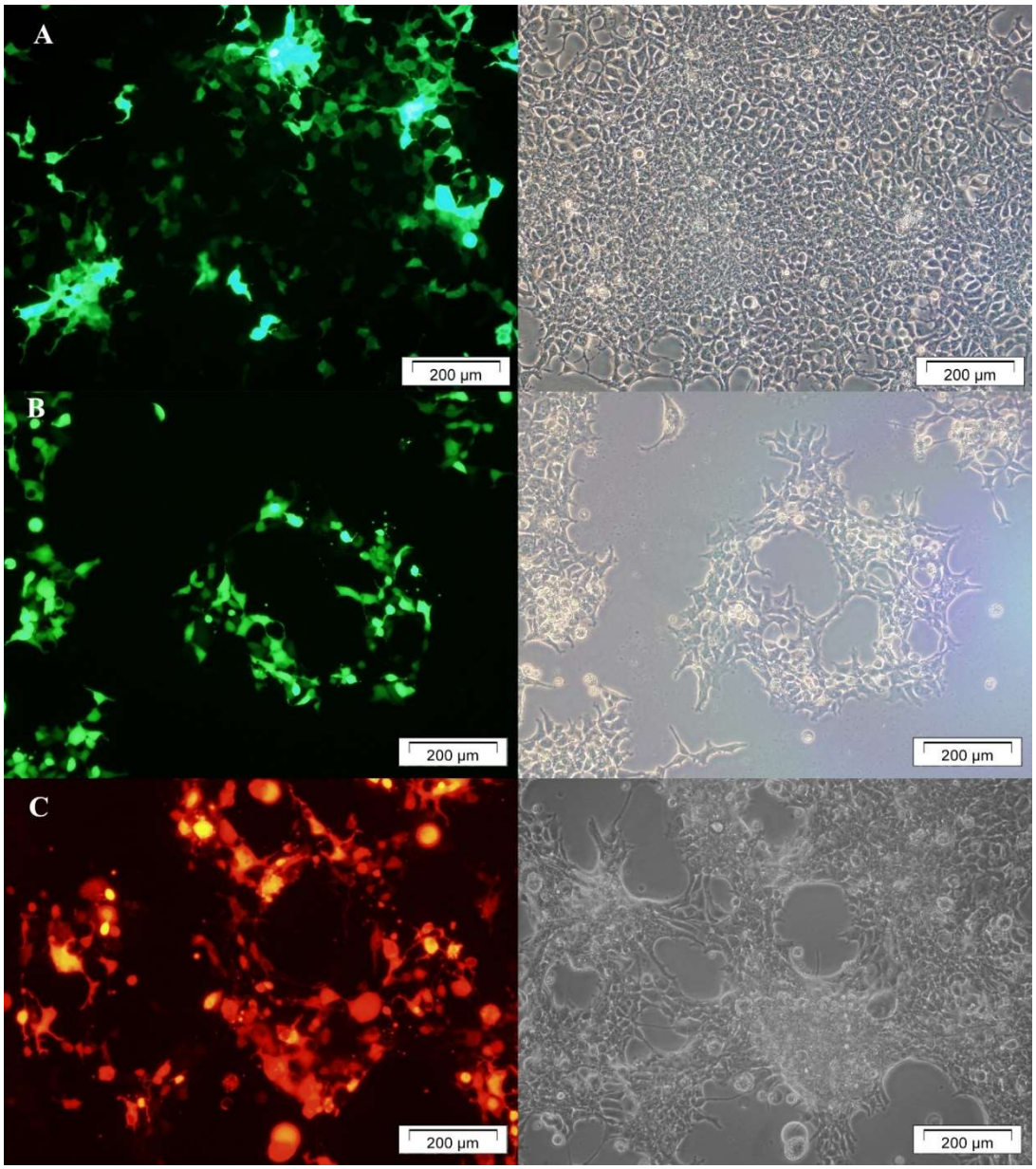

Figure 2:7 Fluorescent and bright field images of HEK293Tcells transduced with pLenti 6/v5-eGFP (A), pWPT-eGFP (B) and pLEX-307-DsRed (C).

A pLenti 6/v5 GFP expression clone was produced and tested for the expression levels of GFP on transduced cells and the level of GFP expression in the cells were found to

be too low and the efficiency of transduction was poor as evident by the low number of cells surviving the blasticidin selection (figure 2.7a), and the relatively poor fluorescent intensity of the cells. The second destination vector tested, the pWPT destination vector had a stronger EF-1 alpha promoter as compared to the CMV promoter on the pLenti 6/v5 destination vector. The transduced cells showed higher expression levels of GFP (Figure 2.7b), but it lacked a selectable marker to select a homogeneous population of cells that express the transgene. The third destination vector tested, the pLEX_307 vector had a stronger EF-1 alpha promoter and a Puromycin selectable marker for selection of transduced cells. This vector showed high level expression of DsRed in cells transduced with pLEX DsRed plasmid (figure 2.7c). These advantages of the pLEX_307 vector meant that it was selected for further studies.

2.3.4 Production of pLEX_307 CCR2 Expression clone and testing CCR2 integration

The expression clone generated due to LR cloning reaction was tested to confirm the successful integration of the gene of interest into the clone. The CCR2 ORF sequence gets integrated between the att sites in the destination vector. This enables the prediction of the entire expression clone sequence. Analysis of the expression clone sequence resulted in the identification of various restriction endonuclease sites within the sequence. A restriction digestion analysis of the expression clone by EcoR1 yielded fragments of the size 1600bp, 2328bp, and 7403bp. Restriction digest of the same clone by the enzyme *EcoRV* yielded fragments of 977bp and 8754bp, while an *AflII* restriction digest yielded fragments of 906bp,1113bp,3750bp and 3962bp (figure 2.8). The above-mentioned restriction enzymes all had restriction sites within the CCR2 ORF from the entry clone and the fragments generated were as predicted by the restriction digest analysis in the pDRAW software used for sequence analysis. This confirmed the integration of the CCR2 ORF into the expression clone which is a result of successful LR recombination of the CCR2 entry clone and the pLEX307 destination vector.

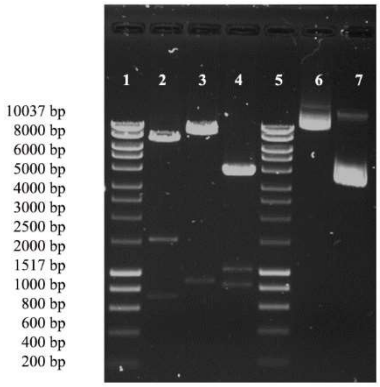

Figure 2:8 Restriction analysis of pLEX307 CCR2 expression clone for CCR2 integration

Lanes: 1: 1kb hyper ladder Marker, 2: pLEX-CCR2 EcoR1 (expected fragments 7403bp,2328bp, 1600bp), 3: pLEX-CCR2 *EcoR V* (expected fragments 977bp, 8754bp), 4: pLEX-CCR2 *Afl III* (expected fragments 906bp, 1113bp, 3750bp,3962bp), 5: 1kb hyper ladder Marker, 6: pLEX-CCR2 expression clone Undigested 9731bp, 9: CCR2 in entry vector undigested 4747bp

The pLEX CCR2 expression clone was sequenced to confirm the successful integration of CCR2 ORF into the expression clone. The sequencing results of the pLEX expression vector when analysed by BLAST returned results confirming the presence of mouse CCR2 sequence within the expression clone (figure 2.9)

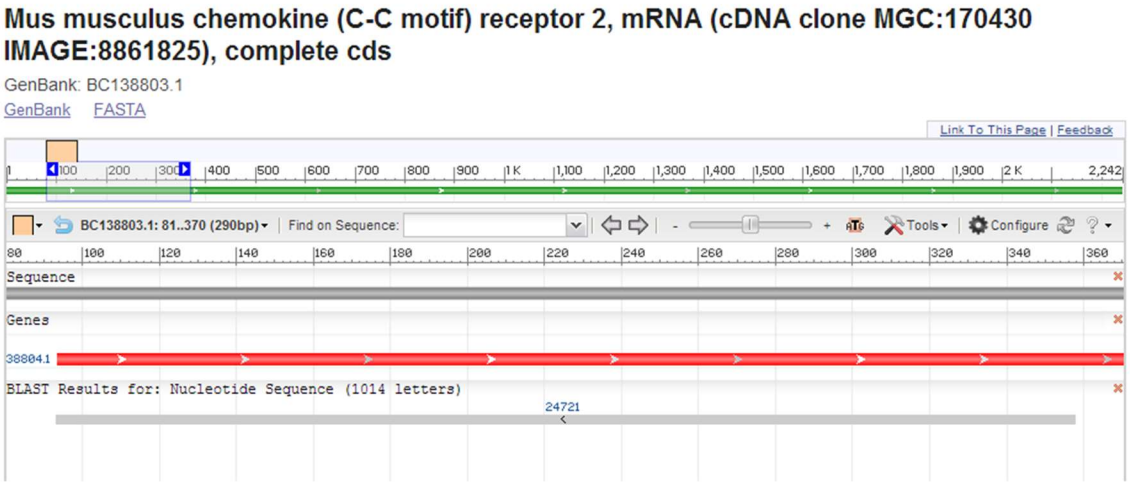

Figure 2:9 Sequencing of pLEX CCR2 expression clone showing successful CCR2 integration.

2.3.5 Production of Lentiviral vectors

Lentiviral vectors with the transgene under three different expression systems pLenti6/V5, pWPT and pLEX307 were produced by transient transduction of HEK293T

cells and were used for subsequent transduction of MSCs. pLenti6/V5 GFP, pWPT-GFP and pLEX307-DsRed vectors were also produced and they served as the controls to assess the transduction efficiency of the vectors. mMSCs were transduced with the above mentioned lentiviral vectors and the transduction efficiency was measured by the number of GFP/DsRed positive cells per random 10x fields (fig 2.10). Statistical analysis (One-way ordinary ANOVA) of the transduction efficiencies using GraphPad Prism software showed significant differences between the groups ($p<0.0001$, $R2=0.9834$).

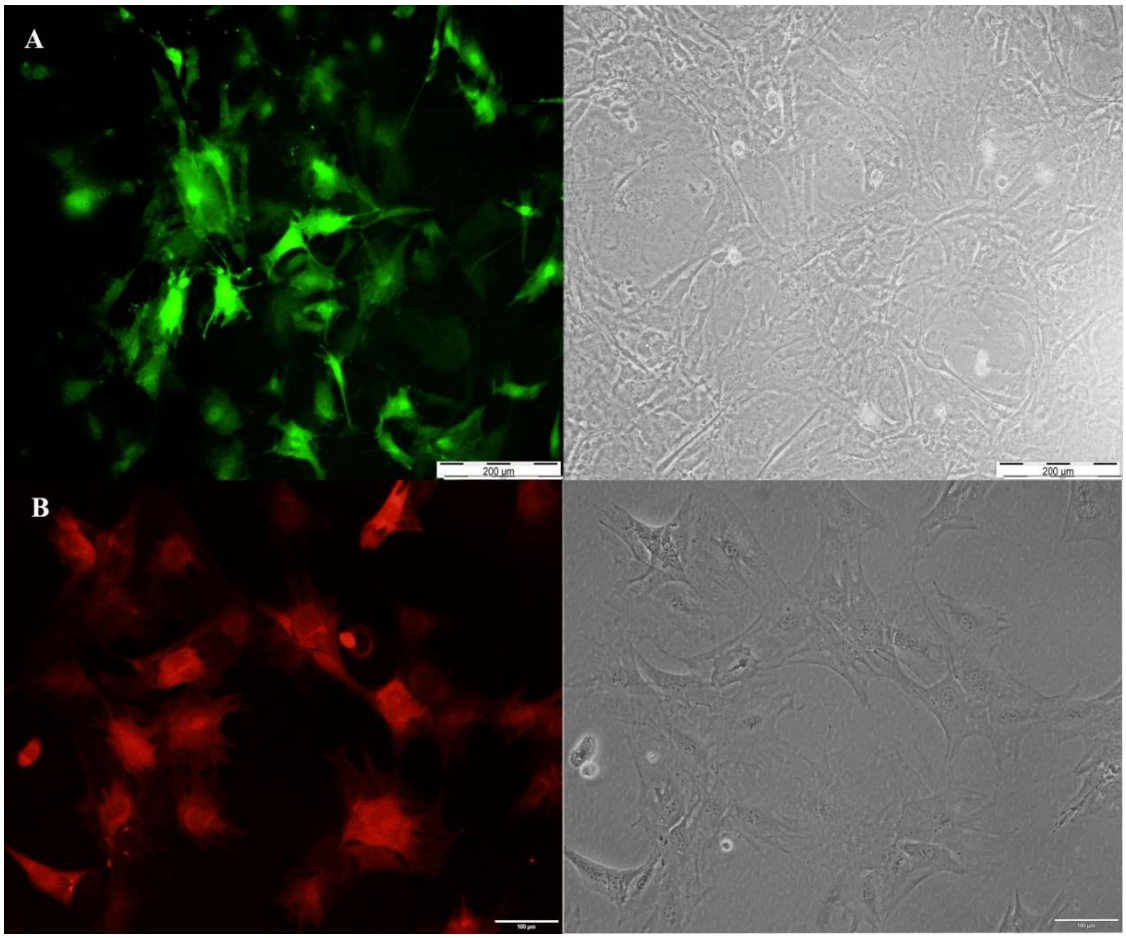

Figure 2:10 Fluorescent and bright field images of mMSCs transduced with pWPT-GFP lentiviral vector (A) and pLEX-DsRed lentiviral vector (B).

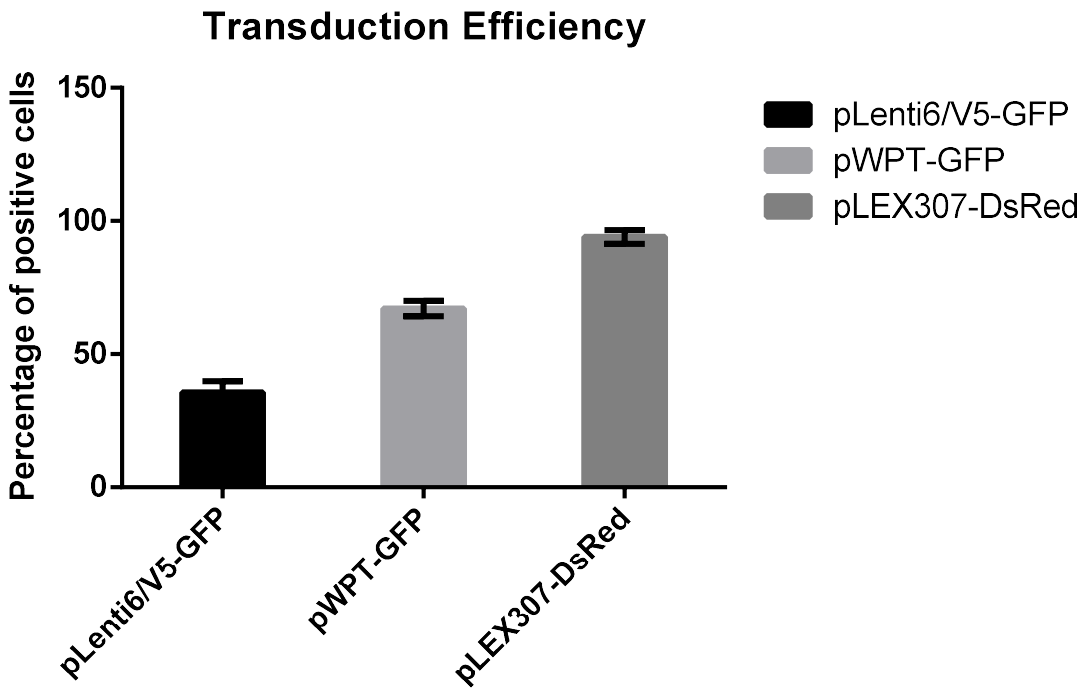

Figure 2:11 Transduction efficiency of three lentiviral vectors tested, measured by the number of GFP/DsRed positive cells per random 10x fields.

2.3.6 Titration of lentiviral vectors

HEK 293T cells were treated with different volumes of lentiviral vectors and the genomic DNA of the cells were extracted after 72 hours for analysing the integration of *gag* sequence into the genome of the cells. The copy number of integrated gag sequence was analysed using a standard curve plotted against *gag* standard DNA (fig 2.12 b). The viral titre was calculated as explained previously and the concentration of lentiviral vector was expressed as transducing units per millilitre (TU/ml). Virus titre was calculated on two doses (1ml and 3ml) of lentiviral vector for the pLEX and pWPT vectors. The titre values of the pLEX vector was significantly higher than that of the pWPT vector at both the concentrations (figure 2.12a). Both the pWPT and the pLEX vector systems tested here yielded very high concentration of lentiviral particles (10^9-10^{10}TU/ml). The titre of pLEX lentiviral vectors were significantly higher than that of the pWPT vector. The high titre of the pLEX vectors along with the presence of a selectable marker (Puromycin resistance) gave it a clear advantage over the pWPT lentiviral vector. Thus, the pLEX lentiviral vectors were selected for genetic modification of MSCs in this study.

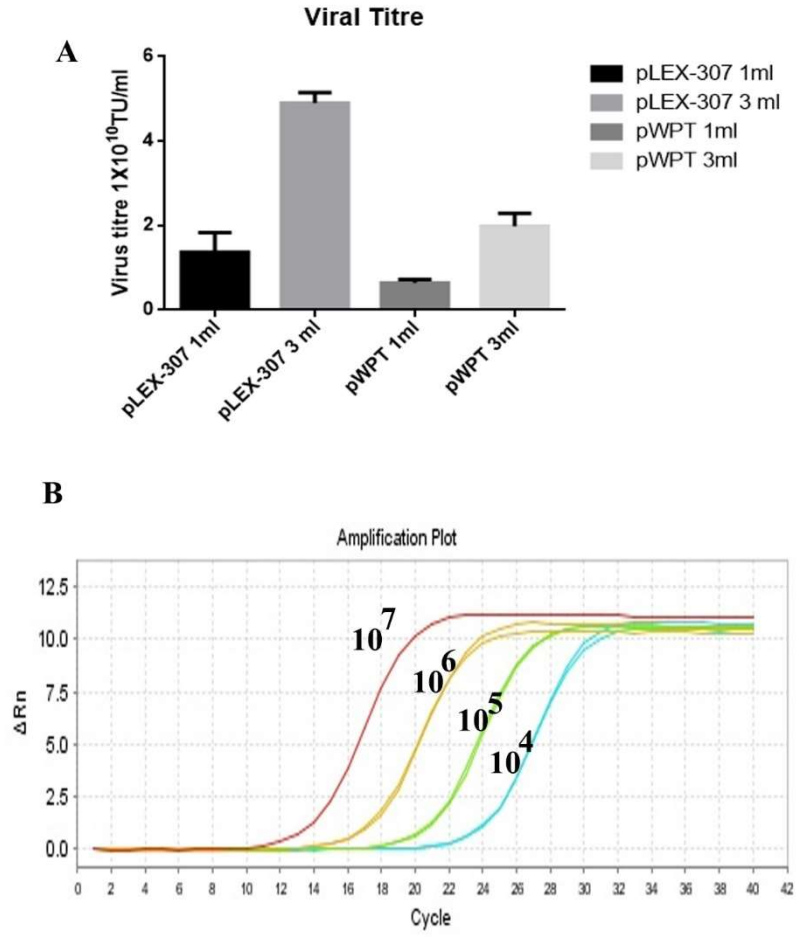

Figure 2:12 Viral titre for pLEX307 Lenti CCR2 and pWPT Lenti GFP(A). Amplification plot of GAG standards of copy numbers ranging from 107 to 104 (B).

2.4 Discussion

MSCs are multipotent[11,12], adult stem cells that have the capacity to self- renew, differentiate into multiple lineages, and have immune suppressive and immunomodulatory properties. They secrete a variety of paracrine factors with roles in inflammation, tissue repair and chemotaxis[1,13,14]. These properties of MSCs made them ideal candidates for treatment of various diseases[15]. Genetic modification of MSCs to improve their therapeutic potential is a constantly emerging area of research with immense potential. Various gene and cell therapy approaches are constantly evolving with the advance in genome editing and genetic modification[16–20].

Viral vectors are one of the most effective modes of gene delivery systems with significant advantages over non-viral gene transfer methods[2]. Lentiviral vectors have clear advantages over other viral vectors as they are efficient in transgene integration and stable transgene expression over long periods without immune activation and toxicity[21]. The development of 2nd and third generation lentiviral vectors have improved the safety concerns relating to the toxicity and pathogenicity of viral vectors in clinical use[4,22].

In this chapter, we have designed, developed and tested three different types of second generation lentiviral vectors. The efficiency of transgene expression and selectable phenotypes of transduced cells were factors in deciding the vector system to be used for further studies. The first vector system was a Plenti6/V5 lentiviral system which had a CMV promoter and a selectable marker of blasticidin resistance. The transduction efficiency of this vector system was poor as compared to the other two systems in terms of transgene expression. This was evident from the number of GFP positive cells that were obtained after transduction of HEK293T cells and mMSCs (fig 2.7,2.9). The second lentiviral vector tested, the pWPT lentivirus, had enhanced transgene expression as compared to the pLENTI6/V5. This can be attributed to the strong EF-1 alpha promoter in this viral system. The efficiency of the EF-1 alpha promoter system compared to other promoters has been reported previously[23,24]. The third viral vector the pLEX lenti, had the highest efficiency of transgene

expression. This vector also had a selectable marker, a Puromycin resistance gene enabling the selection of transduced cells. The higher expression levels of transgene in cells transduced with the pWPT and the pLEX vectors can be attributed to the strong mammalian promoter in the construct. MSCs transduced with the pLEX lentiviral vector showed persistent high level sustained expression of the transgene. This high level of transgene expression along with the presence of selectable marker meant that the pLEX vector system was selected for further MSC modification experiments. High level consecutive expression of transgenes is critical in ex vivo transplantation of genetically modified MSCs for preclinical studies and clinical applications.

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Chapter 3

Optimisation of Lentiviral Transduction and Genetic Modification of Mesenchymal Stem Cells

CHAPTER 3

3 Optimisation of Lentiviral Transduction and Genetic Modification of MSCs

3.1 Introduction

Mesenchymal Stem Cells (MSCs) are self-renewing, plastic adherent, multipotent, immuno- modulatory adult stem cells that can be isolated from various tissue sources including bone marrow[1–4]. MSCs are capable of homing to injured tissues and secrete various paracrine factors that make them ideal candidates to be used in therapeutic applications in diseases with current unmet medical need.

One of the major limitations in MSC therapy is the survival and retention of the transplanted cells at the target site. Various studies showed that MSCs transplanted systemically were distributed predominantly into the lungs and spleen immediately after injection than rather than to the desired target tissues[5,6]. Locally administrated MSC have minimal bio-distribution to distant organs. However, the retention of transplanted MSCs at the target site was found to be diminished over time requiring the need to transplant higher dose of cells to provide therapeutic efficacy[7]. This decreased retention of transplanted cells and associated issues with viability when transplanted into ischemic tissues in combination with the accessibility of target sites for local delivery of stem cells proved to be challenging in different disease models such as myocardial infraction and critical limb ischemia.

Genetic modification of MSCs to overcome these shortcomings has been explored in various disease models[8–12]. Genetic modification of MSCs to enhance their survival[13], bio-distribution / retention at target sites[14,15] along with the possibilities to improve the efficacy of MSCs by altering their angiogenic potential[13,16], potential to secrete growth factors[11,17] and chemokines opened up a new era in the development of Advanced Therapeutic Medicinal Products (ATMPs). Although the therapeutic use of genetically modified MSCs is still in its infancy, the potential of the concept to develop highly effective, targeted therapies with tailored properties will open a new era in

the use of stem cell therapy. The fact that cell therapy products are grown ex vivo prior to transplantation allows for the opportunity to expose the cells to vectors encoding products which may enhance therapeutic efficacy. This approach has challenges in that it accentuates the regulatory issues to be overcome and increases the complexity of the product. In future, genetically modified cells may be used in combination with medical devices and biomaterials which will usher in a new era of complex and hybrid therapeutic technologies. The focus of this thesis is the use of genetic modification alone to enhance the therapeutic effect of MSCs when used to treat critical limb ischemia.

There are a large variety of vectors which may be used to modify cells with a range of properties such as longevity of expression or ability to transduce quiescent cells. Lentiviral vectors are effective gene transfer vectors and they can provide stable long term expression of transgene for a variety of cell types. They can infect and replicate within actively dividing cells as well as non-dividing cells.

MSCs have been genetically modified using a variety of vector systems in the past and this has been reported in the literature. Genetic modification by lentiviral vectors has proved to be the most efficient and stable method due to the properties of lentiviral vectors relative to other viral vectors and non-viral modes of genetic modification[18–21]. Lentiviral transduction of mesenchymal stem cells is a straight forward process wherein lentiviral particles carrying the gene of interest are added to the MSC culture media. The viral particles enter the target cell, crosses into the nucleus of the cells where they integrate into the host chromosome.

This occurs through binding of the viral env glycoprotein with the cell surface releasing the viral nucleoprotein complex (VNC)into the cell. VNCs are transported in to the nucleus of the cell for effective integration of viral genome with the host chromosome. The nuclear membrane is removed only during mitosis and on all other times, transport between nucleus and cytoplasm is mediated by karyopherins or

importins that serve as active molecular transport between nucleus and cytoplasm. For a viral vector to integrate into host chromosome of a non-actively dividing cell, it needs to be able to import its VNC into the nucleus. This is done through the nuclear pore which has high specificity in allowing molecules larger than 40kd to go pass through. Any molecule that passes through the nuclear pore is marked by a nuclear localisation signal. The nuclear localisation signal recognises transport of materials to and from the nucleus. For a lentivirus to infect a non-actively dividing cell, the genetic material needs to be transported into the nucleus of the cell. The VNC is recognised by transport molecules like karyopherins. The VNC-karyopherin complex gets transported into the nucleus through the nuclear pore and into the nucleus. The formation of VNC-karyopherin signal renders the complex a nuclear localisation signal[22].

Several methods to improve the efficiency of lentiviral transduction are available, including addition of chemicals to improve the cellular uptake of the viral particles. Even without the addition of chemicals that aid the cellular uptake of the viral particles, the lentiviral vectors can effectively enter dividing and non-dividing cells and cross into the nucleus and provide long term expression of transgene. This occurs following integration into the host cell chromosome.

In this chapter, we describe the genetic modification of MSCs using lentiviral vectors and confirm the transgene expression at the transcript and protein level.

3.2 Materials and Methods

3.2.1 Genetic modification of MSC to over express CCR2

3.2.1.1 Mesenchymal cell culture

Early passage plastic adherent C57BL/6 Mouse MSCs were obtained from collaborating researchers at NUI Galway and were cultured in Iscove's Modified Dulbecco's Modified Eagle's Medium (Iscove's-DMEM) supplemented with 10% (v/v) FBS (HyClone™ R6, HyClone Logan, Utah, USA), 1% Penicillin streptomycin solution and 1% L-Glutamine. Cells were routinely observed under the microscope to assess their growth and morphology. Medium was replaced every 3 days. The cells were passaged at 80% confluency and used for lentiviral transduction.

3.2.1.2 Mesenchymal stem cell characterisation

The International Society of Cellular Therapy (ISCT) defines Mesenchymal Stem Cells as plastic adherent, colony forming, fibroblast like cells that are self-renewing and have the potential to differentiate into adipocytes, osteocytes and chondrocytes, while selectively expressing specific surface markers. Surface markers that define MSCs vary from species to species and are also based on the source of their isolation[23–27]. MSCs used in this study were from C57BL/6 mice and the characteristic surface markers that define C57BL/6 Mouse MSCs are positivity for CD90, CD105, CD 140a, CD140b, Sca-1 and negative for CD34, CD45 and CD11b.

The antibodies and the isotype controls used were are outlined as follows,

CD 90.2: BD Pharmingen APC Rat Anti-Mouse CD90.2; Clone 53-2.1; cat:553007; conc: 0.2mg/ml.

Isotype control: BD Pharmingen Alexa-Fluor®647 Rat IgG2a,k isotype control; cat:557690; conc:0.2mg/ml.

CD105: BD Pharmingen PE Rat Anti Mouse CD105; Clone MJ7/18; cat:562759; conc:0.2mg/ml.

Isotype control: BD Pharmingen PE Rat IgG2a,k isotype control; clone R35-95; cat:553030; conc: 0.2mg/ml.

SCA-1: BD Pharmingen PE Rat Anti-Mouse Ly-6A/E(SCA-1); Clone:D7; cat:553108; conc:0.2mg/ml.

Isotype control: BD Pharmingen PE Rat IgG2a,k isotype control; clone R35-95; cat 553030; conc: 0.2mg/ml.

CD34: BD Pharmingen Alexa-Fluor®647 Rat Anti-Mouse CD34; Clone: RAM34 cat:560233; conc: 0.2mg/ml.

Isotype control: BD Pharmingen Alexa-Fluor®647 Rat IgG2a,k isotype control; cat:557690; conc:0.2mg/ml.

CD45: BD Pharmingen PE Rat Anti-Mouse CD45; clone: 30-F11; cat: 553081; conc:0.2mg/ml.

Isotype control: BD Pharmingen PE Rat IgG2b,k isotype control; clone A95-1; cat:553989; conc:0.2mg/ml;

CD11b: BD Pharmingen PE Rat Anti-Mouse CD11b; clone: M1-70; conc:0.2mg/ml; cat: 553081.

Isotype control: BD Pharmingen PE Rat IgG2b,k isotype control; clone A95-1; conc:0.2mg/ml; cat:553989.

The procedure for the surface marker characterisation is as follows.

mMSCs were cultured in Iscove's medium (Sigma Aldrich) supplemented with 10%FBS (Invitrogen R6), 1% Penicillin streptomycin solution (Gibco, Grand Island, New York, USA) and 1% L-Glutamine (Gibco, Grand Island, New York, USA). Cells were grown in T175 flasks (Sarstedt) and allowed to reach 70-80% confluency before trypsinizing. Once the cells were ready to be analysed, the media was carefully aspirated off and the flasks were washed with 5ml of PBS to remove any debris or floating cells. This was followed by trypsinisation using 4ml of trypsin-EDTA (Gibco, Grand Island, New York, USA) for 4-5min. The trypsin was then neutralized with 8ml of complete mMSC medium and the cells were transferred to a 15ml centrifuge tubes. The cells were spun at 400g for 5min at room temperature and washed twice

with FACS buffer and passed through a 40µm cell strainer to remove any clumps. A cell count was performed and 100,000 cells were seeded in a 96 well V bottom plate (Nunc) in triplicates for each marker and corresponding isotype control. Triplicates were allotted for un-stained control for gating. The cells were stained with each antibody at a concentration of 50ul/well in FACS buffer and incubated at 4°C for 30 min. The cells were spun twice at 400g for 5min with repeated resuspension in FACS buffer to remove unbound/excess stain. After the second spin, the pellets were re-suspended in 200µl FACS buffer and transferred to follow cytometry tubes and kept in ice until analysis. The flow cytometric analysis was carried out using a Becton Dickinson(BD) FACS CANTO multicolour flowcytometry machine (San Jose, California, USA). The gating parameters were set using an unstained control cell group as follows; forward scatter (FSC) = 20, side scatter (SSC) = 390 and; APC-cy7 (633/780/60-A red filter for detection of viability) =560, PE (488/585/42-A) = 365, APC (633/660/20-A) =522. After completing the gating strategy, 5µl of 1/500 dilution of a viability dye; DRAQ7 (Biostatus, UK) was added to each sample immediately before the samples were loaded on to the flow cytometer for analysis. Gating strategy involved gating out the MSC population, followed by live/dead cell isolation, isolation of single cells, and final gate is applied to identify the PE stained or APC stained cells. Data analysis was performed using FlowJo V.10 flowcytometry data analysis software (FlowJo LLC, Oregon, USA).

3.2.1.3 Lentiviral transduction of MSCs

Transduction was carried out in C57BL/6 Mouse Mesenchymal stem cells between passages 1 and 5. Actively dividing non-confluent cultures of MSCs were used for transduction. Cells were cultured in 15cm dishes prior to transduction. Before the addition of viral vector, the media was aspirated off and the cells were washed with sterile PBS. Roughly 15ml of un-concentrated lentiviral vector for CCR2 was added per 15ml flasks for efficient transduction. Lentiviral vector carrying DsRed served as controls. The dishes were swirled to ensure proper distribution of viral vector and incubated overnight in a CO_2 incubator at 37^0C, 5% CO_2. The media was replaced by 15ml Complete growth media (Iscove's modified DMEM with 10%FBS and L-glutamine) after 24 hours. Expression of the transgene was visualised in cells

transduced with lentiviral vectors carrying DsRed using an inverted microscope with a fluorescent attachment (Nikon Eclipse TS100) 48 hr post addition of viral vector. The cells were trypsinized if desired and selection process was initiated.

3.2.1.4 Selection of CCR-2 over expressing MSCs

pLEX307 Lentiviral particles carrying CCR2 and pLEX307 Lentiviral particles carrying DsRed carries a puromycin resistance gene that gets integrated into the host chromosome along with the transgene conferring puromycin resistance to transduced cells. This enables the selection of transduced cells by culturing them in medium containing puromycin. A puromycin sensitivity study was performed with MSCs cultured in increasing concentrations of puromycin from 0.1 µg/ml to 1 µg/ml. The concentration at which all the cells were observed dead within one passage was considered as the optimum concentration for puromycin selection of transduced cells. In this study, all MSCs exposed to concentrations under 0.7 µg/ml survived even after multiple passages in puromycin containing media. So, a concentration of 0.7 µg/ml of puromycin was selected as the ideal selectable antibiotic concentration. Mesenchymal stem cells transduced with lentiviral particles carrying CCR2 or DsRed genes were trypsinized and cultured in medium containing puromycin (0.7µg/ml) (Invitrogen) for selection. Un-transduced cells served as the control, as they do not have puromycin resistance. The cells were monitored daily and the selection media was replaced every 3 days. The Puromycin selection process was stopped when all the cells in the un-transduced control group were killed by puromycin. The surviving transduced cells were washed well to remove all dead cells and cellular debris. The cells were trypsinized and plated onto fresh culture dishes and analysed for transgene expression.

3.2.2 Confirmation of CCR2 over expression on MSCs

3.2.2.1 Confirmation of CCR2 over expression by Q-PCR

CCR2 expression in MSCs at the transcript level was analysed using quantitative real time PCR. Total RNA from $1X10^6$ transduced and un-transduced cells were isolated using an Isolate II RNA mini kit (Bioline, London, UK) following manufacturer's instructions.

Pure RNA with A260/A280 ratio between 1.9-2.1 was typically obtained. The purity of the RNA samples was checked using nanodrop (Nanodrop ND-1000) as well as a bioanalyzer (Agilent technologies Inc, USA) to ensure high purity of RNA samples. All RNA samples used for PCR reactions were of high purity with RNA integrity numbers >9.

The CCR2 transcripts present in the RNA samples were quantified using a one-step reverse transcription and PCR amplification method using a SensiFAST^{TM} SYBR Hi-ROX Kit from Bioline.

Each sample was run with and without reverse transcriptase enzyme to ensure there was no contamination and amplification of genomic DNA during the steps. A concentration of 20ng/µl of RNA (5 µl) was used per reaction and all the samples were run in triplicates. The total volume of each reaction was 20 µl. The PCR master mix volume was 15 µl. There were two master mixes, one with the reverse transcriptase enzyme and the second one without reverse transcriptase enzyme. The second master mix serves as the background control where no amplification is expected. A water sample was also used with and / or without reverse transcriptase enzyme to ensure there is no amplification of the primers. CCR2 primers used were designed with primer blast online tool for *Mus musculus* CCR2 ORF and commercially synthesised by Sigma Aldrich, St. Louis, Missouri, USA. The sequence for the CCR2 primer used was as follows,

CCR2 Forward Primer: GCCTCCACTCTACTCCCTGG

CCR2 Reverse Primer: GAGCAGGAAGAGCAGGTCAG

Mouse Drosha and TBP primers (IDT technologies, Coralville, Iowa, United States) served as the normalisers for the reactions. Normalisers were also used with and / or without reverse transcriptase enzyme in the master mix.

The sequence for the mouse TBP primers are as follows

Mouse TBP Forward primer 5'-CCAGAACTGAAAATCAACGCAG-3'

Mouse TBP Reverse primer 5'-TGTATCTACCGTGAATCTTGGC-3'

The master mix for the reaction was as follows,

CCR2 +RT Master mix	**Volume Per reaction (µl)**
2x SensiFAST SYBR Hi-ROX Mix	10.0
10µM Forward primer CCR2	0.4
10µM Reverse primer CCR2	0.4
Reverse Transcriptase	0.2
RNAse Inhibitor	0.4
H_20	3.6
Drosha / TBP +RT Master mix	**Volume Per reaction (µl)**
2x SensiFAST SYBR Hi-ROX Mix	10.0
10µM Forward/Reverse primer Drosha/TBP	1.2
Reverse Transcriptase	0.2
RNAse Inhibitor	0.4
H_20	3.2

Parameters for RT-Q-PCR

Cycle	Temp	Time	Notes
1	95^0C	3min	Polymerase activation
40	95^0C	5 sec	Denaturation
	60^0C	15 sec	Annealing / Extension

The RQ values were calculated as follows:

The expression of CCR2 in transduced cells was expressed as fold change as compared to the un-transduced sample.

ΔCT = CT Test - CT Normaliser

$\Delta\Delta CT$ = ΔCT of Transduced cell group– ΔCT Un-transduced cell group

RQ (fold change) =$2^{-\Delta\Delta CT}$

The expression of CCR2 in transduced cells was expressed as fold change as compared to the un-transduced sample.

3.2.2.2 Confirmation of CCR2 over expression by Flow cytometry

CCR2 expression levels of lentiviral transduced MSCs were analysed using the BD FACS Canto flow cytometer. Transduced, selected MSCs as well as un-transduced MSCs were grown to 70-80% confluency, and trypsinized using 0.25% Trypsin. The cells were pelleted down and washed twice in sterile PBS to remove trypsin and residual medium, filtered through a 40µm cell strainer to remove any clumps. The cells were counted and re-suspended at $5x10^6$cells/ml. The cells were F_C blocked with 20µl (10µg) (rat IgG2b anti-mouse CD16/CD32, BD Bioscience) FC block/10^6cells, incubated at 4^0C (OR RT) for 10mins. 100µl of cell suspension (10^6cells) was transferred to a flow cytometry tube and stained with 10ul of PE labelled CCR2 antibody (Monoclonal anti mouse CCR2 R&D Systems, Minneapolis, USA). A PE labelled isotype control antibody (Rat IgG2b Isotype control, R&D Systems Minneapolis, USA) was used as the negative control to identify the level of non-specific background signal from the CCR2 antibody. The cells were incubated at room temperature in the dark for 20mins to allow the antibody binding. The cells were washed with 1ml FACS buffer and re-suspended in 0.3ml FACS buffer. To enable live / dead staining of the cells 5ul of 1/100 dilution of DRAQ7 dye (Biostatus) was added to the cell suspension before they were analysed in FACS Canto.

The gating strategy for the identification of $CCR2^+$ cells are shown in the figure 3.1

Briefly, the gating strategy involved identification of live and dead cells using DRAQ7 dye that stains only dead cells under the APC-CY7 channel 633/780/60 Fig3.1(a), followed by the identification of the single cells using the FSC-H/FSC-A scatter plot (b), plotting a scatter plot for the single cells using SSC-A/FSC-A plot (c), Identifying and selecting the CCR2 positive cells falling under the PE channel 488/585/42-A (d). The CCR2 positive population are represented in a histogram (e), before a final gating to show the live CCR2 positive cells (f). The CCR2 positive cell groups were compared with that of the isotype control and the percentage of CCR2 positive cells in the population were identified.

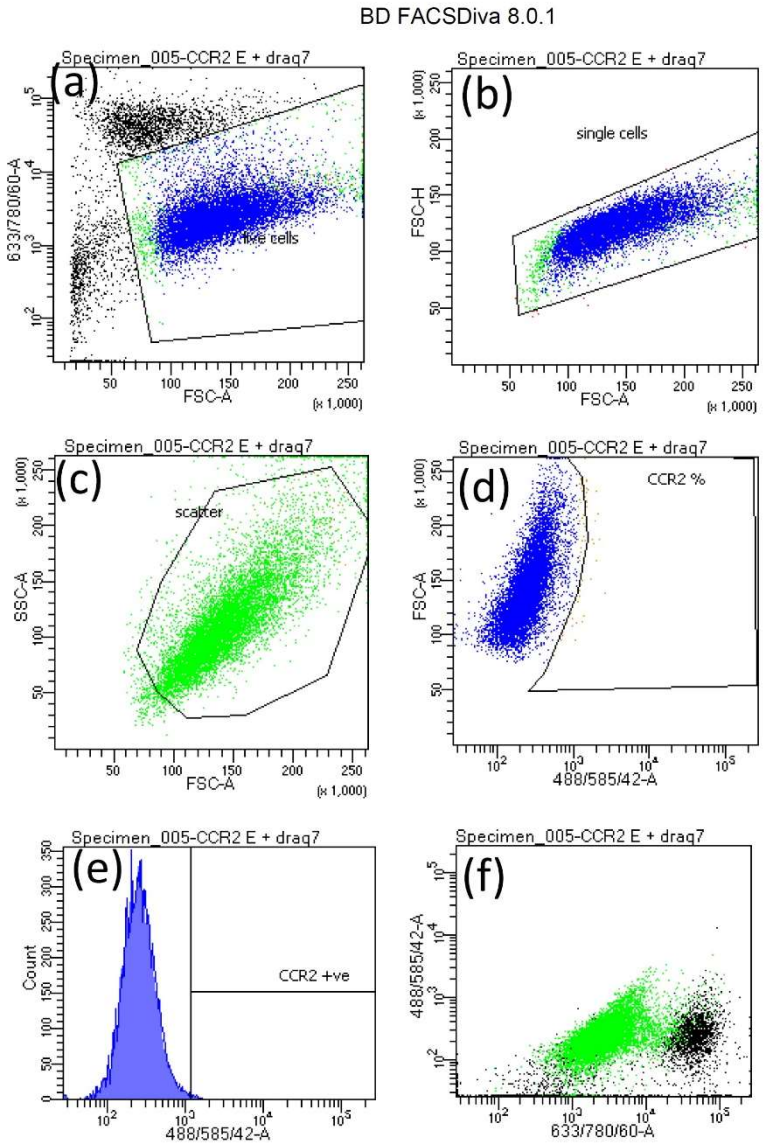

Figure 3:1 Representative gating strategy for the detection of CCR2 expressing MSCs

3.2.2.3 Confirmation of CCR2 over expression by ICC

Immunocytochemistry for CCR2 was performed **to** confirm the over expression of CCR2 receptor on the cell surface. Staining for CCR2 was performed in cells grown on Poly-L-Lysine coated glass slides. The cells grown on glass slides were fixed and permeabilized by acetone before the staining process.

CCR2 over expressing MSCs, un-transduced MSCs and RAW macrophages ($1X10^5$ - $2X10^5$ cells) were seeded on to glass slides and incubated overnight in a CO_2 incubator at 37^0C, 5% CO_2. The following day, media was aspirated off and the slides were washed gently in PBS three times. The cells were fixed by immersing the slides in ice cold acetone at-20^0C for 20 min. The slides were washed thrice in PBS. Unspecific staining was eliminated by incubating the slides in ICC blocking buffer (Affymetrix eBioscience) for 1hr at room temperature. The blocking buffer was aspirated off and primary antibody anti CCR2 antibody (Abcam, UK) diluted in blocking buffer was added to the slides. The slides were transferred to a humidified chamber and incubated at 4^0C overnight. The slides were washed thrice next day with PBS after decanting the primary antibody. Alexa Fluor conjugated secondary antibody (Donkey Anti-Goat IgG H&L Alexa Flour 488, Abcam) was added to the slides at the recommended dilution and the slides were incubated for 2 hours in dark at room temperature. After incubation**,** the secondary antibody is washed off by rinsing the slides with PBS thrice. The slides were air dried and mounted under a glass cover slip with Fluoromount-G with DAPI (Affymetrix eBioscience) mountant. The slides were photographed under a fluorescent microscope (Olympus IX71 Inverted Fluorescent microscope with EXFO Xcite120 fluorescence illumination system) and CCR2 staining was recorded.

3.3 Results

3.3.1 Genetic Modification of Mesenchymal Stem Cells

3.3.1.1 Lentiviral transduction of MSCs

Lentiviral transduction of MSCs to obtain CCR2 over expressing MSCs and control vector (DsRed) was performed without the addition of any chemical agents to increase the transduction efficiency. The cells were found to express the transgene as early as 24 hours after transduction was carried out. This was evident from the DsRed positive cells in the control group.

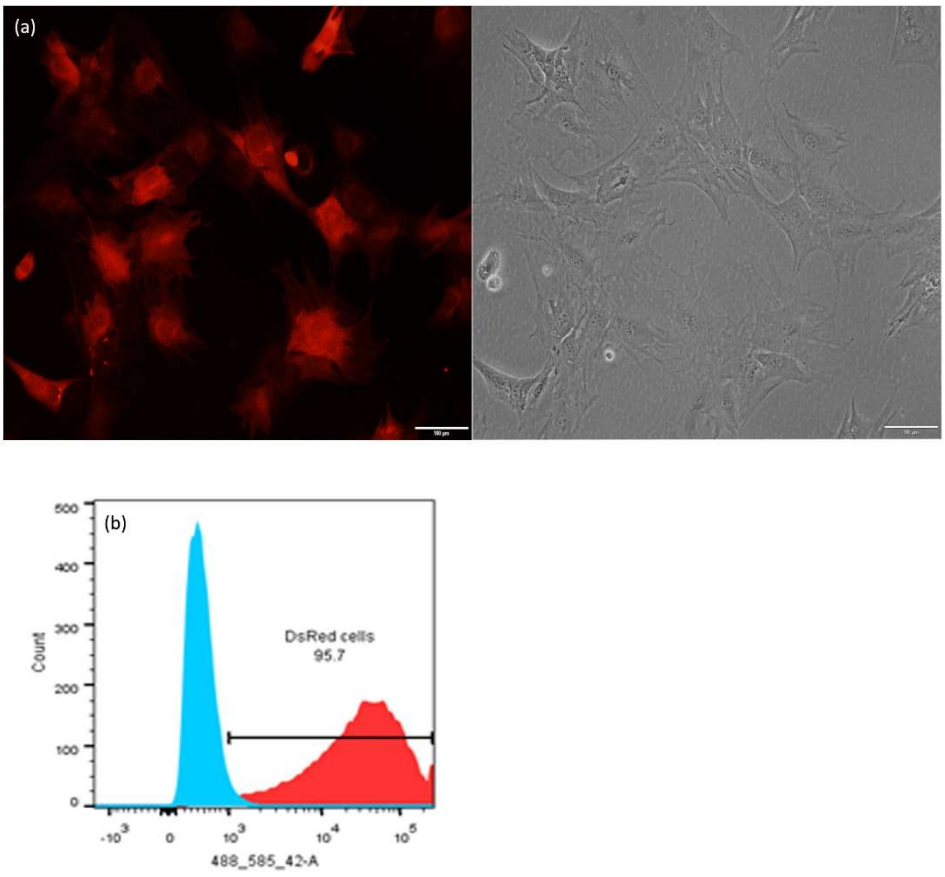

Figure 3:2 Fluorescent and bright field images of mMSCs transduced with pLEX-DsRed lentiviral vector post selection (a), Histogram following flow cytometric analysis showing the percentage of DsRed positive cells (red) in comparison with isotype control (blue) (b)

The number of DsRed positive cells significantly increased at 48 hours when the virus media was replaced by complete growth medium. Addition of viral vector demonstrated no toxicity to cells as visualised in routine microscopic examination. The absence of lentiviral mediates toxicity, the availability of the selectable marker (puromycin resistance gene) for transduced cells and the stable expression of transgene for longer passages meant there was no requirement to perform extensive optimisation analyses for effective transduction. The viability of the cells was calculated by staining the cells with DRAQ7 dye and analysing them via flow-cytometry. There was no change in viability of the cells following lentiviral transduction (fig3.3d). Expression of DsRed was confirmed by fluorescence microscopy (fig 3.2a) and flow-cytometry (fig3.2b), when the over-expression of CCR2 was confirmed by Q-PCR, flow-cytometry, and by immunocytochemistry for CCR2.

3.3.1.2 Selection of CCR-2 over expressing MSCs and DsRed expressing MSCs

Transduced and un-transduced MSCs were cultured in selection media containing puromycin. Transduced cells were puromycin resistant as the lentivirus transfers the puromycin resistance gene to these cells. The cells were observed routinely under a light microscope (Nikon Eclipse TS100) to assess the morphology. The selection process was stopped when the entire un-transduced cells were killed (7-10days of selection). The surviving MSCs were transgenic MSCs and they were passaged, cryopreserved and/or used for further studies.

3.3.2 Confirmation of CCR2 over expression by MSCs

3.3.2.1 Confirmation of CCR2 over expression by Q-PCR

Comparative Q-PCR analysis for CCR2 transcriptome in MSCs transduced with lentiviral vector carrying CCR2 and un-modified MSCs showed significantly increased expression of CCR2 in MSCs transduced with lentiviral vector carrying the CCR2 gene. The over expression of CCR2 was stable and continuous over several passages. The control group with DsRed transduced MSCs and un-transduced MSCs did not show

any increase in CCR2 expression. There was an average increase in CCR2 expression by 36-fold in the CCR2 modified group as compared to the un-transduced control (fig 3.4b). This proved that the lentiviral vector was effective in increasing the expression levels of CCR2 and is expressed over several passages as one would expect from a lentiviral vector. The results were expressed as standard error of the mean of the gene expression from 12 transduction experiments where the CCR2 expression is given as relative to the un-transduced control. The data shows MSCs transduced with lentiviral vector for CCR2 in the pLEX lentiviral vector backbone under EF-1α promoter showed significant increase in the expression of the transgene. Multiple comparison between the groups using Tukey's multiple comparison test shows significant differences ($P < 0.0001$) between the CCR2 and DsRed cell groups, and between CCR2 and un-transduced MSCs, but no significant difference was observed in terms of CCR2 expression between the DsRed group of cells and the un-transduced control.

3.3.2.2 Confirmation of CCR2 over expression by Flow cytometry

Flow Cytometric analysis of MSCs showed more than 90% of the MSCs transduced with lentiviral vector carrying CCR2 express CCR2 on their surface. In contrast, less than 5% of un-transduced MSCs expressed CCR2 on their surface (Fig 3.3) from 7 different set of experiments.

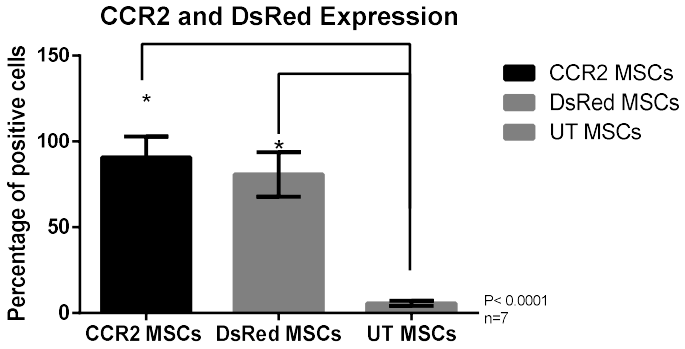

Figure 3:3 Percentage of cells expressing CCR2 and DsRed in MSCs transduced with lentiviral vector carrying CCR2 and DsRed compared to un-transduced MSCs

The data is represented as histogram showing CCR2+ cells (red) within the population. Histogram shows positive staining for CCR2 antibody (red line) and isotype control (blue line) (fig3.4c). The percentage of DsRed positive MSCs were also found to be around 80 percentage of the total population (fig 3.3). These were significantly higher than the native CCR2 expression seen on unmodified MSCs.

3.3.2.3 Confirmation of CCR2 over expression by ICC

Immunocytochemical staining for CCR2 on MSCs showed increased expression of CCR2 on MSCs treated with lentiviral vector for CCR2, in comparison to un-transduced MSCs (fig 3.4a).

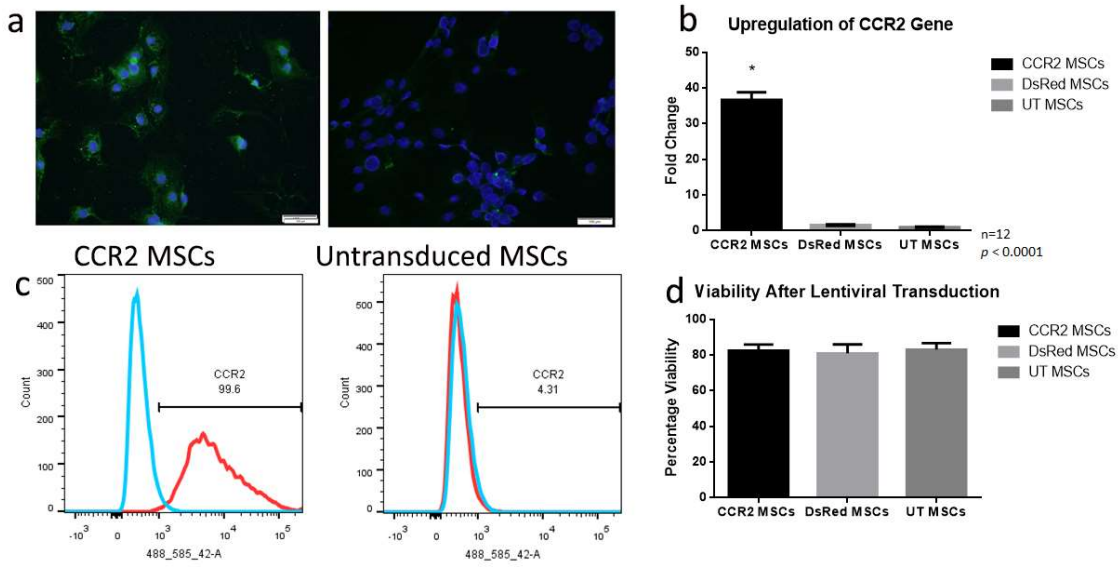

Figure 3:4 Confirmation of CCR2 overexpression in lentiviral transduced MSCs.

(A): Immunocytochemistry of CCR2 over expressing MSCs (left) and un-transduced MSCs (right). (B): QRT-PCR showing the relative fold change in upregulation of CCR2 transcript in MSCs transduced with lentiviral vector for CCR2 in comparison to un-transduced MSC control and MSCs transduced with lentiviral vector for DsRed. (C): Representative Flow cytometry analysis for CCR2 surface marker expression on MSCs showing increased number of cells expressing CCR2 in MSCs transduced with lentiviral vector for CCR2 in comparison with un-transduced MSCs. (d): Viability of the cells were not affected by lentiviral transduction as shown from the percentage of viable cells analysed by flow-cytometry.

CCR2 receptors were stained green (Alexa Fluor 488) and the nucleus was stained blue (DAPI). CCR2 was seen localised to the entire cytoplasm and cell surface in both cells. CCR2 over expressing MSCs showed a marked increase in the CCR2 staining.

3.4 Discussion

Mesenchymal stem cells are self-renewing, multipotent, adult stem cells that can be isolated from bone marrow, cord blood, adipose tissue and can be grown attached to tissue culture plastic. Bone marrow derived MSCs are shown be immune-modulatory, chemotactic, angiogenic, and differentiate into multiple lineages. They play vital role in tissue response and repair and secrete various paracrine factors which are proven to play important roles in tissue homeostasis, regeneration and repair[3,28,29].

Therapeutic potential of MSCs are widely known and they are proven to be excellent candidates for use in therapy of various diseases including cardiovascular diseases. As discussed earlier in this chapter, the limitations of MSCs in terms of their survival and persistence in disease tissue to provide extended or enhanced therapeutic efficacy is an emerging concern amongst researchers. Various methods to alter the functions of MSCs including, co culture, priming and genetic modification are explored in various settings[9,15,20,30–33]. In this Study our aim was to develop lentiviral modified mesenchymal stem cells that over express chemokine receptor, CCR2 that can be used for targeted therapy of critical limb ischemia.

Lentiviral vectors are proven to be efficient gene transfer vectors due to their advantages over other viral vectors such as ability to integrate into dividing and non-dividing cells, stable and high level long term gene expression, low toxicity and the ability to integrate the transgene into the host genome. In this chapter, we have used lentiviral vectors that were designed and validated in our lab to genetically modify MSCs to over express chemokine receptor CCR2. We have designed and validated a second-generation lentiviral system carrying the transgene gene under an EF-1α

promoter and carrying a selectable marker for puromycin resistance, enabling the efficient selection of transgenic cells. Lentiviral vectors we have used to modify bone marrow derived mMSCs did not show any toxicity to the cells and the viability of the cells post transduction was found to be similar to that of un-transduced control (fig3.4d). The lentiviral vectors we used in the current study were pLEX CCR2 and pLEX DsRed. The transduced MSCs were selected by culturing them in media containing puromycin resulting in a homogeneous population of MSCs expressing the transgene.

Q-PCR analysis of the CCR2 transcriptome showed the lentiviral modified MSCs overexpressing CCR2 (CCR2 MSCs) had 40-fold increase in CCR2 expression in comparison with the un-transduced MSCs control (fig3.4b). This was consistent over different passages as reported widely with other lentiviral vectors. The stronger EF-1α promoter enables significant increase in transgene expression as reported before[34].

Flow-Cytometry analysis for the expression of CCR2 receptor showed 90% of the cells in the CCR2 transduced, selected group were positive for CCR2. However, the DsRed expression levels were found to be around 80% of the total population (fig3.3). There were no noticeable changes in the morphology of the cells at any time during the entire transduction and selection process. Viability of the cells were no different than that of the un-transduced control MSCs as evident from the flow-cytometry analysis using the live/dead stain Draq7. There were no significant changes in the percentage of CCR2 or DsRed positive cells after multiple passages.

Immunocytochemical staining of the CCR2 receptor showed increased localisation of CCR2 in MSCs transduced with CCR2 lentiviral vector, compared to un-transduced control.

In summary, the pLEX CCR2 and pLEX DsRed lentiviral vectors were efficient in stable long term transgene expression without any toxicity or loss of viability to the cells.

The over expression of the transgene was confirmed at the transcriptome level and protein level by quantitative RT-PCR and Flow-Cytometry/ Immunocytochemistry respectively.

These findings fall are consistent with previously reported advantages of lentiviral vectors as efficient gene transfer vectors with minimal or no toxicity to target cells, while providing stable long term transgene expression.

3.5 References

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Chapter 4

Characteristic and Functional Assessment of CCR2 Overexpressing Mesenchymal Stem Cells

CHAPTER 4

4 Characterisation and Functional Assessment of CCR2 Overexpressing MSC

4.1 Introduction

Mesenchymal stem cells are fibroblast like, plastic adherent, adult stem cells that can differentiate into multiple lineages[1,2]. They are self-renewing and immunomodulatory[3,4]. MSCs are also known to have chemotactic properties which enable their homing to sites of inflammation and injury where they aid in regeneration and repair, by secreting autocrine and paracrine factors[5–7]. These secreted factors have a role in recruiting other immune cells and in the repair of damaged tissue[8,9]. The migration potential of MSCs is primarily due to the presence of various growth factor receptors and chemokine receptors[2,10]. They also express adhesion factors on their surface[11–13]. MSCs express various adhesion molecules and chemokine receptors which aid in their migration potential to various sites of inflammation and injury[10,11,13–16]. Although the mechanisms by which these receptors and ligands mobilise MSCs are not yet completely understood, the potential to utilize these receptor ligand interactions to improve homing and migration potential of MSCs for use in therapeutic applications are widely being investigated in various disease models[17–22]. Genetic modification, pre-conditioning, and alterations of culture conditions are the most commonly used modes for increasing the homing potential of MSCs. Conditioning of MSCs can be achieved by pre-treating with various cytokines[23–27]. Changes in culture conditions are achieved by either culturing MSCs in hypoxic conditions[28,29] or co-culturing with immune cells[30] .

MSCs express a wide range of chemokine receptors on their surface, which provides them the potential to migrate towards sites of infection and injury. Chemokine receptors are G-protein coupled receptors. The chemokine super-family is sub-divided into four sub families of chemokines namely; CXC, CC, C, and CX3C chemokines. The receptors for these chemokines are named as CXCR, CCR, CR and CX3CR. Chemokines bind to several chemokine receptors and they have multiple possible ligands[31]. MSCs are known to express a variety of chemokine receptors and secrete multiple chemokines[2,11,32,33]. Figure 4.1 illustrates the chemokines secreted by MSCs, the chemokine receptors expressed by MSCs, and chemokines that can bind

to or activate MSCs. The expression levels of these chemokines and receptors vary in nature; hence chemokine expression is generally heterogeneous on MSCs.

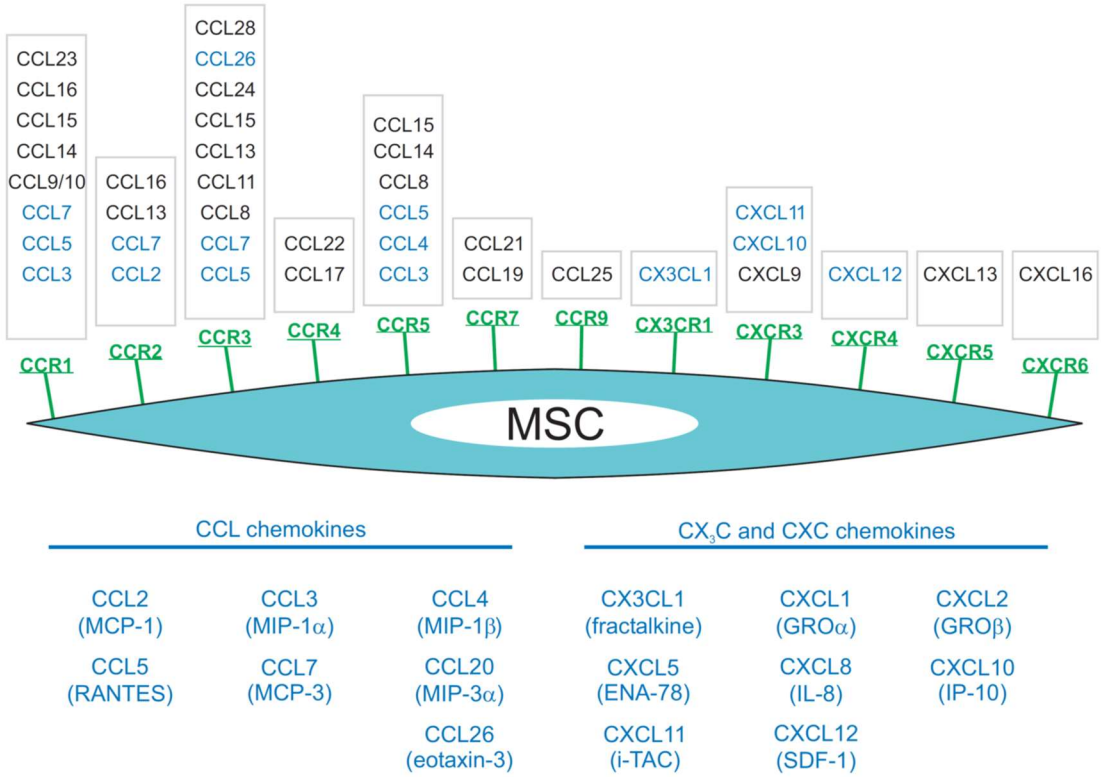

Figure 4:1 Schematic representation of expression of chemokine receptors (green), chemokines secreted by MCSs (blue) and chemokines that stimulate MSCs

The mechanisms by which MSCs migrate towards infection and injury have been extensively studied. The homing potential of MSCs can be attributed to the expression of a range of chemokine receptors and adhesion factors along with the interaction of MSCs with endothelial cells and growth factors. Various receptors expressed on MSCs like CCR1, CCR2[13,32,34], CCR4, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6 have all been shown to play a role in migration[14,35,36].

Adhesion molecules like integrins and growth factors like PDGF-AB, IGF-1, EGF also play a role in the migration of MSCs[13]. MSCs that are pre-treated with cytokines such as IL-6 and HGF have shown increased expression levels of CXCR4 and thereby improved migration[13,37].

CCR2 is generally expressed on immune cells such as monocytes, macrophages, basophils, eosinophils and subsets of T lymphocytes[38–40]. CCR2 plays an important role in immune cell recruitment to sites of infection or injury. MCP1 / CCR2 ligand receptor interaction is well studied in various disease models including cancer[41–45]. The CCR2 positive immune cells are also proven to be protective against various infections[46,47]. Although immune cells do not always express CCR2 on their surface, the native expression of CCR2 on T cells isolated from mouse peripheral tissue were found to be only 8%[48].

Migration of CCR2 expressing MSCs towards MCP-1 is mediated by an intracellular adaptor molecule FROUNT. When MCP-1 binds to CCR2, it activates a cascade consisting of phosphatidylinositol 3-OH kinase (PI3K). FROUNT binds with activated CCR2 and forms clusters at the cell front during chemotaxis resulting in polarisation of MSCs which leads to rearrangement of the cytoskeleton[34,49].

In the present study, we use a lentiviral vector to genetically modify C57BL/6 mouse MSCs to overexpress CCR2. This chapter aims at characterisation and functional assessment of CCR2 overexpressing MSCs in comparison with unmodified and control transduced MSCs.

To assess the effect of genetic modification on the stem cell properties of MSCs, their differentiation potential as well as the expression of MSC specific surface markers were analysed. CCR2 over expressing MSCs, unmodified MSCs and control transduced; DsRed expressing MSCs were analysed for their ability to differentiate into adipocyte and osteoblast lineages. Functional properties of CCR2 overexpressing MSCs were analysed by their ability to migrate towards a gradient of MCP1 in an *in vitro* setting.

MSCs generally express a variety of surface markers, which are used for their identification, isolation and selection. However, the surface markers can vary depending on the source of cells and there is species to species variation as well. Due

to this heterogeneous expression of surface markers, International Society of Cellular Therapy (ISCT), has published guidelines for selection and isolation of MSCs. ISCT guidelines defines a cell as an MSC when they, are plastic adherent, with ≥95% of the cells within the population expressing CD73, CD90, and CD105; only ≤2% of the population expressing CD34, CD45, CD11b, or CD 14, CD79 or CD19 and HLA-II. In addition to these positive and negative markers, the population should also possess the capacity to differentiate into adipocytes, osteocytes and chondrocytes[12,50]. In some cases, negative selection of mesenchymal stem cells is preferred to eliminate the risks of contamination or binding of antibodies in the final cell preparation. Binding of antibodies to certain receptors could activate certain unwanted pathways that can compromise the purity of the cell prep with potential changes in cellular function and properties[51,52]. Combination of positive and negative selection is the most commonly used characterisation method which enables the selection of high purity MSCs that have increased clonogenicity. The MSCs used in this study are bone marrow derived mouse MSCs from C57/BL6 strain of mice. Bone marrow derived C57BL/6 mouse MSCs are generally positive for CD90, CD105, CD 140a, CD140b, Sca-1 and negative for CD34, CD45 and CD11b[53]. Although there are no exclusive markers to identify mouse MSCs, a combination of these positive and negative markers is commonly utilized in their characterisation. Markers like SCA-1 and CD90 are strain dependent and sometimes mouse MSCs are negative for CD90 or CD105[54].

4.2 Materials and Methods

4.2.1 Characterisation of MSC Surface Markers

The objective of this experiment was to analyse the expression of MSC specific surface markers on CCR2 overexpressing MSCs, unmodified MSCs and control transduced MSCs. Passage numbers and batches were kept the same between all three groups used here. That is, the same batch of MSCs which were used to generate CCR2 overexpressing MSCs and DsRed expressing MSCs were used. The passage numbers were kept constant by expanding the unmodified cells to the same passage as that of CCR2 MSCs and DsRed MSCs. MSCs were analysed for their expression of positive markers; CD105, CD90, SCA-1 and negative markers; CD34, CD45, CD11b.

The antibodies and the isotype controls used were as described previously (section 3.2.1.2)

The procedure for the surface marker characterisation is as follows.

CCR2 overexpressing mMSCs and unmodified mMSCs were cultured in Iscove's medium (Sigma Aldrich) supplemented with 10%FBS (Invitrogen R6), 1% Penicillin streptomycin solution (Gibco, Grand Island, New York, USA) and 1% L-Glutamine (Gibco, Grand Island, New York, USA). Cells were grown in T175 flasks (Sarstedt) and allowed to reach 70-80% confluency before trypsinizing. Once the cells were ready to be analysed, the media was carefully aspirated and the flasks were washed with 5ml of PBS to remove any debris or floating cells. This was followed by trypsinisation using 4ml of trypsin-EDTA (Gibco, Grand Island, New York, USA) for 4-5min. The trypsin was then neutralized with 8ml of complete mMSC medium and the cells were transferred to a 15ml centrifuge tubes. The cells were spun at 400g for 5min at room temperature and washed twice with FACS buffer and passed through a 40µm cell strainer to remove any clumps. A cell count was performed and 100,000 cells were seeded in a 96 well V bottom plate (Nunc) in triplicates for each marker and corresponding isotype control. Triplicates were allotted for un-stained control for gating. The cells were stained with each antibody at a concentration of 50ul/well in FACS buffer and incubated at 4°C for 30 min. The cells were spun twice at 400g for 5min with repeated resuspension in FACS buffer to remove unbound/excess stain. After the second spin, the pellets were re-suspended in 200µl FACS buffer and transferred to follow cytometry tubes and kept in ice until analysis. The flow cytometric analysis was

carried out using a Becton Dickinson(BD) FACS CANTO multicolour flowcytometry machine (San Jose, California, USA). The gating parameters were set using an unstained control cell group as follows; forward scatter (FSC) = 20, side scatter (SSC) = 390 and; APC-cy7 (633/780/60-A red filter for detection of viability) =560, PE (488/585/42-A) = 365, APC (633/660/20-A) =522. After completing the gating strategy, 5µl of 1/500 dilution of a viability dye; DRAQ7 (Biostatus, UK) was added to each sample immediately before the samples were loaded on to the flow cytometer for analysis. Gating strategy involved gating out the MSC population, followed by live/dead cell isolation, isolation of single cells, and final gate is applied to identify the PE stained or APC stained cells. Data analysis was performed using FlowJo V.10 flowcytometry data analysis software (FlowJo LLC, Oregon, USA).

Representative Gating strategy for MSC surface marker analysis

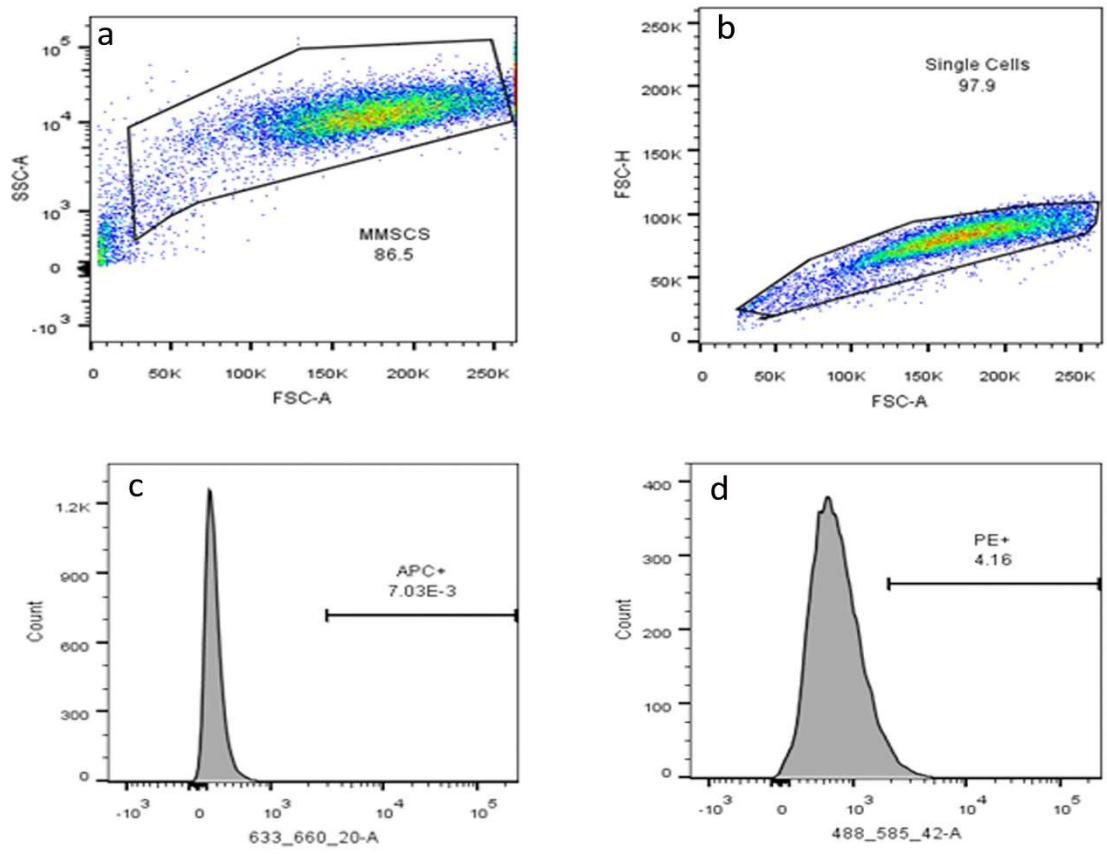

Figure 4:2 Representative gating strategy for MSC surface marker analysis

MSCs are selected based on their FSC-A/SSC-A profile (a). Single cells are identified by FSC-A/FSC-H profile (b), Gates were set for APC positive cells (c) and PE positive cells (d).

4.2.1.1 *In vitro* Differentiation potential of genetically modified MSCs

Mesenchymal cells can differentiate into adipocytes, osteoblasts and chondrocytes under suitable culture conditions. There are also reports that they can also differentiate into myocytes, astrocytes, neural progenitor cells, although this is not a requirement for the characterization of these cells. Although human MSCs are expected to differentiate into adipocytes, osteoblasts and chondrocytes, clonal variations and variations in MSC cultures could lead to the inability to differentiate into all three lineages[1]. Pittenger *et al* reported that, cell density, spatial organisation, clonality, and presence of cytokines or growth factors can also limit differentiation capacity of hMSCs. In this chapter, we have tested the differentiation capacity of genetically modified and unmodified bone marrow derived MSCs from C57BL/6 strain of mouse. Although MSCs are known to differentiate into adipocytes, osteoblasts, and chondrocytes under suitable culture conditions, there are variabilities reported in their differentiation capacity based on the cell source, culture conditions, age, and the species from which the cells are isolated. Murine MSCs are particularly variable in their surface receptor expression and differentiation potential when compared to human MSCs[55,56].

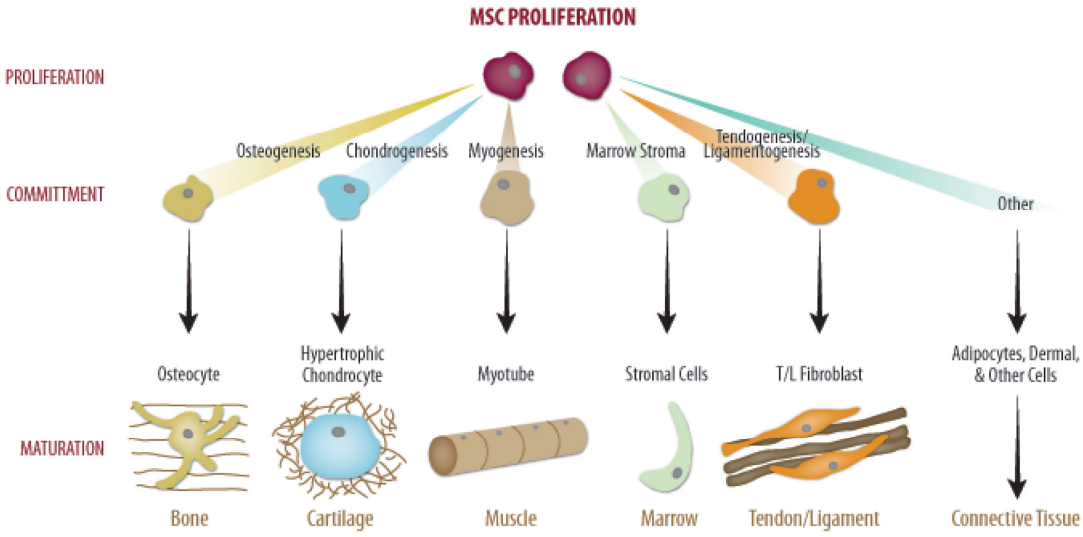

Figure 4:3 Schematic representation of MSC proliferation, differentiation, lineage commitment and maturation into different cell types.

4.2.1.2 Adipogenic differentiation of MSCs

For adipogenic differentiation of MSCs, cells are cultured in 24 well plates, allowed to grow overnight before treating them with adipogenic media. Adipogenic differentiation of MSCs requires treatment of cells with dexamethasone, insulin, indomethacin and 3-isobutyl-1-Methyl-Xanthine (IBMX) in FBS containing media and is detected by the presence of lipids vacuoles detected by oil-red O staining.

Induction media and maintenance media for adipogenic differentiation were prepared (Appendix).

The differentiation assay was performed for CCR2 overexpressing MSCs, unmodified MSCs and DsRed expressing control transduced MSCs.

The cells were trypsinized, counted and seeded at $4X10^4$ cells per well (2ml) into a 24 well plate. Three wells were used as control and three were used for adipogenic differentiation. The cells were grown until confluent to reach confluency and the media was replaced every 3-4 days. Once the cells were confluent, 2ml per well of adipogenic induction medium was added to the test wells. The control wells received normal growth medium. After 3 days, the medium in the test wells were replaced by 2ml maintenance medium and left for one day. Media change was done carefully without disrupting the confluent cell layer or any fat that had begun to deposit over the cells as vacuoles. The media change cycles were repeated until 3 cycles of induction and maintenance are completed. The cells were left in the final maintenance medium cycle for 5-7 days. The cells were fixed in formalin for Oil Red 0 staining.

Adipogenesis was identified by lipid filled vesicles that are stained bright red by Oil Red 0 staining

The staining protocol is as described below.

Oil Red 0 stock solution was prepared by mixing 0.3gm of Oil Red 0 stain in 100ml of 99% isopropanol. Working solution of Oil Red 0 stain was prepared by mixing Oil Red

0 stock solution and water (6:4). The working solution is stable for only 2 hours. The solution was allowed to stand for 10 min and filtered through a Whatman no.1 filter paper. The media was removed carefully and the wells were washed twice with PBS before fixing in formalin, the excess formalin is discarded in formalin waste container. The wells were rinsed with distilled water. Working solution of Oil Red 0 stain was pipetted on to the wells to cover the cell layer and allowed to stand for 5 min before discarding the stain. Excess stain was washed off with 2ml per well 60% isopropanol. The plates were rinsed with tap water until water ran off smoothly. 1/5 diluted (in water) haematoxylin was added to the wells and incubated for 1 min. the plates were washed in warm water and covered with water until photographed.

4.2.1.3 Osteogenic differentiation of MSCs

For MSCs to differentiate into osteocytes, incubation of confluent monolayer of cells with ascorbic acid, dexamethasone, β glycerophosphate in medium containing FBS is required. This will lead to the accumulation of intra cellular calcium and increase in the production of alkaline phosphatase. The media composition is outlined in the Appendix.

Osteogenic differentiation assay was performed for CCR2 overexpressing mMSCs, unmodified mMSCs and DsRed expressing control transduced mMSCs.

The cells were trypsinized, counted and seeded at $4X10^4$ cells per well (2ml) into a 24 well plate. 3 wells were used as control cells and 3 were used for osteogenic differentiation. The cells were grown until confluent to reach confluency and the media was replaced every 3-4 days. Once the monolayer was confluent, medium in the test wells of each cell type were replaced with 2ml of osteogenic medium. Media in the control cells were replaced by complete MSC medium. Media change is repeated every 3 days; the media was changed carefully without disturbing the monolayer. Cells were harvested between 10^{th} and 12^{th} day or before the monolayer

peels. Control and test wells were harvested for alizarin red staining to detect calcium.

The staining was performed as follows.

Alizarin red solution was prepared by dissolving 2 gm of Alizarin red in 100ml of distilled water. The pH of the solution was adjusted to 4.1 to 4.3 with 1% ammonium hydroxide.

The cells were washed twice with 1ml PBS; fixed in 95% ice cold methanol (1ml) for 10min, and washed with 1ml distilled water. The cells were stained with 0.5ml of 2%Alizarin solution for 5min; washed twice with 0.5ml distilled water, left to dry until photographed under the microscope. Few drops of distilled water were added to the wells before imaging under the microscope (Olympus CKX41).

4.2.2 Assessment of migration potential of CCR2 overexpressing MSCs

To assess the migration potential of MSCs, a trans-well migration assay is performed in a chemotaxis chamber with upper and lower wells separated by a porous membrane. The tissue culture insert with a permeable membrane at the bottom serves as the upper chamber. This insert is immersed in the lower chamber that contains serum free media with or without chemoattractant depending on the experimental design. The cells are grown on the membrane inside the trans-well insert and the insert is immersed into the tissue culture plate which contains the media along with chemoattractant at the desired concentration (figure 4.4).

The trans-well insert with the cells and chemoattractant is incubated in the CO_2 incubator, the cells will begin to migrate towards the chemokine containing media, the pore size of the trans-well membrane will not allow the cells to pass through them, so the migrating cells are trapped in the porous membrane. The trapped cells can be stained, visualised and counted under a microscope to quantitatively assess cell migration.

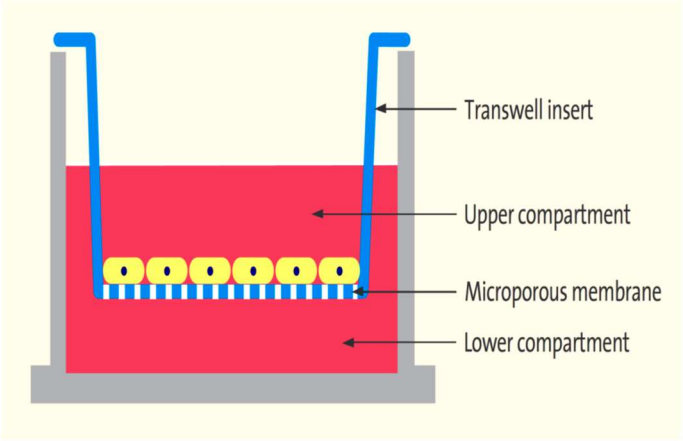

Figure 4:4 Schematic representation of a chemotaxis chamber.

Migration potential of CCR2 over expressing mMSCs, unmodified mMSCs and control transduced, DsRed expressing mMSCs were tested *in vitro* by assessing the migration of cells towards a gradient of recombinant mouse MCP-1 in a chemotaxis migration assay.

Chemotaxis assays was carried out using 24 well plate Falcon® permeable trans-well support with 8.0µm transparent PET membrane (Corning Biosciences). Transduced and un-transduced cells were grown in T175 flasks and were serum starved by culturing in serum free medium for 24 hours prior to the migration assay. The cells were routinely monitored to ensure they do not grow more than 80% confluent during serum starvation. The trans-well insert membrane was activated by adding serum free media (Iscove's media) to both the upper and lower chambers of the insert and incubating the inserts in a CO2 incubator overnight. 50,000 cells were seeded on to the upper chamber of the trans-well insert in a maximum volume of 200µl. Recombinant MCP-1 was added to serum free media in the lower chamber of the insert in a maximum volume of 750µl. The concentration of MCP-1 used in the lower chamber ranged from 5ng/ml to 100 ng/ml. Serum free media without any MCP-1 chemoattractant served as the negative control while complete media (media with 10%FBS) served as the positive control. Another control well with same concentration of MCP1 in the upper chamber and lower chamber was also used to negate the effect of random motility (chemokinesis) induced my MCP1. All conditions were tested in triplicates. The trans-well inserts with cells and chemo-attractants

were incubated for 24 hours in a CO_2 incubator. After incubation, the inserts were carefully removed, media was aspirated off and the membrane inside the trans-well inserts were carefully washed with PBS to remove any unbound cells. The edges of the insert were gently swabbed with a cotton bud to remove excess cells adhering to the surface of the insert. Care was taken not to damage the membrane or touch the outside of the membrane facing the chemoattractant containing media. The membrane is immersed in 4% PFA for 15 min at room temperature to fix the cells bound to the membrane pores. The insert was washed twice in PBS. Crystal violet stain (25% in water) was added to the inserts and incubated for 15 min at room temperature. Inserts were washed with water 5-6 times to remove the excess stain. Inserts were air dried and the membrane was cut out carefully. The membrane was cut out mounted on to a glass slide before visualising and photographing them for cell counting under the microscope. Total number of cells per membrane were counted from five fields as represented in figure 4.5a.

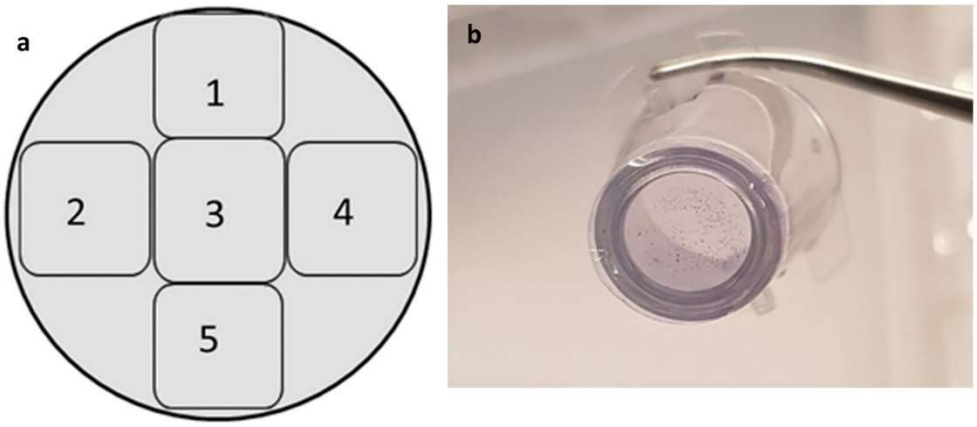

Figure 4:5 Schematic representation of five fields of the trans-well insert membrane imaged (a), A trans-well insert with stained cells visible on the membrane (b)

4.2.3 Statistical Analysis

Statistical analysis was performed using GraphPad Prism V6.0.1 statistical software (Graphpad software, Inc. California, USA). Results were represented as mean± standard deviation (SD). For the analysis of multivariable data, one way or two-way analysis of variance (ANOVA) with suitable multiple comparison tests were carried

out which are specified in each graph. Data represented was considered statistically significant if the p value was lower than 0.05.

4.3 Results

4.3.1 Analysis of MSC surface Markers

Surface marker analysis of MSCs was carried out to analyse if the lentiviral modification of MSCs altered the surface marker expression. Genetically modified mMSCs overexpressing CCR2 and unmodified mMSCs were analysed for their characteristic surface markers. Unmodified MSCs were tested before they were used for genetic modification during characterisation experiments (Chapter3), and they were proven to be positively expressing CD90, CD105 and Sca-1 while negative for the expression of CD34, CD45 and CD11b.

Post, genetic modification, flow cytometric analysis confirmed these MSCs retained the same surface marker expression profile as during pre-transduction (figure 4.6: 1 and 2). CCR2 overexpressing mMSCs and unmodified MSCs were negative for the leukocyte common antigen CD45 which regulates cell growth and differentiation. They were also negative for the expression of haematopoietic stem cell marker CD34, and CD11b. which shows there were no haematopoietic cells within the population. CCR2 overexpressing mMSCs and unmodified mMSCs showed high level (>90%) expression of CD90 (Thy-1) which is involved in signal transduction, T cell activation co-stimulation, adhesion, differentiation and cell migration. Both cell types were positively expressing CD105 (Endoglin) which plays a role in cell adhesion, migration, and vascular remodelling. CD105 is expressed by stromal cells and other marrow derived cells, so the percentage of CD105 expression cannot be considered as a factor determining purity of MSCs. Both CCR2 overexpressing mMSCs and unmodified MSCs expressed high levels of SCA-1 (Stem Cell Antigen-1). SCA-1 is involved in signal transduction and is expressed by a mix of stem cell progenitor cells. SCA-1 is highly expressed on bone marrow derived MSCs from C57BL/6 strain of mice and is not expressed on human MSCs. Thus, SCA-1 can be considered as a unique marker for characterisation, isolation and identification of bone marrow derived mMSCs[57].

In summary, both genetically modified and unmodified mMSCs retained the expression of positive and negative surface markers over passaging and genetic modification. This provides evidence that neither lentiviral transduction nor overexpression of CCR2 has any effect on the expression of these surface markers on mouse MSCs.

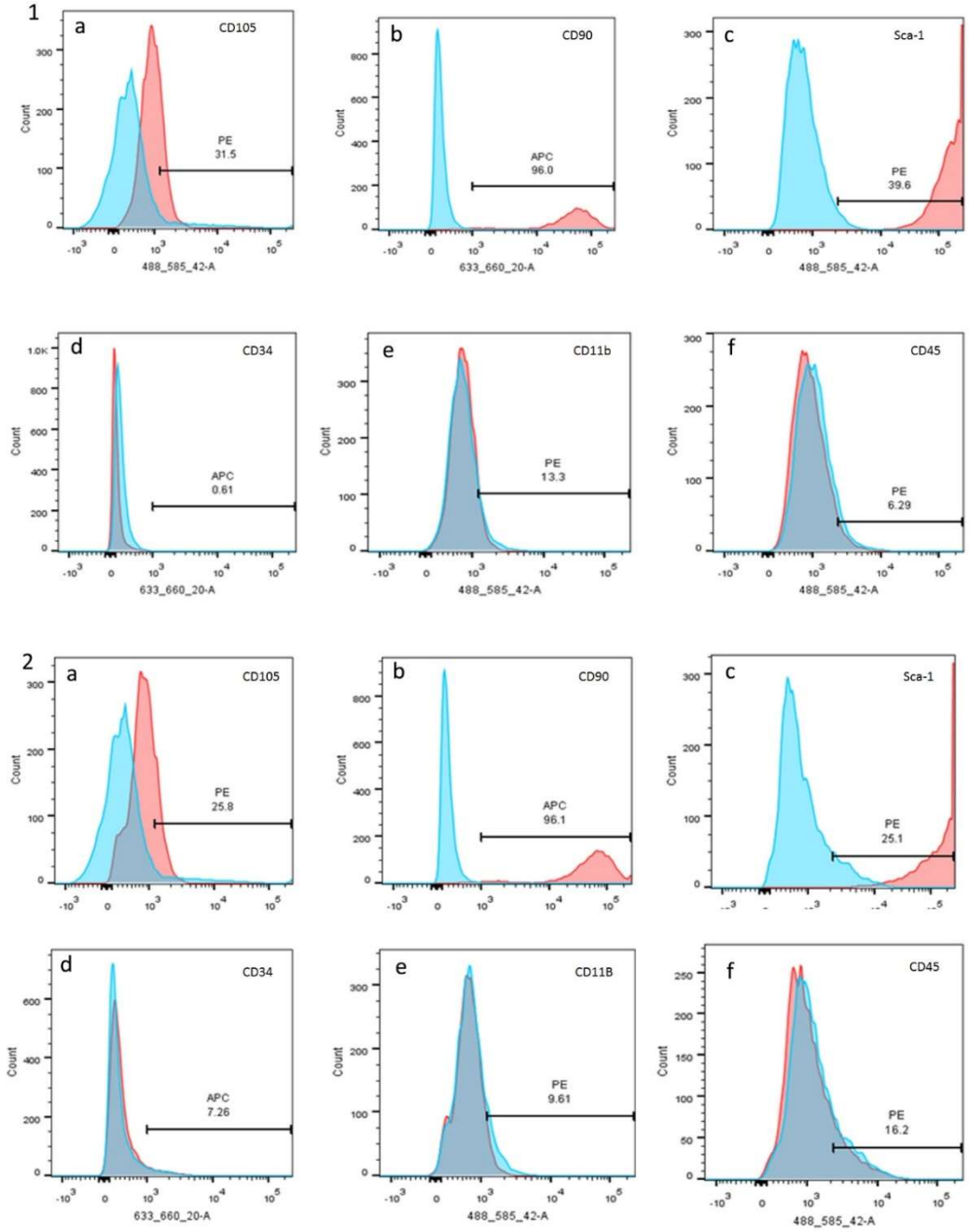

Figure 4:6 Analysis of MSC surface markers *showing, expression of CD105, CD90 and Sca-1 on CCR2 overexpressing MSCs (1) and unmodified MSCs (2). Both CCR2 overexpressing MSCs and unmodified MSCs were negative for the expression of CD34, CD11b and CD45. Red histogram corresponds to specific antibodies mentioned, while blue histogram corresponds to isotype controls.*

4.3.2 *In vitro* Differentiation potential of genetically modified MSCs

Mesenchymal stem cells are known for their ability to differentiate into multiple lineages under suitable culture conditions and in the presence of specific growth factors. Differentiation potential of CCR2 overexpressing mMSCs, unmodified mMSCs and control transduced, DsRed expressing mMSCs to form adipocytes and osteoblasts were studied. All cells types could differentiate into adipocytes as evident from the presence of lipid vacuoles intracellularly, which were subsequently stained with oil red O stain (figure 4.7). The control wells were treated with normal MSCs growth media and they did not produce any lipid vacuoles. CCR2 overexpressing mMSCs, unmodified mMSCs and DsRed expressing mMSCs showed the presence of lipid vacuoles.

Osteogenic differentiation assay was performed in cultured confluent cells that have formed monolayer. CCR2 overexpressing mMSCs, unmodified mMSCs and DsRed expressing mMSCs were assayed for their ability to differentiate into osteoblasts. The experiment was carried out in triplicates. Control cell group received complete mMSC media while test wells received osteogenic induction media. The assay was carried out for 14 days and the cells were fixed and stained for analysis of differentiation. Differentiation of mMSCs into osteoblasts was confirmed by the presence of intracellular calcium deposition, and was visualised by alizarin red staining. CCR2 overexpressing mMSCs, unmodified mMSCs and DsRed expressing mMSCs differentiated into osteoblasts as evident from alizarin red staining (figure 4.8). The cells were imaged under bright field microscope at x40 magnification.

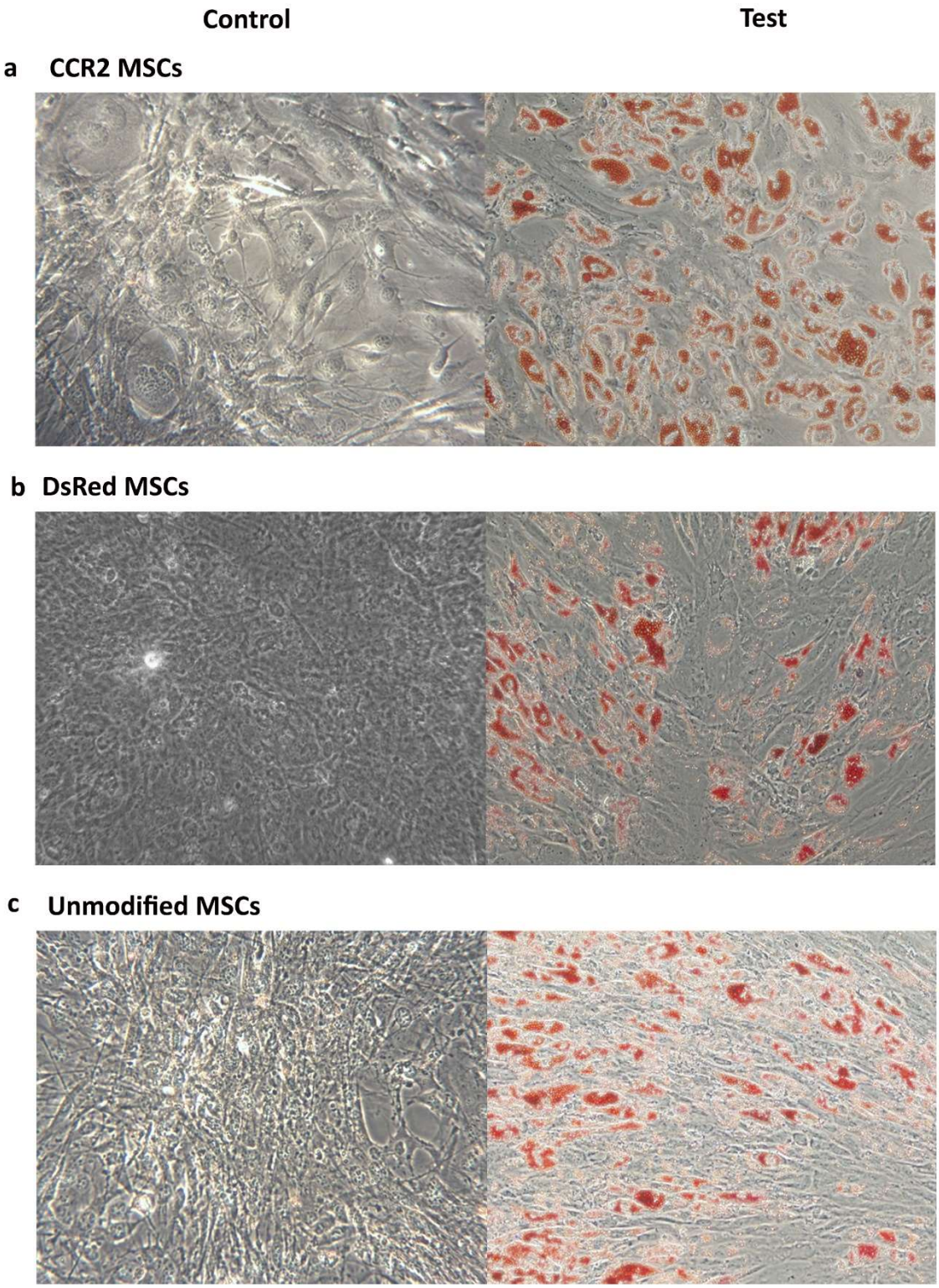

Figure 4:7 Adipogenic differentiation of C57BL/6 mMSCs

CCR2 overexpressing mMSCs, control transduced DsRed expressing mMSCs, and unmodified mMSCs were cultured in adipogenic medium for 21 days. Accumulated lipid vacuoles were stained and photographed under a bright field microscope x200 magnification.

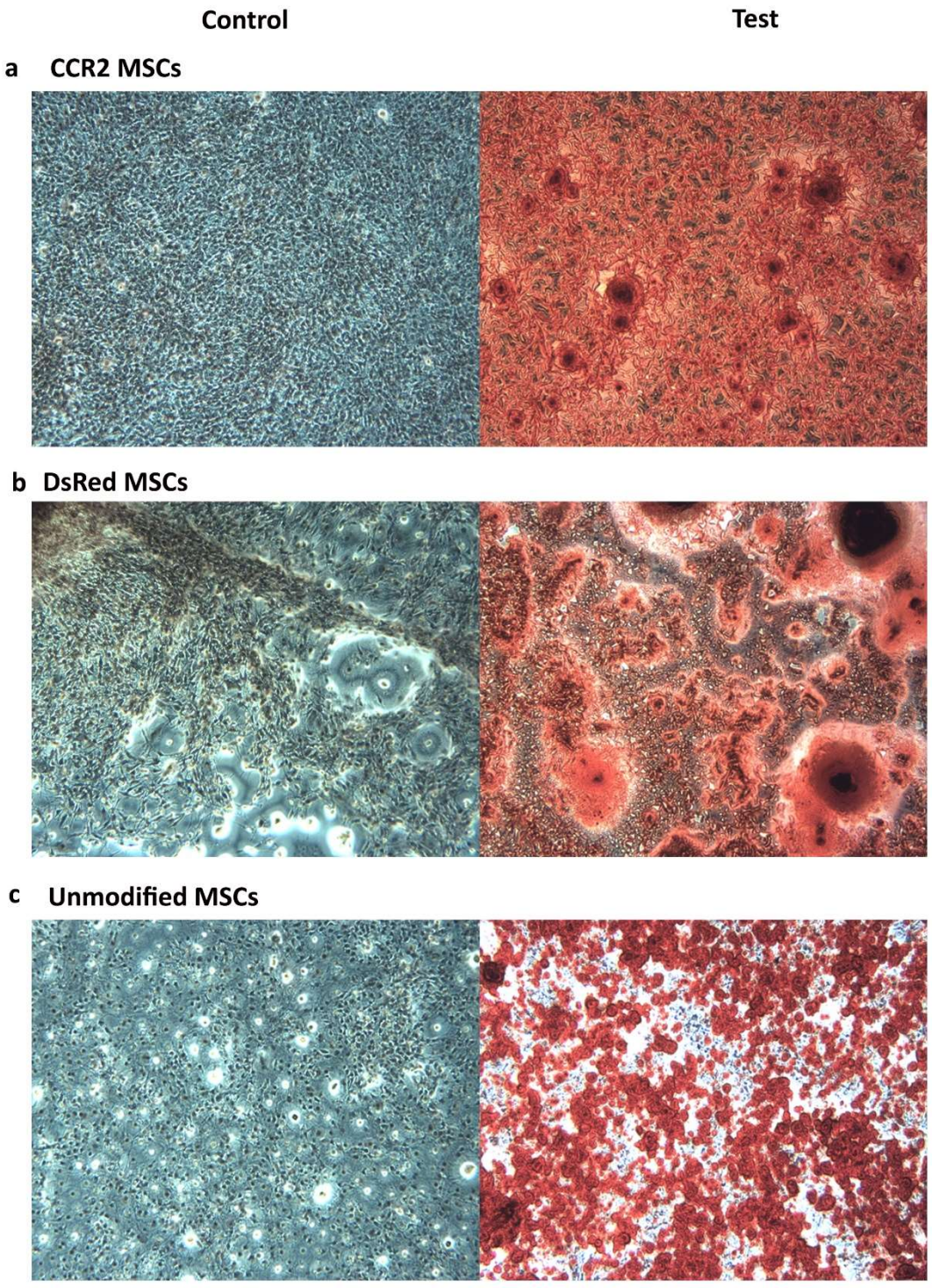

Figure 4:8 Osteogenic differentiation of C57BL/6 mMSCs

Osteogenic differentiation of CCR2 overexpressing C57BL/6 mMSCs (a), control transduced DsRed (b) expressing C57BL/6 mMSCs, and unmodified C57BL/6 mMSCs (c). Cells were cultured until confluent before treating them for two weeks with osteo-inductive media (test). The extracellular calcium deposition is stained with alizarin red and photographed at x40 magnification. Control groups received normal mMSC growth media and did not show any extracellular calcium deposition.

4.3.3 CCR2 Overexpressing MSCs Show Increased Migration Potential

To assess the migration potential of MSCs towards a gradient of MCP-1; CCR2 over expressing MSCs, un-modified MSCs and control transduced MSCs (expressing DsRed) were used in the study. Migrated cells that were trapped in the trans-well membrane was stained using crystal violet and photographed and counted under a microscope at X40 magnification (figure 4.9 A and B). The total number of cells per insert was counted from five fields per membrane as represented in figure 4.5. Data was represented as average number of cells counted per membrane ± standard deviation.

An experiment was set up with increasing concentrations of MCP-1 ranging from 5ng/ml to 100ng/ml to establish the optimum concentration of MCP-1 at which the cells migrated effectively. The highest number of cells was found to be migrating towards media containing serum (positive control), while the negative control, which was serum free media with no chemoattractant added showed little or no cell migration. There was no migration observed in the chemokinesis test wells. This observation proved the assay was not compromised by MCP priming of the cells. The cells that were trapped in the membrane were thus confirmed to be chemotactic and not chemokinetic. A concentration of 10ng/ml of MCP-1 showed significant increase in number of cells migrating in comparison with all the other concentrations of MCP-1 tested (figure 4.9 d). There was a significant increase in cell migration for CCR2 overexpressing MSCs at 10ng/ml when compared with unmodified and control transduced MSCs (Figure 4.9d). There was average of 45±19 CCR2 overexpressing MSCs per membrane (n=14) as compared to 15.5±9 unmodified MSCs (n=14) and 9.7±5.5 DsRed MSCs (n=7). Increasing the concentration of MCP-1 did not increase the total number of cells migrating. There was a statistically significant increase ($p< 0.0001$) in the cell migration between the CCR2 overexpressing MSCs and unmodified MSCs at 10ng/ml, 15ng/ml, 25ng/ml and 100ng/ml concentrations of MCP1; but the highest number of cells were recorded at a concentration of 10ng/ml MCP1. The migration potential of CCR2 MSCs was found to be the highest in comparison with un-modified cells and the control transduced cells. A concentration of 10ng/ml of

MCP-1 was found to be the most effective concentration of chemoattractant that caused the cells to migrate.

To compare the migration potential of CCR2 overexpressing MSCs with that of un-modified MSCs, and control transduced MSCs, trans-well migration assays were repeated subsequently against 10ng/ml of MCP-1. The average cell count from these experiments showed significant increase in migration of CCR2 overexpressing MSCs in comparison with unmodified MSCs and control MSCs (n=3) (fig 4.8e). There were 36.15±10.22 CCR2 overexpressing MSCs, 13.05±9.72 unmodified MSCs and 9.15±5.3 DsRed MSCs detected per membrane. These values were found to be significant between CCR2 overexpressing MSCs and Unmodified MSCs; and between CCR2 overexpressing MSCs and DsRed MSCs ($P<0.0001$); But not between unmodified MSCs and DsRed MSCs (figure 4.9e).

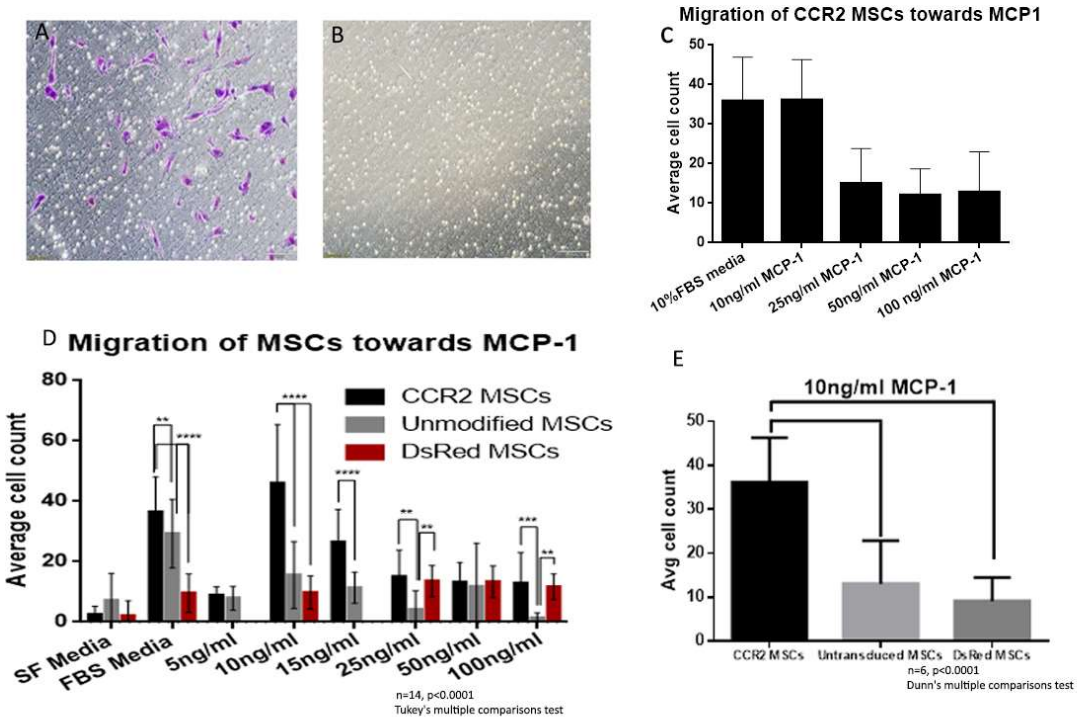

Figure 4:9 *In vitro* Migration potential of CCR2 overexpressing mMSCs

Representative images of migrating MSCs (a) trapped in trans-well membrane stained with crystal violet and control membrane which shows no cell migration (b). Increasing concentration of MCP1 did not increase the total number of migrating cells (c). Migration of CCR2 MSCs, unmodified MSCs and control transduced DsRed MSCs towards an increasing gradient of MCP1 (d). Migration of CCR2 MSCs, unmodified MSCs and control transduced DsRed MSCs towards 10ng/ml MCP1 (e).

4.4 Discussion

In this chapter, we investigate the effect of genetic modification of MSCs using lentiviral vectors on the characteristic surface markers of C57BL/6 mMSCs and on the differentiation potential of genetically modified mMSCs into adipocytes and osteoblasts. We also investigate the migration potential of CCR2 overexpressing mMSCs, unmodified mMSCs and control transduced, DsRed expressing MSCs towards a gradient of MCP-1.

Mesenchymal stem cells express a variety of surface receptors and adhesion factors. They also secrete several chemokines along with numerous other paracrine factors which have important roles in the function and characteristics of MSCs. The role of these receptors and secreted factors in deciding the fate and function of MSCs, both *In vivo* and Ex vivo are extensively studied.

Chemokines are small peptides which are activators and chemo attractants. They are known to play an important role in inflammation, infection, cell activation, chemotaxis, differentiation and cell survival[58]. MSCs secrete a broad variety of chemokines and express different chemokine receptors on their surface[59–61]. MSCs exhibit a high degree of heterogeneity expression of chemokine receptors, surface markers and secreted chemokines[13]. Source, species, age, culture conditions all define the secretory profile and surface marker expression levels on MSCs[1,56].

MSC Characterisation

In the characterisation experiments performed, surface marker profile of bone marrow derived C57BL/6 MCS used in this study were found to be positive for CD90, CD105, SCA-1 and negative for CD11b, CD34 and CD45. Peister *et al* reported variance in surface marker expression, differentiation potential and proliferation rates of MSCs isolated from marrows of different inbred strains of mice. The negative expression of CD90 and positive expression of CD34 in C57BL/6 mouse MSCs were also reported in the same study[56]. However, CD45, CD11b markers were negative in for those cell types, while SCA-1 was stained positive. Phinney *et al* reported positive

staining for CD11b and CD45 in MSC preps from Balb/c mice[55], while Sung *et al* showed similar expression profile for surface markers of C57BL/6 MSCs to that is obtained in this chapter[62]. Another comparative study of murine MSCs from different strains of mice over 50 passages by Meirelles *et al* showed the cells were differentiating into adipo and osteo lineages while positively expressing SCA-1 along with CD44M CD229, CD49e[53]. Although all the MSCs from Peister's study differentiate into adipo, osteo and chondro lineages, chondrocyte differentiation was found to be varied among different strains with the quality of chondrocyte differentiation poor in the C57 strain of mice. Chondrocyte differentiation was difficult to achieve in our study, and this is a general observation for C57BL/6 MSCs isolated within our lab in relation to chondrogenesis from mMSCs from this strain of mouse. This discrepancy observed from the same strain of mice could be attributed to the different modes of isolation of bone marrow, differences in the initial seeding densities, differences in culture media used, differences in the age and passage number of MSCs. The differences in the *in vitro* characteristics of MSCs observed could explain varying results obtained through use of mMSCs *in vivo* experiments using mouse MSCs. Ability of cells used in preclinical studies to differentiate into desired cell populations, secrete desired paracrine factors, interact with specific ligands could all play a decisive role in a successful outcome. Hence the use of the right cell type from the right species, strain and source is of utmost importance.

MSC Migration

The migration potential of MSCs can be attributed to the expression of chemokine receptors expressed on the cell surface along with the presence of chemokines or ligands that binds to the receptors. Although there are several methods to improve the homing mechanism of MSCs, including altering culture conditions or addition of priming factors leading to increased receptor expression on the cell surface. Genetic modification is another approach that is used to improve the migration potential of cells.

Mesenchymal stem cells are known to express various chemokine receptors. Our experiments have shown there is very low level of native CCR2 expression on normal

MSCs (5%). Baseline expression levels of CCR2 is found to be similar in humans and mice. In mice CCR2 is homogeneously expressed on monocytes, 2-15% T cells, 3-10% CD4 T cells express CCR2 and 110-40% of CD8 T cells[48]. However, CCR2 is critical for migration of immune cells such as macrophages. Monocytes and macrophages play an important role in angiogenesis and arteriogenesis[63–65]. Macrophages express CCR2 along with other chemokine receptors that functions in recruitment of these cells to site of infection or injury. MCP-1 is expressed in ischemic injury. The infiltration of macrophages to the site of ischemic injury is detrimental for vascular remodelling, angiogenesis and arteriogenesis[31]. The importance of MCP-1 in promoting angiogenesis is demonstrated in a rabbit model of hind limb ischemia. When MCP-1 was infused into the proximal end of ligated femoral arteries, high collateral density was observed after 7 days[66]. Maximum endothelial cell and smooth muscle cell proliferation was observed 3 days after femoral artery ligation which corresponds to monocyte adhesion and migration through collateral arteries[67].In the present study, CCR2 overexpressing mMSCs generated through lentiviral transduction were tested for their enhanced migration potential towards a gradient of MCP1. CCR2 binds with MCP-1 and isoforms of MCP-1. This interaction has been extensively examined in our research group. The mechanism by which bone marrow derived MSCs migrate towards MCP-1 was identified by previous researchers in our group. They concluded that the migration of bone marrow derived MSCs towards MCP-1 is through the binding of MCP-1 to CCR2 expressed on the surface of MSCs. MCP-1 induced significant migration of BMMSCs and re-localised F-actin. Chemotaxis of MSCs were initiated when MCP-1 binds to CCR2 leading to the release of Gα and Gβγ subunits from the Gαβγ complex on G-protein coupled receptors. They also demonstrated that MSC migration towards MCP-1 exhibit dose dependent inhibition, where increase in the concentration of MCP-1 is shown to inhibit migration[68]. CCR2/MCP1 ligand receptor interaction is known to be involved in inducing cell migration, where CCR2 mediated chemotaxis reassembles the cytoskeleton with the activation of a cluster forming protein called FROUNT[34,49].

The concept of overexpressing chemokine receptors to improve the migration potential of MSCs has been explored and published widely. The most widely explored chemokine receptor combination is that of CXCR4 and SDF-1. Culture expanded MSCs

are known to decrease the level of CXCR4 making them less migratory towards SDF-1[11,69]. Using genetic modification to overexpress CXCR4, Cheng *et al* delivered CXCR4 overexpressing MSCs intravenously to rats 24 hours after coronary occlusion and reperfusion and found high number of CXCR4 overexpressing MSCs homing to the site of injury than unmodified MSCs. These genetically modified CXCR4MSCs enabled higher left ventricular function post myocardial infraction compared to control group or saline group[17]. In another experiment, MSCs overexpressing CCR1 were injected intramyocardially, accumulated in the myocardium at significantly higher levels and promoted reduction of infarct size , increased capillary density, prevented cardiac remodelling and restored cardiac function in 4 weeks[18]. CXCR4 overexpressing MSCs were also used in treatment of acute lung injury[70] where CXCR4 overexpressing cells are transplanted to the bone marrow of rat. CXCR4 MSCs improved lung function, mobilised MSCs from the marrow and prevented further lung damage. SDF-1 /CXCR4 receptor ligand interactions have become widely used in many disease models to mobilise cells thereby improving cardiac function and lung function. It is also known to improve cell engraftment and promote angiogenesis in ischemic muscle[22,71].

Genetic modification of MSCs to achieve enhanced therapeutic angiogenesis is explored in different diseases, including critical limb ischemia. Genetic modification involving viral vectors presents its own hurdles in terms of regulatory requirements when translating into clinic. Sendai Virus mediated genetic modification of MSCs to overexpress angiopoietin-1 on MSCs showed improved blood flow recovery and increased capillary density compared to normal MSCs. Sendai viral modified MSCs overexpressing angiopoietin-1 also showed enhanced expression of p-Akt conferring increased survival of trans planted MSCs[72]

In conclusion, we have generated genetically modified mMSCs with stable overexpression of CCR2 on the surface. A control lentiviral transduced group of mMSCs expressing DsRed were also generated, we have characterised these genetically modified mMSCs for their characteristic surface markers by flow cytometry and concluded that the genetic modification had no effect in the surface marker expression of these cells. CCR2 overexpressing mMSCs, DsRed expressing control transduced mMSCs and unmodified MSCs were positive for CD90, CD105 and

SCA-1, while they all showed negative expression of CD34, CD45 and CD11b. Genetically modified mMSCs were assayed for their ability to differentiate into adipocytes and osteoblasts. Both genetically modified MSC groups (CCR2 overexpressing mMSCs and DsRed expressing mMSCs) as well as unmodified mMSCs could differentiate into adipocytes and osteoblasts. The migration potential of genetically modified MSCs were assayed using a chemotaxis assay and found that the CCR2 overexpressing mMSCs showed significantly enhanced migration towards MCP-1 in a dose dependent manner. Increasing concentration of chemoattractant had a negative effect on the migration of these cells as proven by previous research from our lab[68]. With stable genetic modification achieving CCR2 overexpression and enhanced migration potential, the *In vivo* efficacy of these modified MSCs will be tested in a mice model of hind limb ischemia.

Potential applications of chemokines and chemokine receptors in mobilising and improving homing and retention of transplanted stem cells are endless. CXCR4/SDF-1 is the perfect example of such a ligand receptor combination. The role of CCR2 and MCP-1 interaction in inducing chemotaxis has only been investigated in *in vitro* experiments. As pathways and factors related to cell migration and chemotaxis of MSCs are explored in detail, the future of next generation therapies looks promising. For new therapies to progress from the preclinical setting to the clinic, research needs to be done to elucidate the mechanism of action of these pathways, receptors, their interaction with associated immune cells and niches in the bone marrow and other organs.

4.5 References

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Chapter 5

Therapeutic Efficacy of Genetically Modified mMSCs Overexpressing CCR2 in a Mouse Model of Hind Limb Ischemia

CHAPTER 5

5 Therapeutic Efficacy of Genetically Modified mMSCs Overexpressing CCR2 in a Mouse Model of Critical Limb Ischemia.

5.1 Introduction

MSCs are known for their pro angiogenic properties[1–3]. The pro-angiogenesis potential of MSCs is attributed to their ability to secrete angiogenic proteins. Bone marrow derived MSCs from BALB/c and C57BL/6 mice are shown to express high levels of angiogenic proteins such as VEG-F, FGF, angiopoietin and secrete MCP-1 after hypoxic pre-conditioning and priming with TNF- α and IL-1β in experiments conducted in our research group (Clara Sanz, unpublished data).Conditioned media from MSCs were shown to contain VEG-F, FGF, Monocyte Chemoattractant Protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, chemokine induced by IFN-γ (MIG), Stromal cell derived factor (SDF-1), Platelet Induced Growth Factor-2 (PIGF-2), Matrix Metallopeptidase-9 (MMP-9) and IGFBP-9. [4–7]

Therapeutic applications of MSCs are widely reviewed in multiple disease models and in several clinical trials[5,6,8–11]. Applications of MSCs in promoting therapeutic angiogenesis are of interest in this study. Due to the therapeutic potential of MSCs, several clinical trials including multiple trials for critical limb ischemia are being conducted across the globe using allogenic or autologous MSCs[8,12,13].

MSCs are also known for their homing capabilities[14–16], whereby they can mobilise from the bone marrow and migrate to tissues and organs to aid in regeneration and repair. Homing properties of MSCs are due to the expression of various chemokines and chemokine receptors, grown factors and growth factor receptors[17–20]. Chemokines and chemokine receptors that aid in the migration of MSCs are potential targets for therapeutic applications. Manipulating secreting of chemokines or expression of chemokine receptors could alter the homing capacity of MSCs.

The efficacy of MSCs in mouse models of hind limb ischemia to promote therapeutic angiogenesis is well documented[21] . However, limitations of MSC therapy in promoting long term efficient regeneration of ischemic limbs is not without complications and challenges. One of the main challenges in the use of MSCs in mice models is the huge variability in mice models available to test the efficacy of cells[22–25]. The cells themselves can vary on their properties to induce therapeutic angiogenesis depending on their, site of delivery, dosage, age, species and tissue source from which they are isolated, and culture conditions. Variability in cells and variability in animal models are not the only challenges in the use of MSCs in treatment of critical limb ischemia. Another major limitation in stem cell therapy is the survival and retention of transplanted cells at the target tissue[26]. To achieve therapeutic angiogenesis, the transplanted cells should survive the hostile ischemic conditions and secrete paracrine factors and remodel themselves to promote therapeutic regeneration. Modifications that enhance the cells migrate, retain, survive and engraft into the injured tissue are advancing research in regenerative medicine and in the development of advanced therapeutic medicinal products (ATMPs).

Several strategies to improve MSC viability, retention and angiogenic potential are widely reported such as genetic modification[27–31]. One widely studied receptor is the CXCR4 chemokine receptor which binds to CXCL12 (SDF-1)[32], a chemokine which is upregulated in ischemic tissue. Genetic modification of stem cells to overexpress CXCR4 are used in various pre-clinical and clinical trials for diseases like myocardial infraction[33,34] and critical limb ischemia[35]. These CXCR4 overexpressing cells have shown improved migration potential to promote efficient recruitment and retention of transplanted cells[19,34–38].

This study focuses on genetically modifying MSCs to overexpress a potent chemokine receptor on their surface. The chemokine receptor overexpressed on MSCs in this study is, CC Chemokine Receptor type-2 (CCR2), which is known to bind with high affinity to Monocyte Chemoattractant Protein-1 (MCP-1), a chemokine secreted in

high levels by ischemic and injured tissue. CCR2 is normally expressed on immune cells like monocytes that enable their migration towards injured tissue, but only low level of CCR2 expression is seen on MSCs. Lentiviral vectors carrying CCR2 was used to genetically modify MSCs, and the genetically modified MSCs were tested for the overexpression of CCR2 at the transcript and protein level. These genetically modified MSCs were found to have the same surface markers as unmodified cells and they differentiated into adipocytes and osteoblasts as the unmodified cells did. The CCR2 overexpressing MSCs were shown to migrate in significantly higher numbers to MCP-1 *in vitro*. This chapter aims to look at the *in vivo* production of MCP-1 in ischemic tissue followed by investigation of the therapeutic efficacy of these CCR2 overexpressing MSCs in providing therapeutic efficacy in a mice model of hind limb ischemia. This chapter also investigates the retention and bio-distribution of CCR2 overexpressing mMSCs injected intra-muscularly to a mouse model of hind limb ischemia.

CCR2 is the receptor for MCP-1 and is an activator of monocytes and is involved in the mobilisation of immune cells to sites of infection or injury. The role of chemokine receptors like CCR2 in enhancing migration of immune cells are well documented.[39–41] CCR2 binds with high affinity to MCP-1 and cells that express CCR2 migrate towards MCP-1 *in vitro* and *in vivo*[39,42–45] . MCP-1 is found to be upregulated in sites of infection and injury[46–48]. . Mice devoid of CCR2 gene were unable to recruit macrophages in an experimental peritoneal inflammation model and were unable to clear bacterial intracellular bacteria used for creating the mice model of inflammation[49]. CCR2 expression is detrimental in combating infections and it is known to have a role in tissue remodelling by recruitment of macrophages, which promotes inflammation mediated remodelling of tissues and blood vessels. CCR2 mediated vascular inflammation and remodelling was observed in a study that induced hypertension in mice by infusion of angiotensin. Wild type and CCR2 knockout mice were induced hypertension and hypertension induced vascular inflammation and remodelling was absent in CCR2 knockout mice, proving the role of CCR2 MCP-1 interactions in inflammation and vascular remodelling[39].

MSCs express small level of CCR2 along with other potential chemokine receptors that play a role in their migration and homing potential[44]. Bone marrow derived MSCs express variety of chemokine receptors including CCR2. However, the expression levels of CCR2 on unstimulated MSCs are very low compared to other chemokine receptors[19]. Although MSCs can be primed by exposing them to various cytokines like TNF-α, their effect on the expression of CCR2 is not well established.

MCP-1 is upregulated in injured and ischemic tissue and is important in the infiltration of immune cells to the site of injury[46]. Hind limb ischemia model in MCP-1 deficient mouse is proven to have abnormal monocyte recruitment and collateral formation and strong reduction in blood flow following femoral artery excision[50]. Another hind limb ischemia study demonstrating the importance of MCP-1 and recruitment of CCR2+ macrophages concluded, there was reduced blood flow recovery, reduced collateral formation after femoral artery ligation. There was loss of chemotactic migration potential in macrophages isolated from MCP-1 deficient mice.

The mechanism by which bone marrow derived MSCs migrate towards MCP-1 was identified by previous researchers in our group. They concluded that the migration of bone marrow derived MSCs towards MCP-1 is through the binding of MCP-1 to CCR2 expressed on the surface of MSCs. MCP-1 induced significant migration of BMMSCs and re-localised F-actin. Chemotaxis of MSCs were initiated when MCP-1 binds to CCR2 leading to the release of Gα and Gβγ subunits from the Gαβγ complex on G-protein coupled receptors. They also demonstrated that MSC migration towards MCP-1 exhibit dose dependent inhibition, where increase in the concentration of MCP-1 is shown to inhibit migration[51].

Migration of CCR2 expressing MSCs towards MCP-1 is mediated by an intracellular adaptor molecule FROUNT. When MCP-1 binds to CCR2, it activates a cascade through activation of phosphatidylinositol 3-OH kinase (PI3K). FROUNT binds with

activated CCR2 and forms clusters at the cell front during chemotaxis resulting in polarisation of MSCs which leads to rearrangement of the cytoskeleton[42,52].

In this study, we are investigating the therapeutic potential of genetically modified mouse MSCs over expressing CCR2 in a mouse model of hind limb ischemia.

5.2 Materials and Methods

5.2.1 Analysis of MCP-1 Secretion in Ischemic Tissue

The current study involves transplantation of murine MSCs overexpressing CCR2 to a mouse model of hind limb ischemia.

To analyse the secretion of MCP-1 in ischemic tissue, Ischemic hind limb muscle tissue was collected from male C57BL/6 mice that had undergone surgical induction of hind limb ischemia. The entire hind limb was harvested after euthanasia by CO_2 asphyxiation. Thigh and calf muscles were separated from bone. The muscle tissue was separated into muscle groups and immersed in 5ml serum free DMEM and incubated at 37^0C in a CO_2 incubator for 48 hours. The MCP-1 in the medium was quantified using ELISA (Raybiotech) for mouse MCP-1 as per manufacturer's instructions. The right hind limb of the mice was not ischemic and it served as a control. The secreted levels of MCP-1 were compared between different muscle groups and between ischemic and non- ischemic legs / muscle groups.

5.2.2 Mouse Model for Hind Limb Ischemia

Male C57BL/6 mice, weighing 20-25gm and 8-10-week-old were obtained from Charles River laboratories UK Ltd. All the animal experiments were approved by the Animal Care Research Ethics Committee at National University of Ireland, Galway. All experiments and procedures were performed as per the protocols and guidelines approved by the Institute Ethics committee and the Health Products Regulatory Authority (HPRA) in Ireland.

The animals were housed in a clean sterile environment with bedding, environmental enrichments, water *ad libitum.* Mice were anaesthetised by intra-peritoneal administration of ketamine (80mg/kg) and xylazine (10mg/kg) and anaesthesia was maintained with isoflurane gas with oxygen. (1-3% Isoflurane in 100% oxygen at a flow rate of 1L/min) for all surgical procedures. Surgical induction of ischemia was performed in the left hind legs of the animal while the right leg served as the internal control with no ischemia. The induction of ischemia was performed as follows.

The double ligation model[22,53] for hind limb ischemia was performed to ligate the femoral artery in two locations in the left hind limb of the mice. A midline incision from the lower abdomen to the medial knee was made to expose the femoral artery and its branches. The proximal and distal ends of the femoral artery and the proximal profonda femoris artery in the groin were dissected and ligated. The second incision was made above the knee to expose the superficial femoral artery and vein. The artery and vein were then separated and the artery was ligated above the saphenous–popliteal bifurcation (figure 5.1). The contralateral hind limb served as an internal control within each mouse.

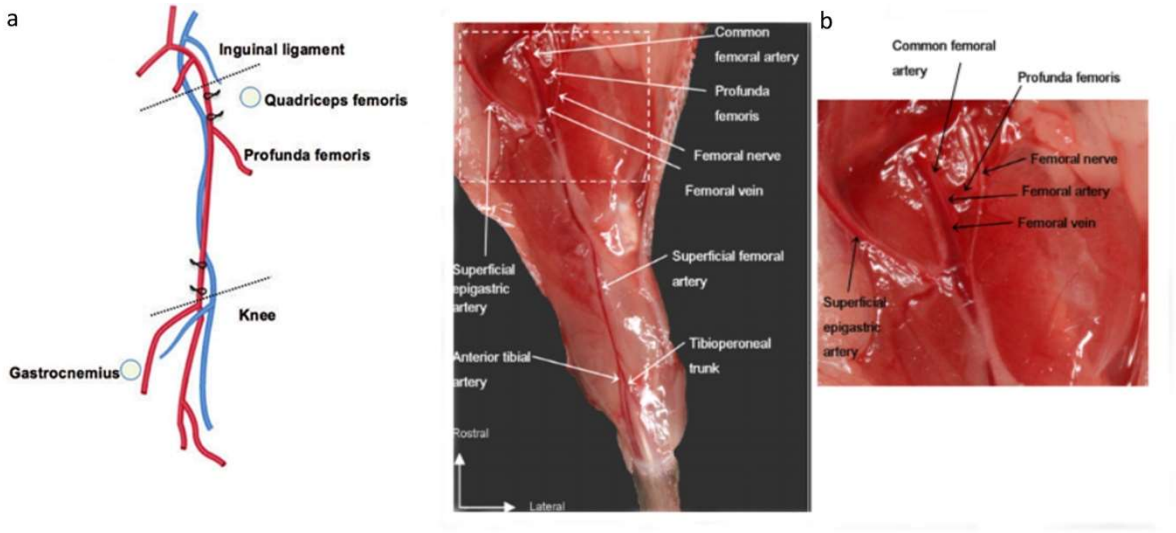

Figure 5:1 Schematic representation of the double ligation model of hind limb ischemia

(a). Vasculature of a typical mouse hind limb showing the femoral artery, profonda femoris, femoral artery and associated vascular structures (b).

Laser Doppler blood flow analysis was carried before and after surgery to confirm successful surgical induction of hind limb ischemia. The overlying skin was closed using a 6-0 Vicryl suture. A total of 48 animals underwent surgical induction of hind limb ischemia. All animals were monitored for signs of distress daily for the first week after surgery and distress assessment as carried out using a post-operative monitoring system.

5.2.3 Laser Doppler Blood Flow Analysis

Laser Doppler imaging to assess the blood flow in the hind limbs of mice were carried out using Moore LDI Laser Doppler imager (Moor Instruments, Devon, UK), before the induction of hind limb ischemia, immediately after induction of hind limb ischemia, on day 3, 7 and 14 respectively (figure 5.3 c). The measurement taken before the induction of ischemia was considered as the baseline blood flow.

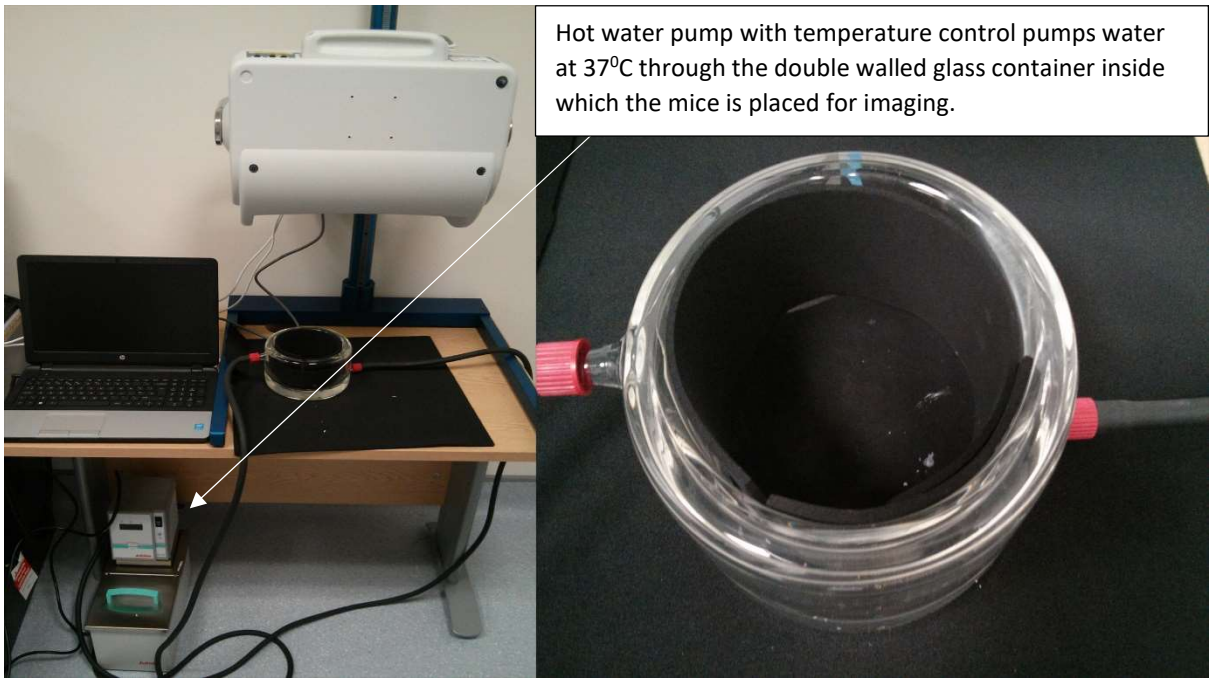

Figure 5:2 Jacketed glass jar and laser Doppler setup

Warm water circulated through the walls of the glass jar inside which the mice were placed before imaging them. The distance between the inner side of the glass jar and the laser Doppler was kept constant for every measurement for the entire duration of the study.

Blood flow measurement was measured immediately after surgery to confirm the successful induction of hind limb ischemia. Even small changes in the body temperature of the animals can have an effect in the blood flow measurement[54]. To

eliminate this variability, the anaesthetised mice were imaged after placing inside a bespoke jacketed glass jar which circulates 37^0C water to maintain constant body temperature (figure 5.2). Laser Doppler imaging was done swiftly, minimising the time that the mice were on the heated surface. Perfusion changes in the lower limbs were recorded and blood flow is expressed as the ratio between ischemic and non-ischemic leg.

5.2.4 MSC Preparation and Intra Muscular Injection

Genetically modified mMSCs overexpressing CCR2 or DsRed expressing control transduced mMSCs were used for the study (chapter 3). Mice were given intra-muscular injections of $1x10^6$ cells on day 3 after induction of ischemia. MSCs were cultured in Iscove's Modified Dulbecco's Modified Eagle's Medium (Iscove's-DMEM) supplemented with 10% (v/v) FBS (HyClone™ R6, HyClone Logan, Utah, USA), 1% penicillin streptomycin solution and 1% L-glutamine (Gibco, New York, USA). The cells were trypsinized, counted and viability checked using trypan blue during counting process. $1x10^6$ cells/ animal were prepared for injection in a total volume of 150µl of sterile PBS (Gibco) in 0.5ml insulin syringes. The cells were aseptically transferred to the syringes inside a Telstar class II biosafety cabinet. The plunger from the syringes were taken out and the cell suspension was pipetted into the syringes to avoid formation of air bubbles or loss of cell volume in the needles. The plunger was replaced and the syringes containing cell suspension were stored in an ice box until injection. The cell suspension was not allowed to stand for more than 30min. in the syringes to avoid clumping of the cells.

The cell preparations were randomised and codes were assigned, before injection. All participants in the study remained blinded for the entire duration of the study and data analysis. A total of 40 animals received intra muscular injections of cells or saline (n=15 for CCR2 overexpressing cells and DsRed expressing cells, n=10 for saline). Animals were anaesthetised by IP injection of ketamine and xylazine (as explained in section 5.2.1), the cell suspension was injected onto three sites (50µl/site) on the thigh muscle (fig 5.3a,b).

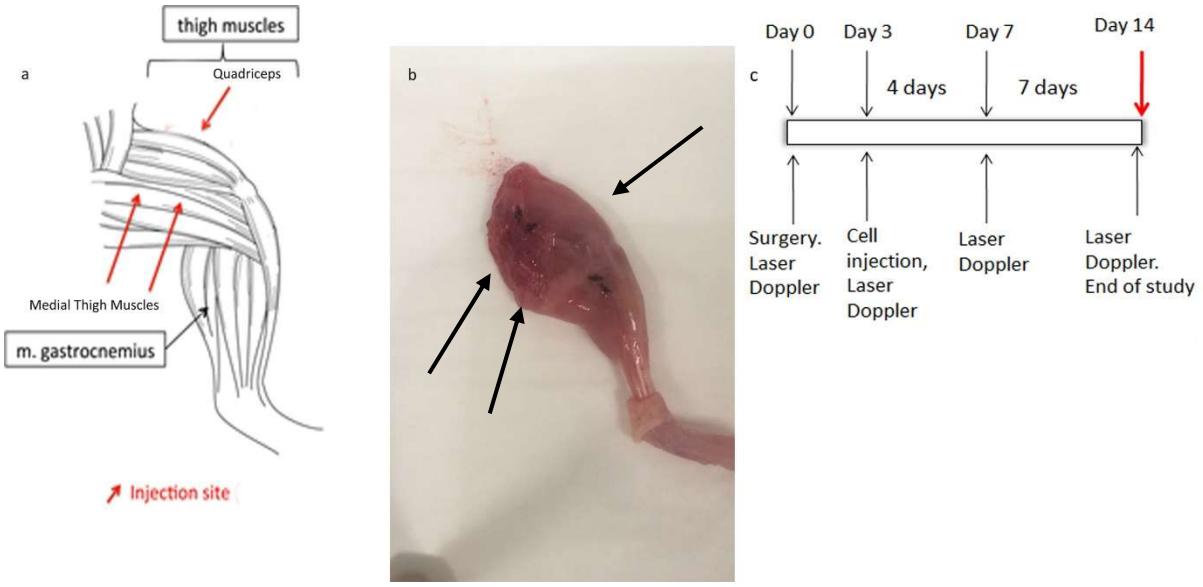

Figure 5:3 Sites of cell injections and *In vivo* study design

Site of cell injection (a): Cells / saline were injected at 3 sites (50µl/site) in the thigh muscles, two injections on the medial thigh muscles and on injection on the quadriceps. Representative image showing the site of injection (arrows) with visible sutures in an excised hind limb. Cells were injected in between the sutures (b). Timeline of study design (c), Laser Doppler imaging done before and after induction of ischemia on day 1, cells were injected on day 3. Laser Doppler imaging was carried out on day 3, 7 and 14.

5.2.5 Functional Scoring of Ischemic Hind Limb

Functional recovery of ischemic leg post-surgical induction of ischemia was measured by semi quantitative assessment of plantar flexion in the ischemic leg[55]. Scorings were carried out on day 3, 7 and 14. A grading scale post ischemic recovery period was used to measure the limb function. A score of 0 represented flexion of toes like that in the non-ischemic foot. A score of 1 indicated plantar flexion but not flexion of toes. Score of 2 represented no plantar flexion of toes while a score of 3 represented dragging of the ischemic limb with loss of control of muscles.

5.2.6 Necrosis Scoring of Ischemic Hind limb

The degree of necrosis on the ischemic limb due to poor blood flow was semi quantitatively measured using a five-point scale[55]. Scorings were carried out on day 3, day 7 and day 14. The severity banding scores are explained in the table below.

Table 5:1 Figure 5:4 Severity scoring for assessment of necrosis

Scale	*Severity*
0	No necrosis or redness
1	Mild redness or cyanosis to tips of toes or toe nails
2	Cyanosis of toes and / or mild necrosis of toes
3	moderate necrosis on 3 or more toes or onset of gangrene on 3 or more toes.
4	Severe necrosis with loss of 3 or more toes
5	amputation of the distal leg, either entire foot or the metatarsals region.

5.2.7 Animal Sacrifice and Tissue Collection

Animals were sacrificed at day 14 by terminal exsanguination under deep anaesthesia followed by cervical dislocation. Post mortem macroscopic examination was conducted in all the animals before collection of tissue samples. Ischaemic, and non-ischaemic hind limbs were harvested from all the animals. Calf and thigh muscles were dissected using a sterile scalpel. For animals that received injections of XenolightDiR labelled cells, ischaemic and non-ischaemic limbs were collected for IVIS imaging. Lungs, liver, kidneys, spleen, intestine, stomach and heart were also collected from these animals for IVIS imaging. The collected organs were kept in dry ice immediately after excision.

5.2.8 Histology and Tissue Processing

Ischaemic and non-Ischaemic calf and thigh muscles harvested were dissected appropriately and were fixed and stored in 10% formalin solution. Calf and thigh muscles of ischemic and non-ischemic limbs were dissected into separate muscle groups. The tissues were transferred to 70% ethanol and stored until processing. Prior to processing the muscle tissues were cut into 5mm sections and placed into labelled cassettes. The cassettes were loaded onto the Leica ASP300 automated tissue processor for routine overnight programme.

The steps in tissue processing involved the following steps.

1. 50% Industrial Methylated Sprit (IMS) for 1 hr
2. 70% IMS for 1hr
3. 90% IMS for 1 hr
4. 95% IMS for 1 hr
5. 100% IMS for 1 hr
6. 100% IMS for 1 hr
7. 100% IMS for 1 hr
8. 100% IMS: chloroform (1:1) for 1 hr
9. Chloroform for 30 min
10. Molten paraffin wax at 60^0C for 1 hr
11. Molten paraffin wax at 60^0C for 1 hr
12. Overnight in paraffin wax (allowed to solidify at room temperature)
13. Molten paraffin wax at 60^0C for 1 hr
14. Molten paraffin wax at 60^0C for 1 hr

Post processing the samples were embedded in paraffin. The embedded tissue specimens were mounted on sectioning stubs and 5µm thick sections were cut on a rotary microtome (Leica). The sections were released into 50^0C water-bath and lifted upon to Super-frost™ microscope slides (Thermo Fischer). Sections were dried at 55^0C overnight to improve the adhesion. Slides were stored in room temperature until staining procedures are carried out.

5.2.9 Haematoxylin and Eosin Staining

Haematoxylin and Eosin staining was performed in 5µm thick tissue sections as follows.

Slides were dewaxed by dipping in xylene twice for 10 min each., residual xylene was removed by dipping the slides twice in 100% ethanol for 2 min each. This was followed by rehydration of tissue sections by sequentially bathing the slides in decreasing dilution of ethanol for 2 min each (95%, 70%, 50% respectively). The slides were then immersed in water for 2 min followed by haematoxylin for 5 min to stain

the nucleus. The slides were washed in running tap water for 4 min to remove the excess stain. The slides were immersed in eosin for 30 sec (with 0.5% glacial acetic acid) to stain the cytoplasm. The excess stain was washed off using in water and the sections were rehydrated by immersing in ethanol (50%,75%,95% and 100%). The slides were washed in cleaning xylene for 2 min. The sections were allowed to air dry before mounting with cover slips using DPX mountant. The slides were left at room temperature to let the DPX dry.

5.2.10 Quantification of Haematoxylin and Eosin Stained Histological Sections

Haematoxylin and Eosin stained tissue sections were analysed for quantification of multicellular infiltrate, adipocyte infiltration and tissue necrosis using a semi quantitative stereology scoring system following the schema of ISO 10993-6 (Biological evaluation of medical devices) (figure 5.4

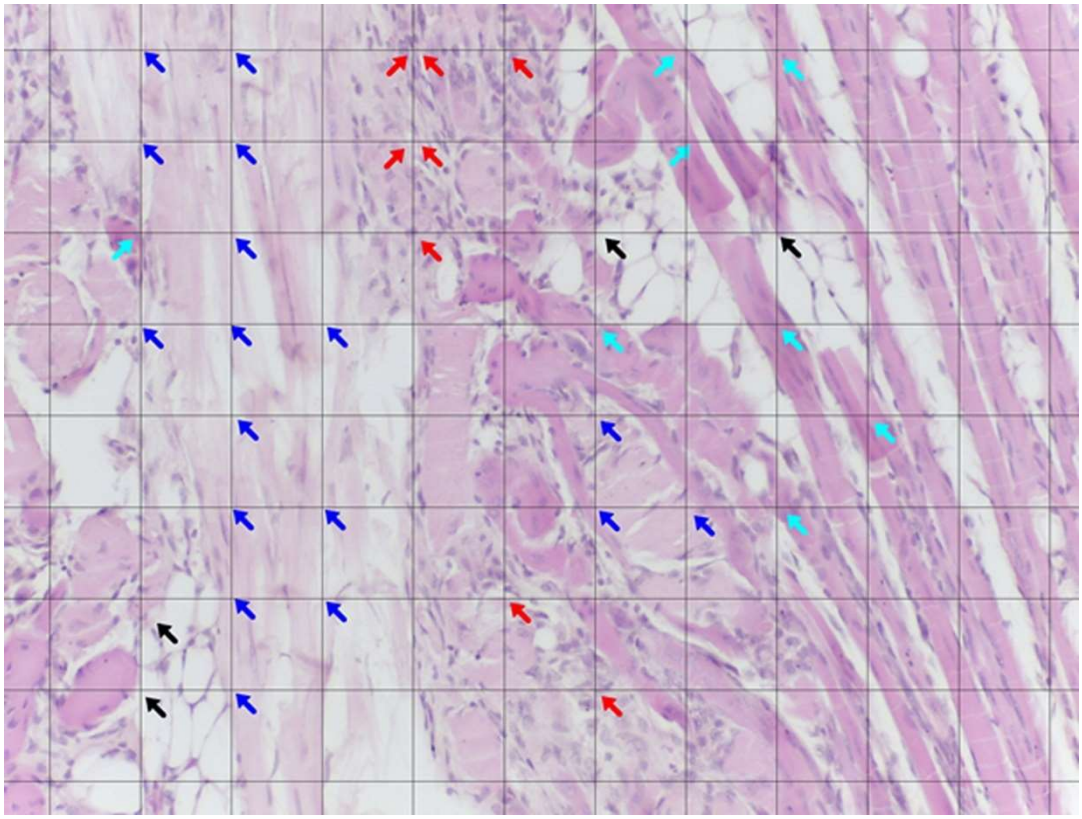

Figure 5:4: Scoring system for histological sections.

Blue arrow represents necrotic muscle fibres, red arrow represents infiltrating inflammatory cells, black arrow represents adipocyte infiltration, cyan arrow represents healthy muscle tissue. Inflammation scoring system (Score 0 = no grids with infiltrating cells, Score 1 =1–5 grids of infiltrating cells, Score 2 = 5–10 grids of infiltrating cells, Score 3 = heavy infiltration >10 grids or presence of packed cells)

Briefly, the histology images were overlaid with an 8x11 grid system and the infiltration score were calculated. A score 0 was given when no grids with infiltrating cells were observed. Score of 1 was given when there were 1–5 grids with infiltrating cells, score of 2 represented 5–10 grids of infiltrating cells, score of 3 is given when there was heavy infiltration on more than 10 grids or presence of packed cells were observed. Muscle necrosis was quantified by counting the average number of necrotic muscle bundles per grid with characteristic nucleus fall out as seen in a typical necrotic muscle bundle. The scoring system for muscle necrosis was the same as explained above.

5.2.11 Lectin Staining for Endothelial Cells

Mouse endothelial cells were stained using GSL-1-B_4 lectin (Biotinylated *Griffonia (Bandeiraea) Simplicifolia* Lectin I) conjugated with Dylight 594 (Vector labs). The sections were dewaxed as described previously and rehydrated in sequential ethanol treatment. The slides were immersed in lectin buffer (Appendix 1) for 5 min, The sections were blocked in 5%FBS in lectin buffer for 30min to avoid unspecific binding, washed twice with lectin buffer (5 min each). The slides were then incubated in GSL-1-B_4 (1:500 dilution in lectin buffer containing 5%FBS) for 1 hr at room temperature protected from light. Negative control slides were treated with 5mM galactose (pre-incubated with GSL-1-B_4 for 1 hr). Slides were washed thrice in lectin buffer for 5 min, mounted with Fluoromount-G with DAPI (Thermo Fisher), and were stored at 4^0C protected from light.

5.2.12 Bio-distribution of Transplanted cells by IVIS Spectrum *In vivo* Imaging

To study the bio-distribution and retention of transplanted cells in animals with hind limb ischemia, advanced fluorescence pre-clinical imaging system, IVIS Lumina LT Series III *In vivo* imaging system (Perkin Elmer, Massachusetts, USA) was used. The IVIS system is equipped with filters that can be used to image reporters that emit from green to near-infrared. For IVIS imaging of transplanted cells, the cells were labelled with a near infrared dye XenolightDiR (1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide, Perkin Elmer) before injection into the muscle tissue.

XenolightDiR has an absorption emission spectrum of 748/780nm. Xenolight labelling of cells was done immediately before cells were prepared for injection.

DiR stock solution was prepared by dissolving 25mg of the dye in 3ml ethanol, a working solution of 320µg/ml was prepared by diluting 199µl of the stock solution in 5 ml PBS. The cells were incubated with the 320µg/ml solution for 30 min at room temperature. The cell suspension was centrifuged at 1000 rpm at 4^0C and the pellet was washed twice in PBS. A total of 10 animals received injections of XenolightDiR labelled cells ($1x10^6$ cells in total volume 150µl at three sites on the calf muscle as describes on figure 5.3.

To assess the bio-distribution/retention of transplanted MSCs, CCR2 overexpressing mMSCs and DsRed expressing mMSCs labelled with near infra-red fluorescent dye XenolightDiR and injected intra muscularly into animals 3 days after induction of hind limb ischemia. $1x10^6$ cells in a total volume of 150µl were injected into three sites on the calf muscle. There were 10 animals that received injection of CCR2 overexpressing mMSCs and 10 animals received injection of DsRed expressing mMSCs. Laser Doppler blood flow was measured before induction of ischemia, immediately after induction of ischemia, on day 3, day 7 and on day 14. Animals were sacrificed on day 14. Ischemic and non-ischemic limbs were collected, all organs such as heart, liver, kidneys, spleen, lungs, stomach and intestines were collected. The organs were imaged to detect the presence of cells in tissues and organ samples. The samples were imaged at excitation wavelength of 710nm and emission wavelength of 760nm (ICG filter) at an exposure time of 0.5 seconds.

Organs and hind limbs from an animal which did not receive any cell or dye injection served as the control during IVIS imaging

5.2.13 Statistical Analysis

Statistical analysis was performed using GraphPad Prism V6.0.1 statistical software (Graphpad software, Inc. California, USA). Results were represented as mean±

standard deviation (SD). For the analysis of multivariable data, one way or two-way analysis of variance (ANOVA) with suitable multiple comparison tests were carried out which are specified in each graph. Data represented was considered statistically significant if the p value was lower than 0.05.

5.3 Results

5.3.1 MCP-1 is Upregulated in Ischemic Tissue

To assess if MCP-1 is upregulated in ischemic tissue, MCP-1 secretion from ischaemic and non-ischaemic muscle tissues were analysed using ELISA. ELISA detected presence of MCP-1 in both ischaemic and non-ischaemic tissue (figure 5.4). However, there was a statistically significant increase in the expression of MCP-1 in the ischaemic tissue when compared to non-ischaemic tissue. Both, calf and thigh muscles were found to be expressing MCP-1. There was a significantly increased expression of MCP-1 in ischaemic thigh muscle when compared to ischaemic calf ($p<0.001$). There was statistical significance in the level of MCP-1 expression between ischaemic and non-ischaemic tissue and between calf and thigh muscles. There was also significant increase in MCP-1 expression between ischaemic thigh and non-Ischaemic thigh (p,0.0001) and between ischaemic calf and non-ischaemic calf muscle ($p<0.05$).

The tissue explants were incubated in medium for 48 hours prior to analysis, this could explain the presence of MCP-1 in non-ischaemic tissue. Ischemia is known to induce the production of MCP-1 in cells and tissues. The increased levels of MCP-1 in ischaemic tissue were expected[56,57]. Similar measurements previously carried out in our lab showed increased levels of chemokines including MCP-1 upregulated in infracted heart explants. The explanted heart was incubated in serum free medium for 48 hours. The resulting medium (containing secreted factors from infracted heart) showed increased migration potential of bone marrow derived stromal cells within 5-10 min of being in contact with MCP-1 in an *in vitro* setting[51].

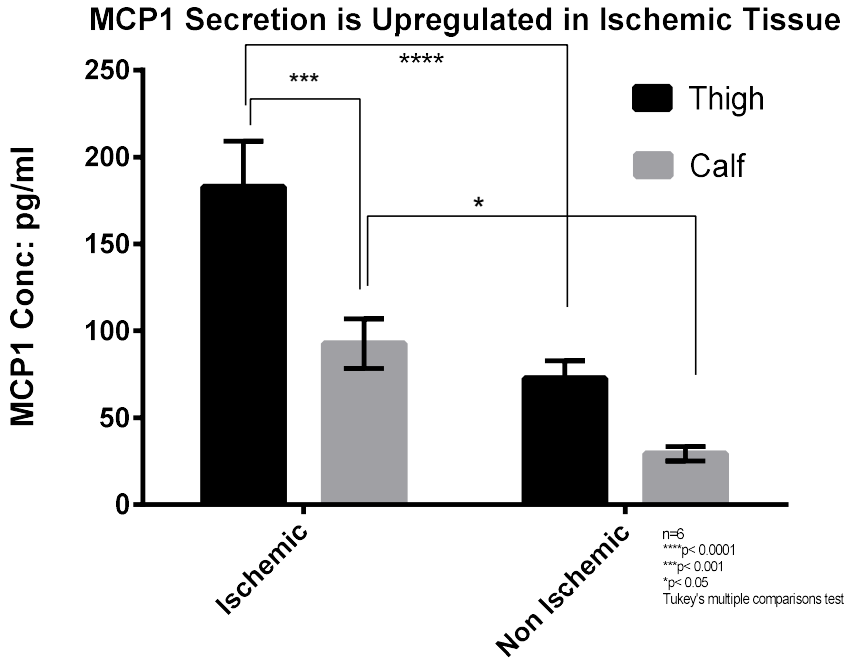

Figure 5:5 : MCP-1 expression in calf and thigh muscles from ischemic and non-ischemic mice limbs.*:*

MCP-1 expression in calf and thigh muscles of mice hind limbs showing increased expression of MCP-1 in ischemic tissue; n=6 $p<0.05$, two-way ANOVA with Tukey's multiple comparison test.

5.3.2 Laser Doppler Blood Flow Analysis Shows Therapeutic Recovery

Laser Doppler blood flow was measured in animals to assess the efficacy of MSCs in promoting limb perfusion recovery. A total of 15 animals received intra muscular injections of CCR2 overexpressing mMSCs, 14 animals received DsRed expressing mMSCs and 10 animals received saline injections.

Laser Doppler imaging was performed before the induction of ischemia to determine the baseline blood flow to help assess therapeutic improvement. It was then performed, immediately after the induction of ischemia on day 0, on day 3, day 7 and on day 14. Laser Doppler blood flow measurement on the non-ischaemic limb (right) served as the internal control. Pre-surgery blood flow was used to assess the endogenous variability in blood flow between the right and left limbs before induction of ischemia. An exclusion criteria was set for animals with more than 20% difference in the blood flow between their right and left limbs prior to surgery. No

such variability was observed in any of the animals used in this study. Measurement of blood flow after surgical induction of ischemia showed a marked reduction in the ratio of blood flow between ischemic and non-ischemic limb to 0.064±0.03 (figure 5.6). This proved the success of the surgical procedure to induce ischemia.

One day 3 after induction of ischemia, perfusion rates were found to be 0.20±0.08. However, there was no statistical significance in the change of perfusion rates between any groups at this point. Intramuscular injection of cells or saline were carried out on day 3.

After injection of cells or saline, at day 7 there was statistically significant improvement in blood flow in animals that were injected with CCR2 overexpressing mMSC s when compared to the ones that received DsRed MSCs ($p< 0.0001$) and with the saline group ($p<001$). Animals that received the injection of CCR2 MSCs showed an increase in recovery by 61%±16, while animals that received DsRed cells showed a recovery of 40%±12%. Saline group of animals showed a recovery of 45%±12%. By day 14, the recovery has progressed to 85%±19% for the CCR2 group, 70%±20% for the control transduced group and 73%±10% for the saline group (figure 5.6). This was significant between CCR2 and DsRed group ($p<0.001$) and between the CCR2 group and saline ($p<0.05$).

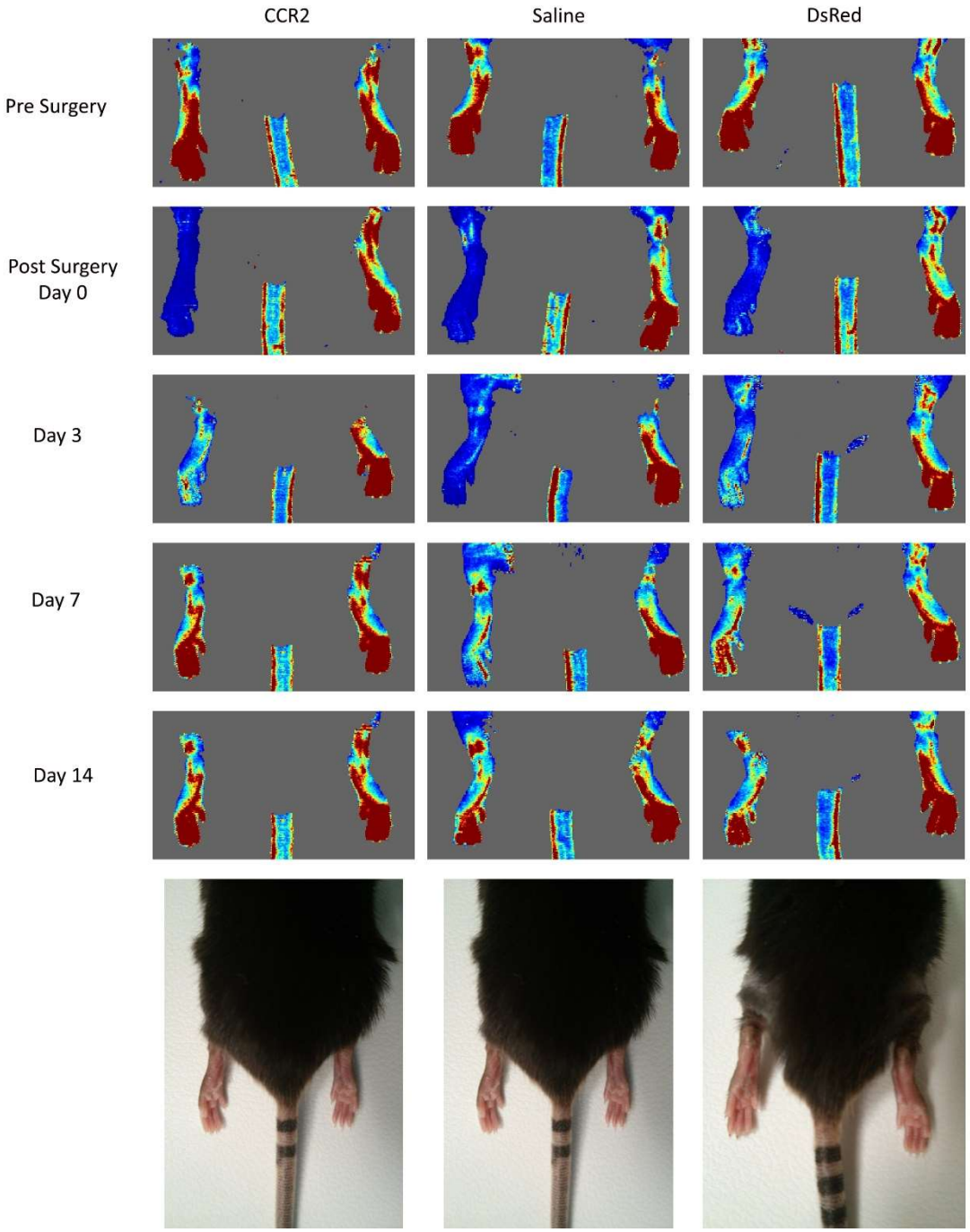

Figure 5:6: Representative images of Laser Doppler blood flow in mice hind limbs

Laser Doppler blood flow in hind limbs of mice, that received, syngeneic transplantation of CCR2 overexpressing mMSCs, DsRed expressing mMSCs, and saline control. Images of mice hind limbs showing no necrosis or tissue damage in any of the groups.

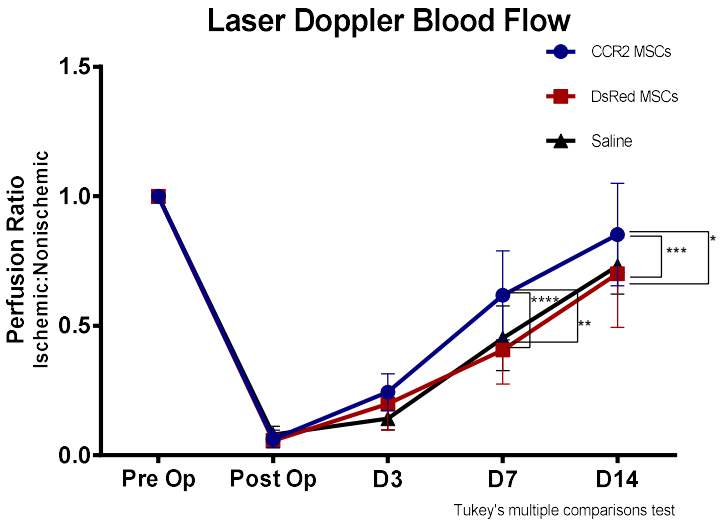

Figure 5:7 Therapeutic assessment of CCR2 overexpressing mMSC s by Laser Doppler blood flow analysis.

Blood perfusion ratio between ischemic and non-ischemic limbs of animals treated with CCR2 overexpressing mMSC s; n=15, DsRed expressing mMSCs; n=14, and saline; n=10. Showing significant improvement in blood flow in the CCR2 group; two-way ANOVA; *p<0.05, Tukey's multiple comparison test.

5.3.3 Assessment of Limb Function in a Mouse Model of Hind Limb Ischemia

Therapeutic assessment of cell therapy was assessed using an ambulatory scoring system for limb usage. The scoring was done in a blinded manner before induction of ischemia, on day 3, day 7 on day 14. A grading scale post ischemic recovery period was used to measure the limb function (as described in 5.2.5) Animals that received the CCR2 overexpressing mMSC s showed improvement in the ambulatory functions at day 14 compared to other groups. Animals that received saline performed worst among the groups. However, there was no significant impairment in limb usage in any groups when compared to studies in our research group involving BALB/c strain of mice which showed significant impairment in limb function and necrosis. There were significant differences between limb functions in animals receiving CCR2 MSCs and saline as well as CCR2 MSCs and DsRed MSCs at day 7 and day 14, but not between animals that received saline and mock transduced DsRed cells (figure5.7).

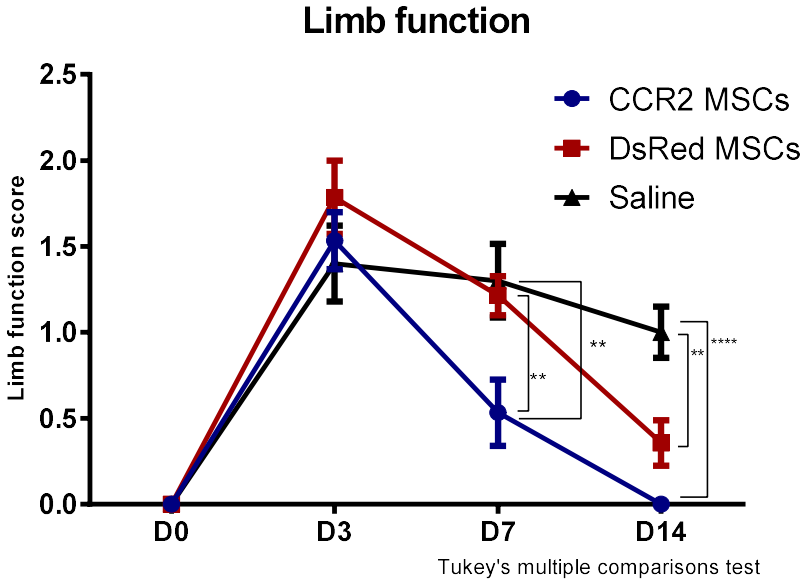

Figure 5:8 CCR2 overexpressing mMSC treated group shows limb salvage.

There was significant improvement in animals that received CCR2 MSCs when compared to saline or mock transduced MSCs at day 7 and 14. P<0.001; two-way ANOVA.

5.3.4 Necrosis and Ambulatory Scoring

Necrosis scoring was done on ischemic hind limbs as per the scoring system,

Scorings were carried out on day 3, day 7 and day 14. A score of 0 represented no necrosis, cyanosis or redness in any area of the hind limb. A score of 1 represented mild redness or cyanosis to the tips of toes or toe nails. Score of 2 represented cyanosis of toes and / or mild necrosis of toes. Number of toes affected were also considered. Score of 3 represented moderate necrosis where more than half the number of toes developed necrosis or the onset of gangrene on 3 or more toes. Severe necrosis with loss of 3 or more toes in the ischemic limb was represented by a score of 4 while a score of 5 was used when there was amputation of the distal leg, either entire foot or the metatarsals region.

The scoring was performed in a blinded manner to prevent bias in assessing the necrosis score. C57BL/6 mice are known to be very resilient to ischemia[22,24,53], the animals in this study did not exhibit any necrosis except for mild blackening of the tip of toe nails.

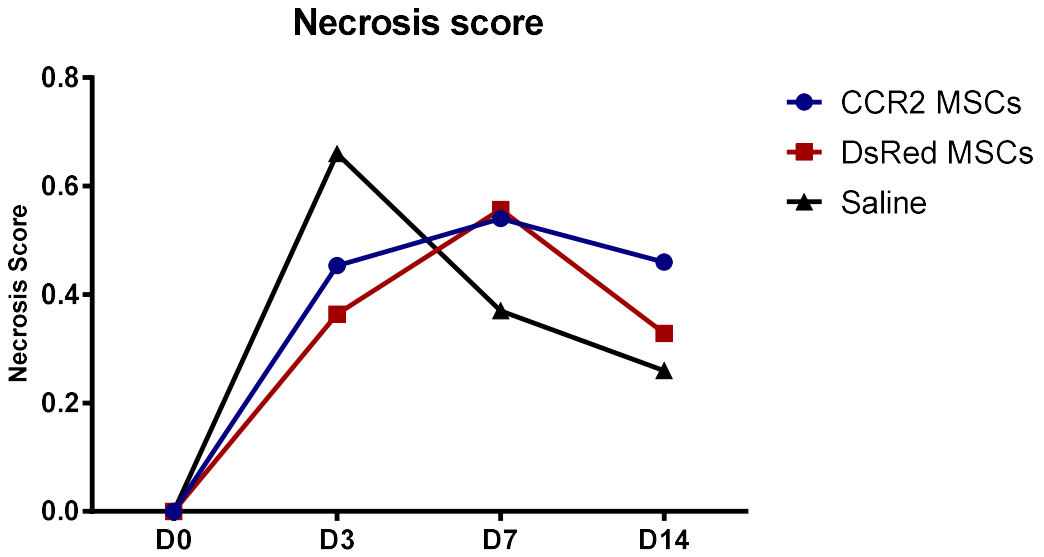

Figure 5:9 Representation of severity in hind limb ischemia.

Necrosis of hind limb as represented by the severity banding. There was no significant severity observed in any groups during the study.

5.3.5 Histology and Tissue Analysis

Tissues samples collected after animal sacrifice were processed and embedded in paraffin blocks before sections were cut for histology.

Histological staining and evaluations were carried out in 5 sections per sample with sections of 5µm thickness where, each section was generated 100µm apart from calf muscles of ischemic and non-ischemic hind limbs. The sections were photographed under a bright field microscope at x100 magnification.

5.3.6 Haematoxylin and Eosin Staining of Muscle Tissue Section

Haematoxylin and Eosin staining of tissue sections showed extensive mononuclear immune cell infiltration in ischemic tissue samples when compared to non-ischemic samples (figure 5.10). Ischemia induced increased infiltration of immune cells. There were necrotic muscle fibres observed in ischemic tissue samples. Significant muscle necrosis, loss of muscle striation and interfibrillar oedema was observed in tissue sections from animals that received DsRed MSCs and saline control (figure 5.10c). These samples also showed intra muscular adipocyte accumulation. CCR2

overexpressing mMSC treated ischemic muscle group had marked increase in perivascular infiltrations of inflammatory cells between muscle bundles and these tissues showed the highest number of immune cell infiltration (figure 5.10c&e) There was no muscle degradation visible in animals that received CCR2 MSCs in comparison to the other two groups, the muscle tissue was not necrotic and there was minimal or no muscle wastage seen (figure 5.10a). Post ischemic muscle regeneration was seen in all the groups. Interestingly, DsRed MSC treated group showed increased muscle wastage and necrotic muscle fibres than animals that received saline injections. Muscle sections from non-ischemic limb showed no increase in immune cell infiltration or muscle necrosis and exhibited normal muscle histology. (figure 5.10 b, d and f). These observations were uniform in all the replicates analysed.

Stereological quantification of Haematoxylin and Eosin stained muscle tissues showed significantly high multicellular infiltration in tissue sections from animals that received injections of CCR2 overexpressing cells and DsRed expressing cell compared to those that received saline injections (figure 5.11a). The inflammation score of these tissue sections were significantly higher than that of animals that received saline injections. Tissue sections from animals that received injections of cells and saline showed the presence of adipocytes, but there was no statistical significance between any of the groups (figure 5.11b). Quantification of muscle necrosis, characterised by collapse of muscle nucleus and loss of muscle striation were highest in animals that received injections of DsRed expressing cells. Muscle sections from animals that received injection of CCR2 overexpressing cells showed least number of necrotic muscle fibres while animals that received injection of saline showed moderate tissue necrosis. There was statistically significant difference between muscle necrosis scores of animals that received CCR2 overexpressing cells when compared to those that received injections of DsRed cells and saline (figure 5.11c).

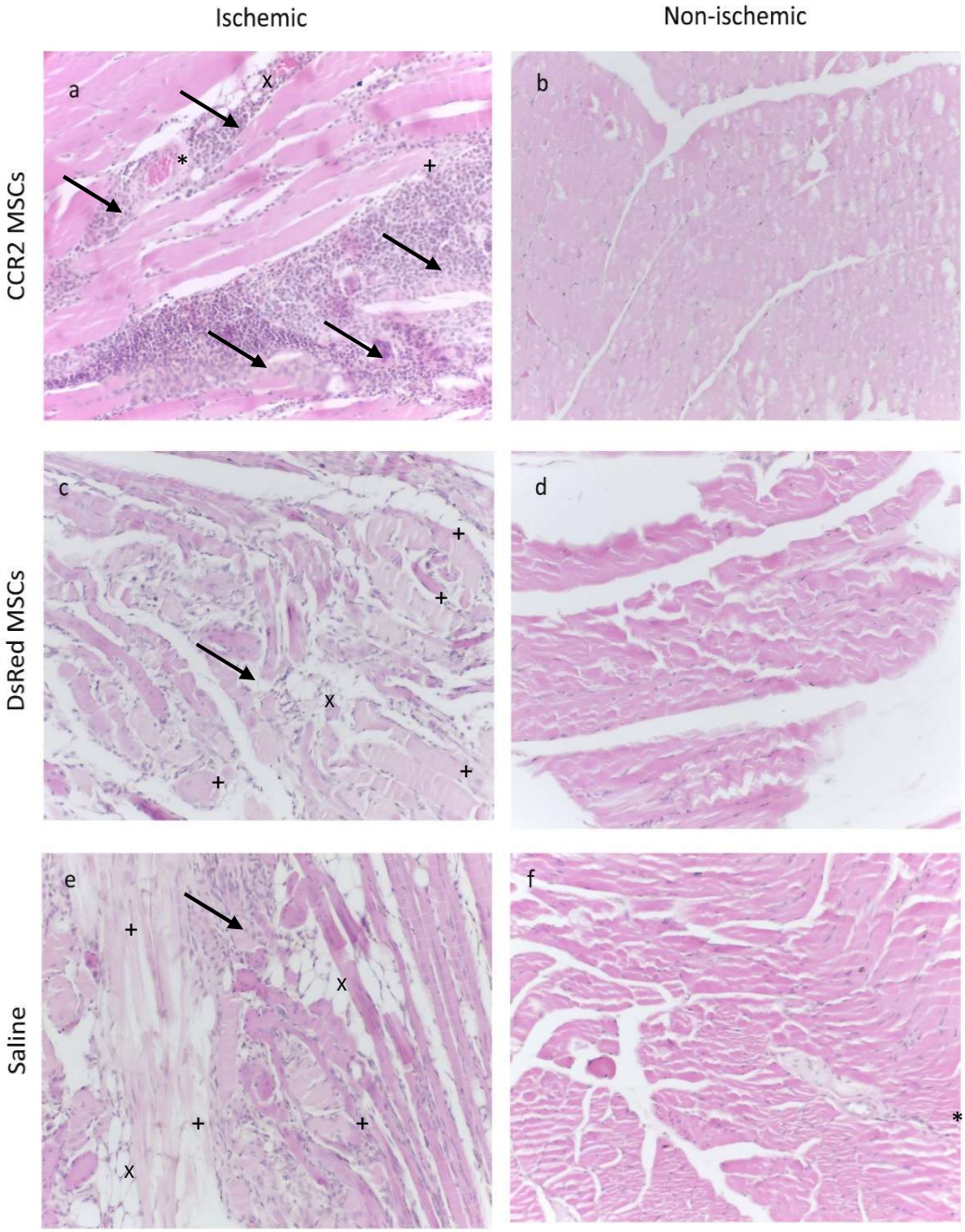

Figure 5:10 Representative images of Haematoxylin and Eosin staining of paraffin embedded tissue sections of calf muscle from ischemic and non-ischemic hind limbs of mice.

Mice injected with; CCR2 overexpressing mMSCs (a&b), DsRed expressing mMSCs (c&d) and saline control (e&f). Black arrows represent infiltrating immune cells, + represents degenerating muscle tissue, x represents intra muscular adipocyte accumulation, * represents blood vessels. X100 magnification.

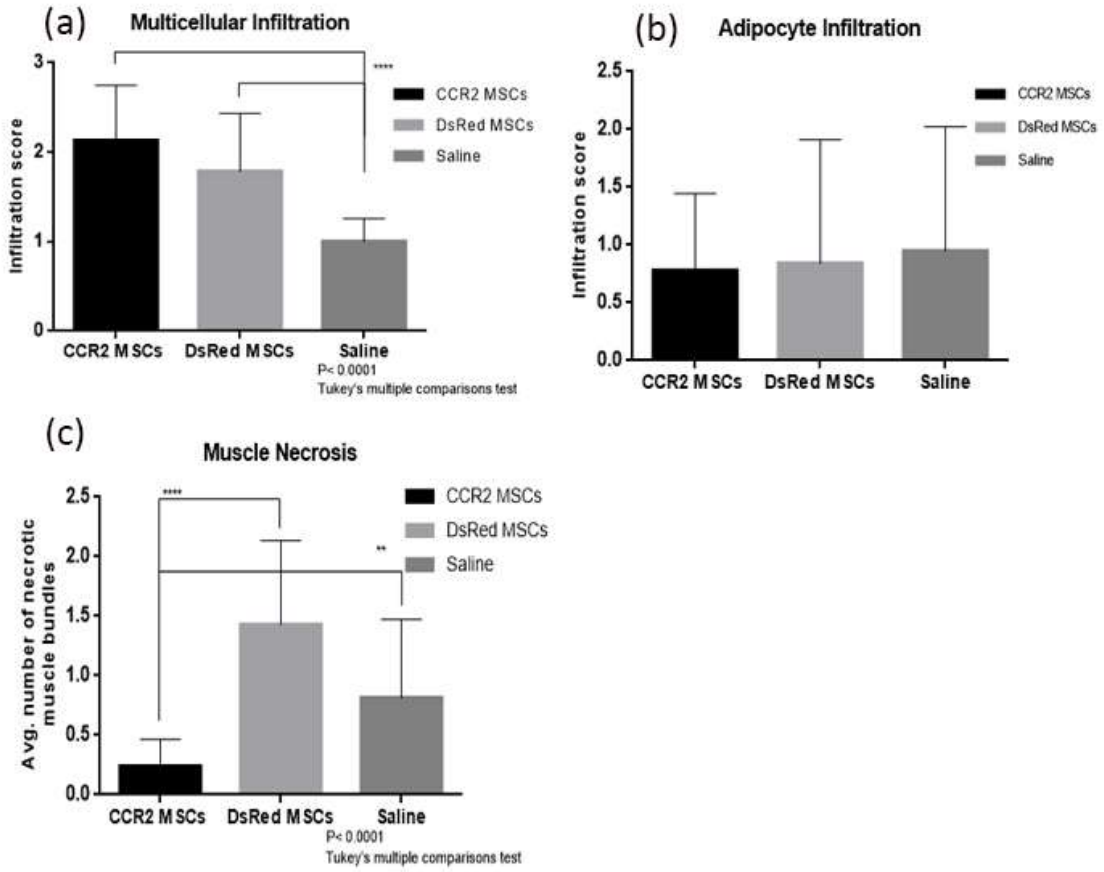

Figure 5:11 : Stereology Quantification of muscle sections.

Quantification of multicellular infiltrate (a), Adipocyte infiltration (b) and muscle necrosis (c)

5.3.7 Lectin Staining for Endothelial Cells

Surgical induction of ischemia will disrupt the blood flow to the distal limb, resulting in initiation of compensatory mechanisms to restore blood flow. One compensatory mechanism that happens after surgical induction of ischemia is the triggering of proximal arteriogenesis, whereby small pre-existing collateral vessels are enlarged and remodelled, leading to the formation of functional vessels trying to bypass blood flow from the occluded site[58]. It will also trigger angiogenesis distally, a process by which endothelial tubules sprouting into capillaries by recruiting smooth muscle cells. This is initiated by ischemia and has limited capacity to increase perfusion of ischemic tissue[59]. Tissues from ischemic and non-ischemic calf muscles were stained using GSL-

1-B4 lectin to assess the capillary density. GSL-1B4 is s lectin which is reported to stain endothelial cells by specifically binding to terminal α-galactosyl residues expressed by endothelial cells[60].

Ischemic and non-ischemic calf muscle tissues collected from animals treated with CCR2 overexpressing mMSCs, DsRed expressing mMSCs and saline were paraffin embedded and sectioned into 5µ thick slices, 100µm apart. The tissue sectioned were stained with lectin GSL-1B4 labelled with Dylight 594 to visualise endothelial cells (red fluorescence). The slides were mounted with a glass slide with Fluoromount-G with DAPI which stains the nucleus of the cells blue.

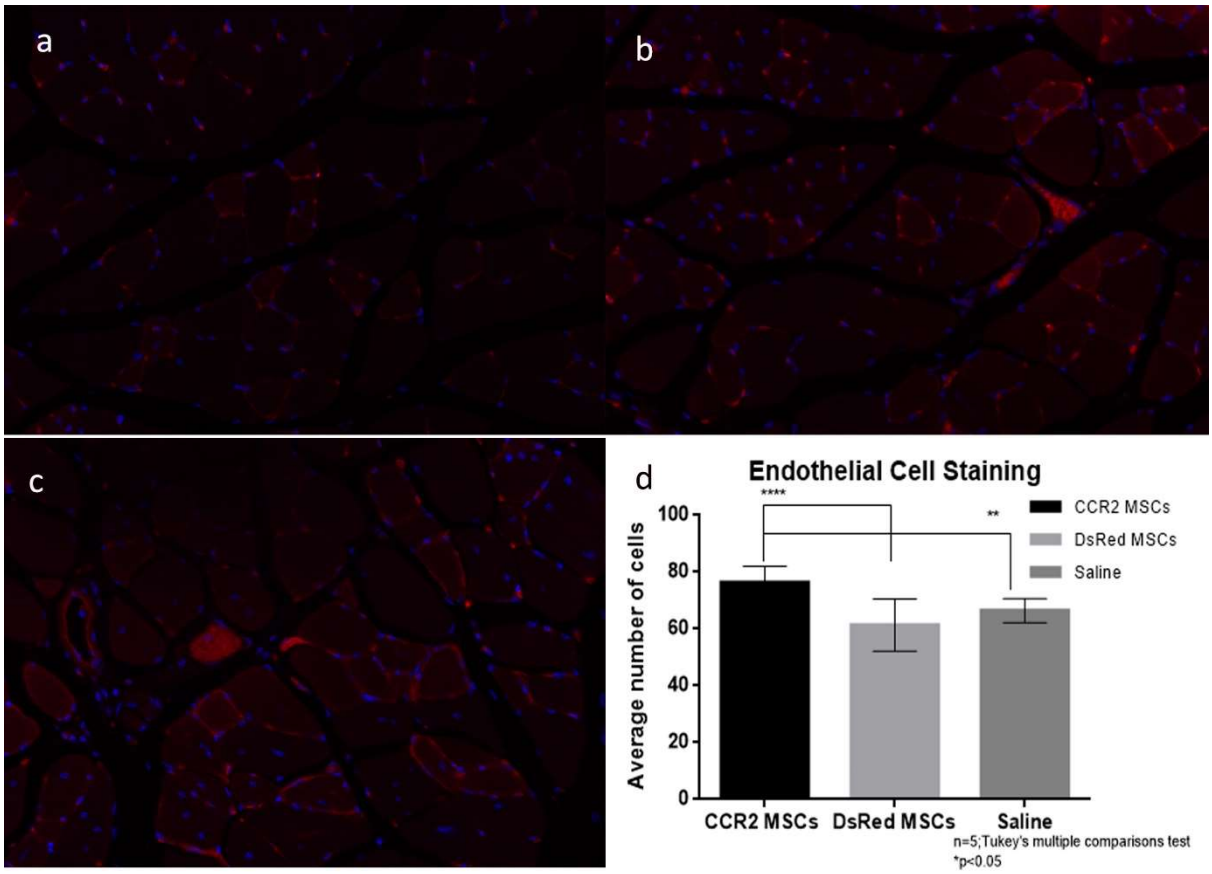

Figure 5:12 Lectin staining and quantification of endothelial cells

Representative images of tissue sections stained with GSL-1B4 lectin for endothelial cell detection. Animals treated with; Saline (a), DsRed Cells (b), CCR2 MSCs (c). Quantitative measurement of endothelial cells in tissue samples on day 14 post injection of cells (d). CCR2 overexpressing mMSC treated group showed significant increase in the number of stained endothelial cells when compared to DsRed expressing mMSC treated group ($p<0.0001$) and saline control ($p<0.05$); ordinary one-way ANOVA with Tukey's multiple comparisons test.

The tissue sections were photographed under a fluorescent microscope (OlympusIX71 with EXFO Xcite 120 fluorescence illumination system) X200 magnification (figure 5.12 a, b and c).

The number of capillaries stained in each tissue sections were counted and data is represented as average number of cells counted per 10x field ± Standard deviation (figure 5.12 d). Analysis of tissue sections for staining of endothelial cells shows there was an increased number of endothelial cells in ischemic muscles compared to non-ischemic muscle. This could be because of the enhanced sprouting of capillaries expected from ischemic induction. Comparison of endothelial cell count between treatment groups show there was a significant increase in the number of endothelial cells expressed in ischemic tissue from animals treated with CCR2 overexpressing MSCs (76.3±5.7) when compared to that from DsRed expressing MSC (61.3±9.2) treated group and animals that received saline (66.4±4.2) injection. There was statistical significance between positively stained endothelial cells from ischemic calf muscles from CCR2 MSC treated group when compared with DsRed MSC treated group ($p<0.0001$), and animals that received saline injections ($p<0.05$)

5.3.8 Bio-distribution / Retention of Transplanted CCR-2 Modified MSCs in a CLI Model

Ischemic and non-ischemic limbs as well as all organs such as heart, liver, kidneys, spleen, lungs, stomach and intestines were collected from animals that received injection of labelled cells 3 days after induction of ischemia. The limbs and organs were imaged using advanced fluorescence pre-clinical imaging system, IVIS Lumina LT Series III *In vivo* imaging system (Perkin Elmer, Massachusetts, USA) to detect the presence of cells in tissues and organ samples. The samples were imaged at excitation wavelength of 710nm and emission wavelength of 760nm (ICG filter) at an exposure time of 0.5 seconds.

There were fluorescence signals from all organs and limbs from animals that received labelled cell injections. However, the fluorescence intensity was clearly high from ischemic hind limbs when compared to non-ischemic hind limbs (figure5.13). Organs and limbs from animal that received no labelled cell injection did not exhibit any

fluorescence under IVIS imaging. Adaptive background fluorescence subtraction was done during capture of all images.

Quantification of fluorescence is calculated based on the radiant efficiency (photons/sec/cm^2/steradian) of each tissue sample; which is the measure of the fluorescence intensity of the sample, eliminating any background fluorescence and excitation wavelength interference.

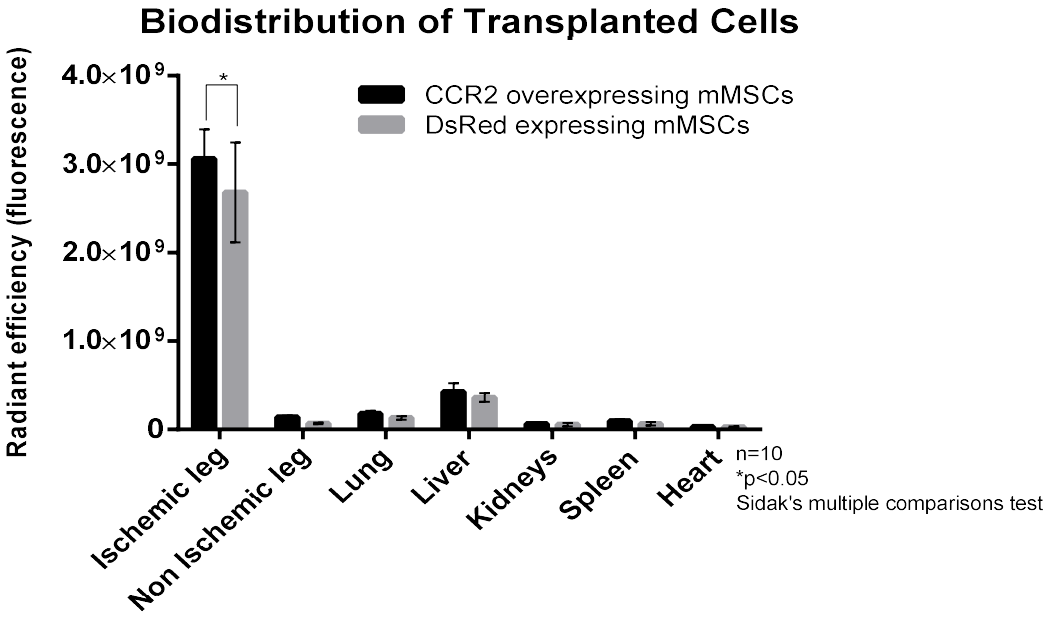

Figure 5:13 Quantification of bio-distribution of transplanted cells by IVIS imaging

Fluorescence intensity from ischemic and non-ischemic hind-limbs as well as organs from animals that received intra muscular injections of CCR2 overexpressing MSCs and DsRed expressing MSCs. Significantly higher fluorescence observed on ischemic muscles of animals that received injection of CCR2 overexpressing mMSCs; $p<0.05$; two-way ANOVA with Sidak's multiple comparisons test.

IVIS imaging of ischemic and non-ischemic legs from animals that received intra muscular injection of 1x10^6 each of CCR2 overexpressing MSCs and DsRed expressing MSCs showed there was an increase in the fluorescent intensity on ischemic limb compared to that of non-ischemic limb.

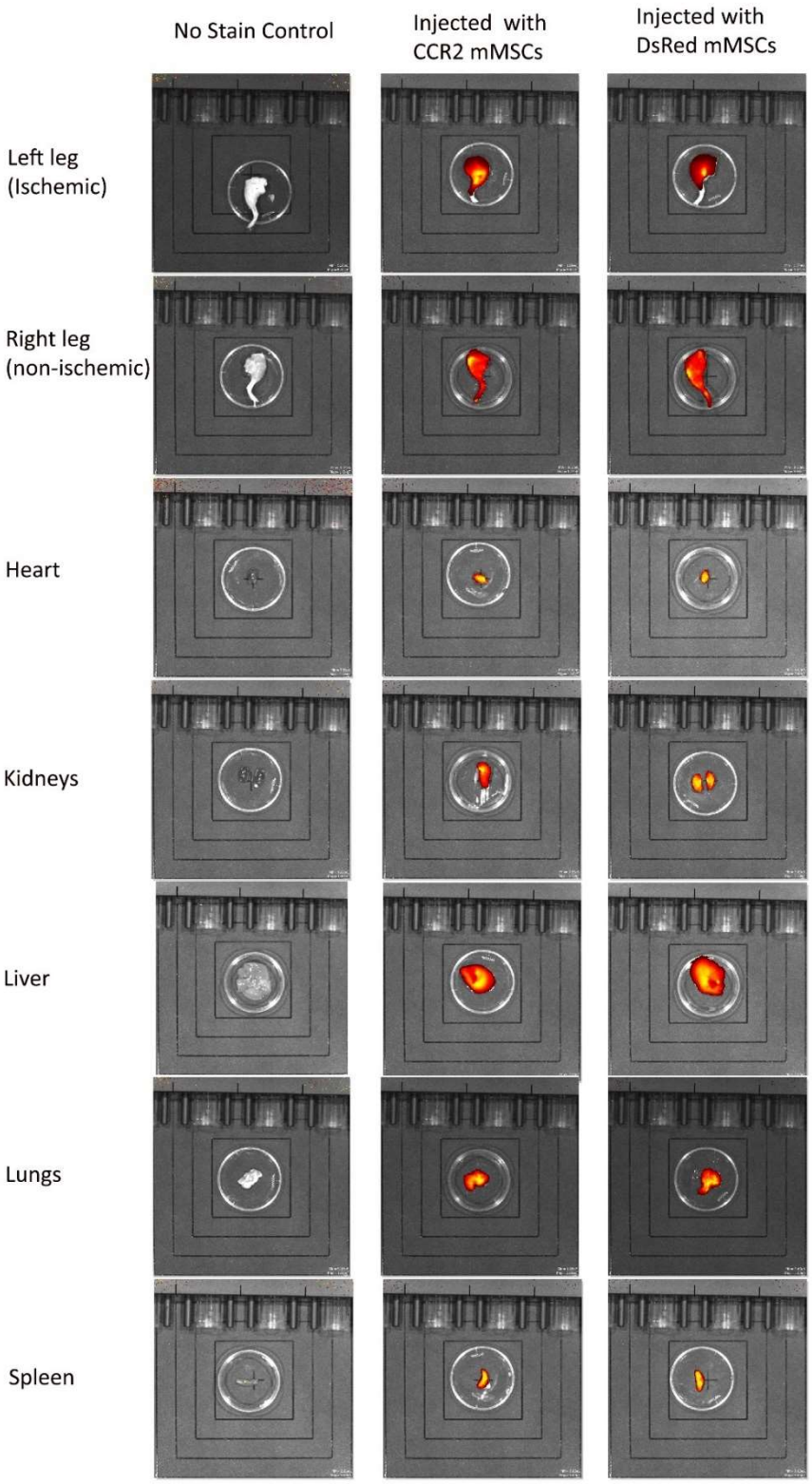

Figure 5:14 IVIS imaging of organs from mice that received cell injections for bio-distribution.

Organs of mice that received injections of CCR2 overexpressing mMSCs, DsRed expressing mMSCs 14 days after hind limb ischemia.

Quantification of radiant efficiency showed the transplanted cells were retained in the ischemic leg in both the animal groups at day 14 post induction of ischemia. However, CCR2 expressing MSCs showed significantly ($p<0.05$) increased retention at the injection site than DsRed MSCs. relatively low fluorescence signals were observed in all the organs examined. However, there was no evidence to prove this fluorescence observed was due to the infiltration of transplanted MSCs. There was an increase in fluorescence in liver compared to any other organ in both the cell groups (figure 5.14). This could be due to the leeching of XenolightDiR from the muscle tissues. As liver detoxifies chemicals and metabolises drugs introduced into the body.

5.4 Discussion

Analysis of MCP-1 secretion in ischemic and non-ischemic calf and thigh muscles from C57BL/6 mice that had undergone surgical induction of ischemia revealed increased secretion of MCP-1 in ischemic muscle than in non-ischemic muscle. There was an increase in the expression of MCP-1 in the ischemic calf muscles than in ischemic thigh muscles. This could be since MCP-1 is upregulated not only in ischemic tissue, but also in injured tissue[46,61]. Therefore, it comes as no surprise that there is upregulation of MCP-1 at the site of surgery. The increased levels of MCP-1 in ischaemic tissue were expected. Similar measurements previously carried out in our lab showed increased levels of chemokines including MCP-1 upregulated by infracted heart explant incubated in medium for 48 hours and showed increased migration potential of bone marrow derived stromal cells within 5-10 min of being in contact with MCP-1 in an *in vitro* setting[51]. Studies in ischemic cerebral tissue have shown similar results, where ischemic tissue showed time dependent secretion of MCP-1, ranging from 200pg/ml at 6 hours, 800pg/ml at 24 hours and 900pg/ml at 48 hours. The level of MCP-1 was reduced to 120pg/ml after 7 days. The same study showed increased migration of rat bone marrow stromal cells to the conditioned medium from ischemic brain. The number of migrating cells were found to be dose dependent with increasing cells migrating with increase in concentration of MCP-1 in medium[62]. These results show similarity with the results obtained in this study, but with far less concentration of MCP-1 detectable from ischemic muscle samples (180pg/ml in

ischemic thigh) and cells migrating in a dose dependent manner but with lesser number of cells migrating at higher concentration of MCP-1.

In this study, we have used a C57BL/6 mouse model for hind limb ischemia by ligating the femoral artery at two sites to negate the effects of high density of collaterals that are native to the C57BL/6 strain. Several reports suggest the variability of mouse models for hind limb ischemia studies have demonstrated the ability of the C57BL/6 strain of mice for high levels of endogenous recovery[25,63–65]. There are several reports that proves the surgical techniques and strain of mice can cause discrepancies in recovery and efficacy of the animal model.[23,65–69]. We have conducted comparative studies which showed in the effect of mouse strain and surgical techniques leading to the optimisation of the double ligation technique used here[22]. Although double ligation model is considered severe in strains like BALB/c, this was well tolerated in C57BL/6 strains of mice. Limb perfusion alone cannot be considered as a measure of recovery as it does not always translate into limb salvage and vice versa. Surgical techniques can influence the outcome of an HLI model. Induction of hind ischemia by single ligation of femoral artery can result in a very weak model in strain like C57BL/6 while double ligation gives the right therapeutic window for a short duration study like this. In this double ligation model of hind limb ischemia, the proximal and distal ends of the femoral artery and the proximal profonda femoris artery in the groin are dissected and ligated. The second incision was made above the knee to expose the superficial femoral artery and vein. The artery and vein were then separated and the artery was ligated above the saphenous–popliteal bifurcation. The model was efficient although it was in a strain of mice with collateral density. There was no significant endogenous blood flow recovery seen in any of the animals. The recovery rates were well below our cut off point of 30% in all animals

Measurement of blood flow ratio between ischemic and non-ischemic limb after surgical induction of ischemia showed a marked reduction in blood flow to 0.064±0.03. This proved the success of the surgical procedure to induce ischemia.

Intramuscular injection of CCR2 overexpressing mMSCs, DsRed expressing mMSCs and saline were carried out on day 3 after induction of ischemia, perfusion rates were found to be 0.20±0.08. There was no statistical significance in the change of perfusion rates between any groups at this point. The recovery of 20% at day 3 is half that of the reported perfusion values seen in our previous experiments with BALB/C strain of mice undergoing double ligation ischemia induction[22] and in our previous study, single ligation of femoral artery in BALB/c and athymic nude mice resulted in perfusion levels of 30±10% and 40±15%, while double ligation in these strains showed no more than 20±5% recovery in blood flow[22].

Another HLI study conducted in C57BL/6 mice investigating the angiogenic properties of platelet rich plasma by sustained release showed increase in perfusion rates of 98±4% recovery after 28 days, with recovery reaching 80% within the first two weeks of study. This study induced ischemia by ligating, cutting and excising entire right saphenous artery and vein, with entire external iliac artery and veins with deep femoral and circumflex arteries along with veins. This model was so severe in its approach. Yet it showed remarkable recovery at the end of the study, with the control group recovering 57±12%[70].

A study comparing four surgical techniques for induction of ischemia in C57BL/6 mice showed 20% recovery at day 3 when total excision of femoral artery and excision of femoral artery in combination with iliac artery was performed. However, single ligation of femoral artery and single ligation of iliac artery showed increased perfusion rates of more than 40% at day 3[71]. Single ligation ischemia induction in C57BL/6 and BALB/c demonstrated that BALB/C shows significantly less endogenous recovery than C57 mice. C57BL/6 strain is shown to have increased collateral density and complex vasculature which aids in speedy recovery when only one ligation was carried out below the profonda femoris to induce ischemia[64]. Laser Doppler imaging had shown there was an increase in the recovery of blood flow at day 3 compared to single ligation models of C57BL/6, and the increase in perfusion rates observed were less than that is reported in studies where single ligation of femoral artery was used

to induce ischemia (20%±8% on day 3), but on the same range as reported in animals that underwent double ligation, irrespective of strain. This is proof that, although there is high density of collaterals in C57/BL6 strain of mice, the double ligation model provided a model with similar level of comparability with other strains of mice.

After injection of cells and saline, at day 7 there was statistically significant improvement in blood flow in animals that were injected with CCR2 overexpressing mMSC s when compared to the ones that received DsRed MSCs ($p< 0.0001$) and with the saline group ($p<001$). Animals that received the injection of CCR2 MSCs showed an increase in recovery by 61%±16, while animals that received mock transduced cells showed a recovery of 40%±12%. Saline group of animals showed a recovery of 45%±12%. By day 14, the recovery has progressed to 85%±19% for the CCR2 group, 70%±20% for the control transduced group and 73%±10% for the saline group (figure 5.7). This was significant between CCR2 and DsRed group ($p<0.001$) and between the CCR2 group and saline ($p<0.05$). The high levels of recovery in all the groups can be attributed to the C57BL/6 strain used in this study. Although the initial rates of recovery until day 3 were found to be comparable to any other established hind limb ischemia mice model, the increase in blood flow on day 7-14 is above that of other strains and is characteristic feature of the C57/BL6 strain. The CCR2 MSC transplanted group showed increased perfusion rates at the end of the study. The DsRed cells performed worst in the study. Even in our *in vitro* migration assays (Chapter4), the DsRed cells underperformed in comparison to unmodified MSCs. This could not be because of the lentiviral transduction, but because of the changes in cellular properties due to the expression of the DsRed protein.

Assessment of limb function and necrosis shows there was no loss of limb function in any of the animals at any stage in the study. The highest severity seen in this study in limb function was on day 3. There were significant differences between limb functions in animals receiving CCR2 MSCs and saline as well as CCR2 MSCs and DsRed MSCs at day 7 and day 14, but not between animals that received saline and DsRed cells (figure 5.8).

Necrosis assessment of the hind limbs showed the animals in this study did not exhibit any necrosis except for mild blackening of the tip of toe nails (figure 5.9). C57BL/6 mice are known to be very resilient to ischemia and hence they did not develop any necrosis unlike BALB/c or athymic strains which develops comparatively high degree of necrosis when underwent single or double ligation[22].

Haematoxylin and Eosin staining of tissue sections showed extensive mononuclear immune cell infiltration in ischemic tissue samples when compared to non-ischemic samples (figure 5.10 and 5.11a). Ischemia induced increased infiltration of immune cells. There were necrotic muscle fibres observed in ischemic tissue samples. Significant muscle necrosis, loss of muscle striation and interfibrillar oedema was observed in tissue sections from animals that received DsRed MSCs and saline control (figure 5.10 c and 5.11c). These samples also showed intra muscular adipocyte accumulation (figure 5.11b). CCR2 overexpressing mMSC treated ischemic muscle group had marked increase in perivascular infiltrations of inflammatory cells between muscle bundles and these tissues showed the highest number of immune cell infiltration (figure 5.10 c & e). This effect could be because of the presence of CCR2 positive cells (CCR2 MSCs) in the muscle, along with the presence of ischemia and injury due to surgery which will increase the influx of immune cells infiltrating. CCR2 is known to recruit macrophages into sites of infection and injury. The role of CCR2 in promoting the infiltration is documented in infections. CCR2 deficient mice showed significantly reduced macrophage recruitment in lungs after *Cryptococcus neoformans* infection when compared to mice with normal CCR2 expression[72]. CCR2 also has a role in determining the Th1 Th2 predominance of infiltrating macrophages. Th1 Th2 preference of infiltrating immune cells are known tom have a role in determining the regenerative properties of immune cells[73].

There was no muscle degradation visible in animals that received CCR2 MSCs in comparison to the other two groups, the muscle tissue was not necrotic and there was minimal or no muscle wastage seen (figure 5.10a). Post ischemic muscle regeneration was seen in all the groups. Interestingly, DsRed MSC treated group showed increased muscle wastage and necrotic muscle fibres than animals that

received saline injections (figure 5.11c). Muscle sections from non-ischemic limb showed no increase in immune cell infiltration or muscle necrosis and exhibited normal muscle histology. (figure 5.10 b, d and f). Hence the CCR2 overexpressing MSCs showed enhanced regenerative potential and muscle regeneration with no muscle loss or oedema in any animals.

Regenerative potential of CCR2 modified MSC treatment is further augmented by the detection of high number of endothelial cell count in ischemic tissue from animals treated with CCR2 overexpressing MSCs (76.3±5.7) when compared to that from DsRed expressing MSC (61.3±9.2) treated group and animals that received saline (66.4±4.2) injection. There was statistical significance between the number of positively stained endothelial cells from ischemic calf muscles from CCR2 MSC treated group when compared with DsRed MSC treated group ($p<0.0001$), and animals that received saline injections ($p<0.05$) (figure 5.12).

Bio-distribution study by IVIS imaging after intra muscular injection, showed increasing fluorescence in ischemic tissue compared to the non-ischemic tissue. This can be attributed to the retention of transplanted cells at the ischemic tissue (figure 5.13). The retention of CCR2 positive cells could be primarily due to the high expression of MCP-1; the CCR2 ligand at the ischemic site. Although MSCs are known to migrate towards MCP-1 without the overexpression of CCR2[51], the CCR2 overexpressing MSCs are shown to be retained higher numbers than control transduced MSCs. CCR2 MSCs are proven to migrate better compared to unmodified MSCs in experiments outlined in chapter 4. Analysis of bio-distribution of transplanted cells in different organs from mice treated with CCR2 over expressing MSCs and control transduced MSCs showed no significant fluorescence in any organs when compared to that of the ischemic leg. However, there was some fluorescence observed in liver and lungs, the two organs where transplanted cells are often found. The quantification of fluorescence levels showed these numbers are extremely low in comparison to what is retained in the target site (figure 5.13). The ischemic limbs

from animals that received injections of CCR2 overexpressing MSCs showed significantly higher fluorescence than that from DsRed MSCs animals.

Related bio-distribution studies where transplantation of culture expanded adipose derived MSCs into immune compromised mouse model and subsequent bioluminescence imaging 8 months after transplantation had shown intramuscular and intra venal delivered MSCs colonised in the liver[74]. In the present study, increased fluorescence intensity in liver explants from animals that received intra muscular injections of MSCs using IVIS imaging. However, the presence of MSCs in liver was not confirmed by histology or PCR based bio-distribution assays. Bio-distribution studies by a high sensitive PCR based assay for the detection of human MSCs injected intra muscularly into immune compromised mice after surgical induction of ischemia carried out by members of our group could not find any residual human cells in any organs 3 months after cell injections[75].

The role of CCR2 and MCP-1 in immune cell infiltration is well known. The autocrine and paracrine role of CCR2 and MCP-1 is proven to enhance angiogenesis[76,77]. The increasing concentration of MCP-1 due to ischemia induces infiltration of immune cells and macrophages that play a role in vascular remodelling and angiogenesis. The upregulation of MCP-1 after induction of ischemia will result in the retention of these transplanted cells in the ischemic site. Moreover, CCR2 / MCP1 recruited macrophages will help in positive remodelling of the tissue rather than increasing inflammation[78,79]. This remodelling of skeletal muscles is seen in histology sections from animals treated with CCR2 overexpressing MSCs. These delivered cells along with the native angiogenic paracrine factors MSCs are known to secrete will drive the enhancement of angiogenesis at the ischemic tissue. These cellular paracrine factors along with the capacity of C57 mice to form collaterals at a higher rate than other strains of mice along with their high underlying vascular density leads to efficient therapeutic angiogenesis and functional improvement in the hind limbs of animals treated with CCR2 overexpressing MSCs.

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Chapter 6

Summary and Conclusion

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6 Summary and Conclusion

Critical limb ischemia (CLI) is an end stage peripheral arterial disease affecting millions of people across the globe and has high mortality rates associated with it. There are no successful treatment options for patients who present with CLI. Conventional therapies like endovascular surgery or bypass grafts have limited success in delivering long term therapeutic outcome. When conventional therapies are not successful patients often end up having amputations of the distal limb.

Stem cell therapies are showing promising results in the treatment of CLI. Adult stem cells such as Mesenchymal stem cells (MSCs) are known for their properties to secrete various angiogenic growth factors and chemokines[1,2]. MSCs secrete a variety of chemokines and growth factors that are known for their ability to promote angiogenesis[3]. MSCs can be isolated from various sources including bone marrow, umbilical cord and adipose tissue. They are known for their immune modulatory properties and for their ability to home to sites of ischemia and injury. Autologous and allogenic MSCs are proven for their ability to induce therapeutic angiogenesis in animal models of hind limb ischemia (HLI)[4,5]. Autologous and allogenic therapy of CLI using MSCs are beginning to generate results in various clinical trials across the world[6–9].

The use of MSCs for CLI treatment is not without challenges. Bio-distribution/ retention of transplanted cells to the site of injury is the major challenge in achieving therapeutic benefit from MSC therapy. Less than 1% of systemically transplanted cells often end up at the target site[10]. Intramuscular delivery of MSCs require multiple injections at the target site to achieve therapeutic efficacy.

This study explores the possibilities to enhance bio-distribution and retention of transplanted MSCs by overexpressing a chemokine receptor on MSCs. The chemokine receptor in focus here is the CC chemokine receptor type 2 (CCR2). CCR2 binds with high affinity to Monocyte Chemoattractant Protein 1 (MCP-1). MCP-1 is reported to be upregulated in ischemia and injury and it serves as the chemokine that aids in the migration of monocytes and other immune cells to the site of injury. These immune cells express CCR2 on their surface enabling their migration to tissues with high level of MCP-1 production. MSCs are known to express CCR2, but in levels. The strategy in this study is to overexpress CCR2 on MSCs using a lentiviral vector, enabling the enhanced retention of, CCR2 overexpressing MSCs at the ischemic site enabling prolonged secretion of angiogenic factors leading to therapeutic angiogenesis.

Chapter 2 details the various lentiviral expression systems tested for efficient transgene integration and expression of CCR2 and, also for the presence of selectable markers enabling the selection of modified cells expressing CCR2. The pLEX307 lentiviral expression system was selected based on the presence of the strong promoter EF-1α which enabled high level expression of CCR2 and based on the presence of puromycin resistance it confers to the transduced cells enabling selection of modified cells in culture medium containing puromycin.

pLEX307 lentiviral construct was shown to generate high titre lentiviral particles enabling high level trans gene expression with no toxicity to cells. This expression system was used to produce lentiviral vectors carrying CCR2 and control vector carrying DsRed for further experiments in this study.

In chapter 3, the ability of pLEX CCR2 lentiviral particles to overexpress CCR2 on mouse MSCs were investigated. pLEX CCR2 lentiviral particles were confirmed to be able to overexpress CCR2 on MSCs. The baseline expression of CCR2 on mMSCs were 5% before genetic modification. After lentiviral transduction, a population of mMSCs that express CCR2 were selected by puromycin selection. The overexpression of CCR2

was confirmed at the transcript level by Q-PCR. There was a 36-fold increase in the CCR2 gene transcript in these modified, selected cells when compared to unmodified cells. The overexpression of CCR2 on lentiviral modified cells at the protein level were confirmed by immune-cytochemical staining for CCR2 and by flow cytometry.

Characterisation and functional assessment of CCR2 overexpressing MSCs were carried out in chapter 4. CCR2 overexpressing MSCs were tested for their stem cell properties and expression of MSC specific surface markers to ensure the lentiviral genetic modification did not alter their stem cell properties and surface marker expression. CCR2 overexpressing mMSCs, control transduced mMSCs expressing DsRed and un-modified mMSCs were analysed for their characteristic stem cell surface marker expression. CCR2 overexpressing mMSCs, control transduced mMSCs expressing DsRed and un-modified mMSCs were found to be positive for the expression of CD90, CD105 and SCA-1, while negative for the expression of CD11b. CD34 and CD45. This confirmed that the genetic modification did not alter the stem cell surface marker expression on these cells. CCR2 overexpressing mMSCs, control transduced mMSCs expressing DsRed and un-modified mMSCs were tested for their capacity to differentiate into adipocytes and osteoblasts. All the cell types tested differentiated into adipocytes and osteoblasts, confirming the genetic modification did not alter their differentiation potential.

CCR2 overexpressing mMSCs, control transduced mMSCs expressing DsRed and un-modified mMSCs were tested for their ability to migrate to a gradient of MCP-1 in an *in vitro* chemotaxis experiment. It was concluded that the CCR2 overexpressing mMSCs migrated at higher numbers to MCP-1 in comparison with control transduced DsRed mMSCs and unmodified mMSCs. It was observed that the CCR2 overexpressing mMSCs migrated to MCP-1 in a dose dependent manner where, their migration was higher at lower concentrations of MCP-1 while increasing the concentration of MCP-1 did not increase their migration. This observation was consistent with the observations made by previous researchers in our group who concluded that the migration of mMSCs towards MCP-1 exhibited dose dependent inhibition of

migration, suggesting a positive feedback loop mechanism where lower concentrations of MCP-1 induced mMSC migration while increasing MCP-1 concentration did not increase cell migration.

In chapter 5, the CCR2 overexpressing mMSCs were tested for their therapeutic efficacy in a mouse model of hind limb ischemia. Ischemia was induced in C57BL/6 strain of mouse by ligating the femoral artery at two locations. Three days after induction of ischemia, the animals received intra muscular injections of $1x10^6$ cells. There were 3 groups of animals in this study, animals received injections of CCR2 overexpressing mMSCs, DsRed expressing control transduced mMSCs, and saline control. Blood flow to the distal limbs were measured using Laser Doppler blood flow analysis before the induction of ischemia, immediately after induction of ischemia, on day 3, on day 7 and on day 14. Animals that received the injection of CCR2 overexpressing mMSCs showed significant improvement in blood flow recovery to their distal limbs on day 7 and day 14 in comparison with animals that received injection of DsRed expressing mMSCs or saline. There was no necrosis or impairment in limb function noticed in these animals. Histological assessment of tissue sections from calf muscles of animals revealed increased immune cell infiltration into the ischemic tissue, with high number of immune cells infiltrating into the muscles of animals that received intra muscular injections of CCR2 overexpressing cells when compared to animals that received injections of DsRed expressing cells or saline. There was ischemia induced oedema, significant muscle loss and adipocyte infiltration in animals that received DsRed expressing cells and saline. Animals that received CCR2 overexpressing mMSCs showed improved skeletal muscle regeneration compared to other groups. Staining for endothelial cells revealed increased presence of endothelial cells in tissue sections from animals that received injection of CCR2 overexpressing cells when compared to animals that received DsRed expressing cells and saline. This concluded that the CCR2 overexpressing mMSCs were efficient in promoting recovery to ischemic limbs by remodelling skeletal muscles and promoted therapeutic angiogenesis by increasing the capillary density at the ischemic tissue thereby enhancing blood flow to the distal limbs.

To assess the bio-distribution / retention of transplanted cells, animals were intra muscularly injected with cells labelled with XenolightDiR, a near-infrared fluorescent dye 3 days after the induction of ischemia. Ischemic and non-ischemic limbs along with organs were collected at day 14 post induction of ischemia. IVIS imaging of limbs and organs revealed increased fluorescence in ischemic limbs compared to non-ischemic limbs indicating the retention of transplanted cells at the ischemic tissue. Animals that received injection of CCR2 overexpressing MSCs showed increased fluorescence in their ischemic limbs when compared to animals that received injections of DsRed cells. This is evidence of increased retention of CCR2 overexpressing cells at the ischemic sites when compared to DsRed cells. Analysis of heart, kidneys, liver, lungs and spleen revealed there was no significant fluorescence in any of these organs when compared to the ischemic limb. However, there was elevated levels of fluorescence in liver and lungs when compared to other organs. This was expected as liver and lungs are the target organs where transplanted cells often end up at.

In conclusion, genetic modification of MSCs to overexpress CCR2 resulted in a population of MSCs that overexpress CCR2 at the transcript and protein level. Lentiviral mediated genetic modification of MSCs enabled stable long term transgene expression without altering stem cell properties of MSCs. The genetically modified MSCs migrated efficiently at higher numbers towards a gradient of MCP-1 *in vitro* when compared to unmodified MSCs and MSCs transduced with a lentiviral vector carrying DsRed. Finally, this study shows genetically modified MSCs overexpressing a chemokine receptor can achieve therapeutic angiogenesis with improved retention of transplanted cells in a mouse model of hind limb ischemia.

6.1 References

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Chapter 7

Appendix

7 APPENDIX I:

7.1 Media and Buffers

Media

Standard complete medium for mouse MSC	
Iscove's modified DMEM	Sigma Aldrich
10% equine serum	Thermo Scientific
10% FBS	Hyclone™, Thermo Scientific
1% P/S	Gibco, Life Technologies

Standard complete medium for HEK293 cells	
D-MEM (high glucose)	Gibco, Life Technologies
2 mM L-glutamine	Gibco, Life Technologies
10% FBS	Hyclone™, Thermo Scientific
1% P/S	Gibco, Life Technologies

Osteogenic differentiation medium	
DMEM (Low Glucose)	Gibco, Life Technologies
100µM ascorbic acid 2-phosphate	Sigma
10mM β-glycerophosphate	Sigma
100nM Dexamethasone	Sigma
10% FBS	Hyclone™, Thermo Scientific
1% P/S	Gibco, Life Technologies
1% L-Glutamine	Gibco, Life Technologies
10% Equine Serum	Hyclone™, Thermo Scientific

Adipogenic differentiation media		
Induction media	DMEM (High Glucose)	Gibco, Life Technologies
	1µM Dexamethasone	Sigma
	10µg/ml Insulin	Sigma
	200µM Indomethacin	Sigma
	500µM 3-Isobutyl-1-Methyl-Xanthine	Sigma
	10% FBS	Hyclone™, Thermo Scientific
	1% P/S	Gibco, Life Technologies
Maintenance media	DMEM (High Glucose)	Gibco, Life Technologies
	10µg/ml Insulin	

	10% FBS	Hyclone™, Thermo Scientific
	1% P/S	Gibco, Life Technologies

Buffers

Lectin buffer (1X for making 1L)	
8766 mg NaCl	Sigma
11.1 mg $CaCl_2$	Sigma
1.26 mg $MnCl_2$	Sigma
2383 mg HEPES	Sigma

FACS buffer
2% FBS
1x PBS
0.05% NaN_3