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THE ROLE OF CC-CHEMOKINES IN ANGIOGENESIS

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A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy
INTRODUCTORY STATEMENT

The studies presented in this thesis are the results of original research carried out while the author was enrolled as a candidate for the degree of Doctor of Philosophy in the Sydney Medical School, University of Sydney. These studies were conducted between March 2012 and December 2015 at the Heart Research Institute, Sydney.

All experimental work carried out for this thesis is entirely my own original work except where stated otherwise in the text. The work presented in this thesis has not been submitted for a degree or a diploma in any other university.

Anisyah Ridiandries

December 2015
“The capacity to learn is a gift;
The ability to learn is a skill;
The willingness to learn is a choice.”
— Brian Herbert, House Harkonnen
Dear PhD (aka The Role of CC-chemokines in Angiogenesis),

I’m sad to say that our journey together has now come to an end. I can’t say it was an easy journey, but in the end (and with a bit of reflection), I can say it was definitely fun. There were times when I wanted to leave you, because you were stubborn and uncooperative, but the lure of the lab always pulled me back in. You have challenged me, pushed me, broken me, but in the end I always got back up and kept pushing on. I am determined to keep going, always. You pushed me to do things I didn’t know I was capable of doing and encouraged me to do the things I wanted to do, and to do them for me, for that I am thankful. You have made me a better, stronger, and wiser person.

These last four years with you have not always gone smoothly, but I’ve been lucky to have a good network of people around me to help me through. Firstly, of course, my supervisor, Dr Christina Bursill, without her guidance, encouragement and support I would not have been able to get through this. Her ability to see the bigger picture when I was too focused, and to see the details when my focus was lost, has helped me to make sense of you, PhD. Her input and advice was always fair and helped to shape the way I thought about my research and its presentation. I am truly grateful for all that she has done, and extremely lucky to have her as a supervisor and mentor.

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Outside of the lab and HRI there are 3 friends that mean the world to me, Sarah Arakelian, Julia Buca, and Desiree Lapan. First, Sarah and Julia, these ladies have been with me since forever. We each have our own busy lives, which meant that our catch ups where few and far between, but when we do meet it’s as if all that time was never lost. When we meet up whether for a whole day or just an hour, I regain focus and determination. Their friendship, even after all these years, reinvigorates me, I truly cherish them both. Desiree, my Dearest, even though she is now 4435 km away I know I can always count on her. She knows me better than I know myself. The honesty and truthfulness in our friendship is paramount, especially during our overanalyses of every situation (haha!). Despite being busy studying med, she always has time for me and I know that no matter where we both are in the world we will both be there for each other. I’m not sure I’ve sufficiently expressed the impact these 3 women have had on me, but let’s just say it’s a lot!

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Finally as I say goodbye to you, I also say goodbye and thank you to everyone at HRI. I have enjoyed all the laughter and cake filled times immensely.

It’s been a long and arduous journey, but at the same time it has been a wonderful experience to take this journey with you. Every action we take in life leads us to this specific point in time and place, this is exactly where you should and need to be.

Always remember, “while there is tea, there’s hope”.

Much love,

Anisyah Ridiandries
CONFERENCE ABSTRACTS ARISING FROM THIS THESIS


Ridiandries A, Bursill CA “Broad-spectrum chemokine inhibition blocks inflammation-induced pathological angiogenesis”, Australian Health and Medical Research Congress, Adelaide, Australia 25-28 November (POSTER)

MANUSCRIPTS ARISING FROM THIS THESIS


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ABSTRACT

Angiogenesis is a fundamental process in which new blood vessels are formed from pre-existing vessels. It is important in growth and development and plays a critical role in postnatal physiological processes such as wound healing and in tissue neovascularisation in response to ischaemia such as that following a myocardial infarction. Despite the importance of angiogenesis for survival, an imbalance in the regulation of angiogenesis causes inflammatory-driven pathological angiogenesis. This leads to the exacerbation of diseases such as cancer to increase tumour growth, or atherosclerosis where neovessels accelerate plaque growth causing instability and plaque rupture. Diseases associated with angiogenesis are highly prevalent globally, with cardiovascular-related disorders and cancer being the leading causes of mortality worldwide. Furthermore, with the increasing incidence of diabetes and the advancing aging populace, the impact of vascular complications associated with impaired angiogenesis are also on the rise, with peripheral vascular disease and impaired wound healing being key contributors to disease complications. Current anti-angiogenic therapies for the treatment of these diseases inhibit both pathological and physiological angiogenesis and cause side effects such as hypertension, bleeding, severe weight loss, diarrhoea and nausea in a large percentage of patients. Thus an alternate therapy which specifically targets the inflammatory-driven angiogenesis whilst preserving hypoxia-mediated angiogenesis may be beneficial to treat these pathologies whilst causing fewer side effects.

One potential target as an alternate therapy are chemokines, small (8 – 10 kDa) chemotactic cytokines that regulate the migration of cells to sites of inflammation or injury. There are four sub-classes: CC, CXC, CX3C, and XC, with CC-chemokines being the largest class with almost 30 ligands and 10 receptors. Increasing evidence suggests CC-chemokines play a key role in inflammatory-driven pathologies associated with angiogenesis. For example a host of CC-chemokines (CCL1, CCL2, CCL3, CCL5, CCL11, CCL13, CCL18, and CCL19) are found in human atherosclerotic plaques, and ApoE−/− mice have reduced plaques in CC-chemokine receptor and deletion studies. Additionally, CC-chemokines CCL2 and CCL5 are increased in breast and prostate cancer, and CCL19 and CCL20 have been shown to promote tumour metastasis. CC-chemokines are thought to promote angiogenesis through two pathways. The first, a direct pathway in which CC-chemokines attach to CC-chemokine receptors on the surface of endothelial cells, this stimulates the PI3K/Akt pathway and increase angiogenesis. For example, CCL2
stimulates the phosphorylation of cytoskeletal proteins, which are essential for endothelial cell migration and neovessel formation. Tubulogenesis *in vitro* is also increased following incubation with CCL2, CCL11 and CCL16. Secondly, CC-chemokines promote angiogenesis through an indirect pathway. CC-chemokines attach to glycosaminoglycans (GAGs) on the surface of endothelial cells. This causes a chemokine gradient to recruit inflammatory cells that release pro-angiogenic cytokines and growth factors such as VEGF to the inflamed site promoting angiogenesis. In contrast, CC-chemokines have no reported implications in hypoxia-mediated physiological angiogenesis. Thus targeting the CC-chemokine class may represent as an alternate therapy to inhibit inflammatory-driven angiogenesis whilst preserving hypoxia-mediated angiogenesis. Single CC-chemokine inhibition studies have previously been conducted in a mouse model of sponge-induced inflammatory angiogenesis, indicating inhibition of CCL3 or CCL5 reduced macrophage infiltration but did not affect angiogenesis. Furthermore, knock-out of CCL2 in mice did not affect monocyte recruitment. Redundancies in CC-chemokines signalling may explain the lack of change in these studies that have targeted only a single CC-chemokine/CC-chemokine receptor. Thus broad-spectrum CC-chemokine inhibition may be an alternate therapeutic approach.

A 35 kDa soluble protein from the Vaccinia virus called ‘35K’ uniquely inhibits the whole CC-chemokine class while having no effect on chemokines from other classes. It has high affinity binding to CC-chemokines preventing them from binding to their cognate receptors. It does this by binding to common structural sites found on every CC-chemokine, promoting broad-spectrum inhibition of the entire class. Previous studies with 35K highlight its ability to inhibit a number of disease pathologies associated with inflammation including acute peritonitis, hepatitis, liver fibrosis and vein graft accelerated atherosclerosis. Similarly, ApoE knock-out mice overexpressing 35K have reduced plaque area and macrophage infiltration in both long and short term studies. However, its role in the regulation of angiogenesis across all pathophysiological contexts is unknown.

The main objectives of the present studies were to determine and compare the role of CC-chemokines in both inflammatory-driven and ischaemia-mediated angiogenesis. To do this, *in vitro* studies were conducted to elucidate the effect of the broad-spectrum CC-chemokine inhibitor 35K on functional angiogenic assays and key angiogenic mediators. Furthermore, the effects of 35K on neovascularisation were explored in four *in vivo* animal models that represent disease pathologies where angiogenesis is important. This includes
the (1) peri-arterial femoral cuff model and (2) hindlimb ischaemia model that represent pathological and physiological angiogenesis respectively, (3) the Lewis lung tumour neovascularisation model and (4) the murine model of wound healing.

Our in vitro studies conducted with isolated 35K protein evaluated the differential effects of CC-chemokine inhibition in inflammation and hypoxia on human coronary artery endothelial cells (HCAECs). The key functional angiogenic assays of proliferation, migration, and tubule formation were used. These represent the steps of angiogenesis in which activated endothelial cells increase proliferation and migration to sites of injury, and then assemble into tubular structures to form new blood vessels. The global inhibition of CC-chemokines by 35K significantly attenuated endothelial cell proliferation, migration, and tubule formation in response to inflammation. However under hypoxia, 35K had minimal effects on proliferation, migration and tubule formation in vitro.

To elucidate the mechanistic action of 35K in vitro, the expression of two key pro-angiogenic markers HIF-1α and VEGF were determined. We show that 35K conditionally regulates VEGF expression, suppressing VEGF protein levels in inflammation but, in contrast, had no effect in hypoxia. Interestingly, we found that 35K suppressed HIF-1α protein levels in both inflammation and hypoxia, although this effect was less pronounced in hypoxia. Given that HIF-1α is post-translationally modulated by prolyl hydroxylases (PHDs) in hypoxia, we determined the effects of 35K on PHDs and found, however that 35K had no effect on PHD1-3 in hypoxia. This suggests that inhibition of HIF-1α in hypoxia by 35K is through an alternate pathway and not through the post-translational modulation of HIF-1α. Overall, the in vitro studies demonstrate that broad-spectrum CC-chemokine inhibition by 35K conditionally regulates in vitro angiogenesis, such that inflammatory-driven angiogenesis is suppressed while minimal to no effects are found on hypoxia-mediated angiogenesis.

To determine if the differential effects of 35K on angiogenesis also occurred in vivo, the peri-arterial cuff model of inflammatory adventitial angiogenesis and the hind limb ischemia models were used. An adenoviral approach was used to systemically overexpress 35K protein. The adenovirus overexpressing 35K (Ad35K) was infused via the tail vein three days prior to surgeries. Adenovirus overexpressing GFP (AdGFP) and PBS were also injected three days prior to surgery as adenoviral and vehicle controls. In the peri-arterial femoral cuff model, mice injected with Ad35K had significantly less adventitial neovessels
and arterioles. Whilst there was a trend for a reduction in adventitial macrophages and CD68 mRNA levels, 35K did not have a significant inhibitory effect on macrophage recruitment, suggesting that the inhibitory effect of 35K on neovascularisation in inflammation is via 35K directly suppressing endothelial cell angiogenesis, rather than the indirect effect via the initial recruitment of macrophages. In contrast, in the hindlimb ischaemia model, blood flow perfusion and capillary density were not affected by CC-chemokine inhibition, indicating that ischaemia-induced angiogenesis was preserved. Furthermore, gene analysis showed conditional regulation of VEGF by 35K in vivo in the two models.

As in previous studies of CC-chemokine inhibition using 35K, chemokine activity was suppressed in the 35K treated mice for both models. Despite the inhibition of chemokine activity, CCL2 and CCL5 plasma levels were increased. Interestingly, we found that 35K induced significant reductions in CCL2 and CCL5 in the femoral artery and hindlimb tissues. Taken together, our CC-chemokine expression data suggests that 35K is binding to CC-chemokines in the tissues of the mice and then sequestering them into the circulation. Alternatively, circulating CC-chemokine levels increase to compensate for the reduced activity, however 35K continues to bind to new CC-chemokines preventing increases in activity leading to increases in circulating (although inactive) CC-chemokines.

The Lewis lung tumour neovascularisation model was used to observe the effect of CC-chemokine inhibition in a system where both inflammation and hypoxia both play a driving role in the exacerbation of tumour formation. As with the previous in vivo study, the mice were injected with Ad35K or controls 3 days prior to injection of Lewis Lung Carcinoma (LLC) cells. Twelve days after LLC injection at the end of the study the tumour was excised to study the effect of CC-chemokine inhibition on tumour angiogenesis and progression. The study did not show any significant changes to tumour growth, or tumour neovascularisation, however we observed slight trends to reduction in tumour size and arterioles formed in the tumours of Ad35K injected mice compared to controls. Furthermore, despite no changes to the expression of CCL2, inflammatory marker (p65), and trending increases in proliferation (Ki-67, c-Jun) and cell invasion markers (MMP2, MMP9), we observed a promising trend of increased expression of key apoptosis markers (p53, caspase 3, caspase 8, and caspase 9). Although these changes were not significant they are promising. Further studies will need to be conducted in a less aggressive tumour
model or with alternate delivery strategies of 35K to determine the effects of broad-spectrum CC-chemokine inhibition in the context of cancer.

Wound healing is a multi-step process requiring strict coordination between inflammation and cell growth in the wound. Angiogenesis plays a critical role in the early stages of wound healing, recruiting inflammatory cells to the wound site and providing a microvascular network to maintain new tissue formation. However, increased inflammation leads to prolonged wound healing and excessive scar formation, with impaired wound healing often resulting in unfavourable outcomes such as amputation. CC-chemokines play an important role in inflammation and angiogenesis, two key wound healing processes. In our final animal model, we sought to examine the effect of broad-spectrum CC-chemokine inhibition in a murine model that mimics the human process of wound healing. Topical application of 35K protein enhanced blood flow recovery and wound closure at the early-mid stages of wound healing. 35K promoted neovascularisation at the critical early stages of wound healing, where angiogenesis is important. 35K-treated wounds also had significantly lower expression of the active p65 subunit of NF-κB, the key transcription factor involved in inflammation. Finally, 35K-treated wounds had reduced collagen content indicative of less scar formation. These findings show that broad-spectrum CC-chemokine inhibition may be beneficial in chronic wounds where inflammation is in excess, in order to promote wound healing and neovascularisation and reduce scar formation.

In summary, the studies presented in this thesis demonstrate for the first time that broad-spectrum CC-chemokine inhibition by 35K attenuates inflammatory-driven pathological angiogenesis whilst preserving ischaemia-mediated neovascularisation in vitro and in vivo. Mechanistically, we found that the key pro-angiogenic growth factor VEGF was conditionally regulated by 35K such that VEGF protein levels were suppressed in inflammatory conditions, but were unchanged in response to hypoxia. While the results were inconclusive in our tumour neovascularisation model, we observed a trend for a reduction in tumour weight, suggesting that further exploration is required. Finally, we found that 35K increased wound neovascularisation and wound closure, and reduced collagen formation in dermal wounds. Overall, 35K may present as an alternate therapy to specifically inhibit inflammatory-driven pathological diseases in which angiogenesis plays a key role and have distinct advantages over current anti-angiogenic therapies that inhibit angiogenesis in all pathophysiological contexts.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>Ad35K</td>
<td>Adenovirus overexpressing 35K</td>
</tr>
<tr>
<td>AdGFP</td>
<td>Adenovirus overexpressing GFP</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BCP</td>
<td>1-Bromo-3-chloropropane</td>
</tr>
<tr>
<td>bFGF/FGF-2</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CsCl</td>
<td>Caesium Chloride</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPX</td>
<td>dibutyl-phthalate in xylene</td>
</tr>
<tr>
<td>EBM-2</td>
<td>Endothelial Basal Medium -2</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescence Isothiocyanate</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HCAEC</td>
<td>Human Coronary Artery Endothelial Cells</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia Inducible Factor 1 Alpha</td>
</tr>
<tr>
<td>HMDM</td>
<td>Human Monocyte Derived Macrophages</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxic Response Element</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>HT-PBS</td>
<td>Heat Treated PBS</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
</tbody>
</table>
KCl  Potassium Chloride
LDS  Lithium Dodecyl Sulfate/decyl Sulfate
LPS  Lipopolysaccharide
MCM/CM  Macrophage Conditioned Media
MCP-1  Monocyte Chemoattractant Protein 1
MgCl  Magnesium Chloride
MMP  Matrix Metalloproteinase
Na₄P₂O₇  Sodium Pyrophosphate Tetrabasic
NaCl  Sodium Chloride
NaF  Sodium Fluoride
NF-κB  Nuclear Factor Kappa Beta
NO  Nitric Oxide
NOS  Nitric Oxide Synthase
OCT  Optimal Cutting Temperature
PBS  Phosphate Buffered Saline
PBST  Phosphate Buffered Saline and Tween 20
PCR  Polymerase Chain Reaction
PDGF  Platelet Derived Growth Factor
PEG  Polyethylene Glycol
PHD  Prolyl Hydroxylase
PI3K  Phosphoinositide 3-kinase
ps  pshuttle
RA  Rheumatoid Arthritis
RNA  Ribonucleic Acid
RPMI  Roswell Park Memorial Institute Medium
SDF-1α  Stromal Cell-Derived Factor 1 Alpha
SEM  Standard Error Mean
Siah  Seven In Absentia Homolog
TBST  Tris-Buffered Saline and Tween 20
TIMP  Tissue inhibitors of MMP
TNF-α  Tumour Necrosis Factor Alpha
tris-HCl  Tris(hydroxymethyl)aminomethane Hydrochloride
VCAM-1  Vascular Cell Adhesion Protein 1
vCK  Viral Chemokine
vCKR  Viral Chemokine Receptor
VEGF  Vascular Endothelial Growth Factor
VEGFR  Vascular Endothelial Growth Factor Receptor
vp  Virus Particles
VSB  Virus Storage Buffer
CHAPTER 1. INTRODUCTION
1.1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels. It is an essential process in development, growth and repair. Under postnatal physiological conditions, angiogenesis is important in tissue neovascularisation in response to ischaemia (such as that following a myocardial infarction and in peripheral vascular disease) and wound healing. However, uncontrolled regulation of angiogenesis can lead to the exacerbation of inflammatory-driven angiogenesis associated diseases such as atherosclerosis and cancer. In these conditions the effects of angiogenesis become pathological, where new blood vessels supply the diseased tissue with growth factors and cytokines promoting growth and accelerating the progression of disease states.

Angiogenesis associated diseases are highly prevalent globally, with cardiovascular-related disorders and cancer being the leading causes of mortality worldwide. Current anti-angiogenic therapies suppress angiogenesis in all pathophysiological conditions and while many are successful in suppressing tumour progression, these are often accompanied by severe side effects, thus highlighting the need for novel therapeutic targets that can inhibit inflammatory-driven angiogenesis while having no effect on ischaemia-mediated angiogenesis.

Key regulators of angiogenesis are small inflammatory proteins known as chemokines. They are divided into four classes: CC-chemokines, CXC-chemokines, CX3C-chemokines and XC-chemokines. Emerging evidence suggests that the CC-chemokine class promote inflammatory-driven pathological angiogenesis, however there is little reported evidence for their role in ischaemia-mediated physiological angiogenesis. Therefore, we propose that broad-spectrum inhibition of the CC-chemokine class may specifically suppress pathological inflammatory-driven angiogenesis, whilst preserving important physiological ischaemia-mediated angiogenic processes. This approach may have advantages over current anti-angiogenic therapies.
1.2. Angiogenesis in pathophysiological states

The regulation of angiogenesis in various pathophysiological contexts involves a number of critical growth factors, cytokines, signalling cascades and cellular processes that are triggered in response to either an inflammatory or ischaemic response and is regulated via two distinctly different yet overlapping pathways (Carmeliet 2000). The pathological angiogenesis pathway seen in tumour and atherosclerotic plaque progression is driven by inflammation. Conversely, the physiological angiogenesis required in response to tissue ischaemia or wound healing is mediated by ischaemia/hypoxia.

1.2.1. Inflammatory-driven pathological angiogenesis

Inflammatory-driven pathological angiogenesis accelerates a number of disease processes, in particular atherosclerosis and cancer (Carmeliet 2000; Moulton et al. 1999; Moulton et al. 2003). Pathological angiogenesis is induced at sites of vascular inflammation that have increased recruitment of monocytes/macrophages. Neovessel formation is stimulated by macrophages through the release of pro-angiogenic factors, including VEGF, bFGF, TNF-α, IFN-γ, that then stimulate excessive neovessel formation (Mantovani et al. 2008). For example, plaque neovessels allow the increased transportation of cells, cytokines and growth factors to the plaque which exacerbate the disease process (Carmeliet & Jain 2000b; Moulton et al. 1999; Moulton et al. 2003). This makes plaque neovessels particularly undesirable. Furthermore, they are also thin walled and prone to haemorrhage making the plaque unstable and prone to rupture (Moreno et al. 2004). Elevated levels of inflammation also have direct effects on endothelial cells and will stimulate neovascularisation. Under inflammatory conditions vessels form dense and highly disorganised networks (Nagy et al. 2009). These new vessels develop from existing vessels in which endothelial cells are recruited from sites adjacent to the injury (Chung & Ferrara 2011). These recruited endothelial cells accelerate angiogenesis either by forming part of the new vessel or by stimulating vessel growth. For example in tumours, fibroblasts aid in the remodelling of the extracellular matrix (ECM) to make way for new vessels and have also been shown to have pro-angiogenic properties (Bhowmick, Neilson & Moses 2004; Kalluri & Zeisberg 2006). Due to the imbalance between the pro- and anti-angiogenic switch, pathological angiogenesis persists and exacerbates the disease,
preventing its resolution. Inflammatory-driven angiogenesis is implicated in several inflammatory diseases such as atherosclerosis, cancer and rheumatoid arthritis.

1.2.2. Ischaemia-mediated physiological angiogenesis

Vascular supply of oxygen is critical to human growth and development. During embryonic growth the cardiovascular system is established early, reflecting on the importance of oxygen supply via the vasculature (Carmeliet 2003). As an adult, postnatal physiological angiogenesis is critical for growth and repair, wound healing, menstrual cycle, and pregnancy to supply oxygen and nutrients to the embryo. During angiogenesis, early vessels are formed by endothelial cells, however as blood flow is established remodelling may occur and involve additional cell types such as endothelial progenitor cells (EPCs) derived from the bone marrow, or smooth muscle cells from surrounding tissue. These vessels form a smooth muscle layer to cope/respond to increased blood flow in the area (Chung & Ferrara 2011). Physiological angiogenesis is primarily triggered when there is a chronic imbalance in tissue oxygen supply versus demand following injury or vessel occlusions, which restrict the supply of blood. The ensuing hypoxic environment induces neovascularisation of the ischaemic tissue. In athero-occlusive diseases this response is crucial for the formation of a coronary collateral circulation (Ware & Simons 1997), which is a critical factor for survival following a myocardial infarction (Sabia et al. 1992) and is associated with improved prognosis in the context of stable chronic coronary disease (Billinger et al. 2002). When sufficient vasculature is achieved, angiogenesis resolves and a balance between pro- and anti-angiogenic factors is re-established. Physiological angiogenesis is therefore an essential regenerative process, which is important in ischaemia-related cardiovascular disease. Additionally, peripheral vascular disease is on the rise due to decreased activity and increased consumption of fatty foods. Peripheral vascular disease is the narrowing of the vessel wall in arteries outside of the cardiac system. It is largely due to atherosclerosis, but can also occur as occlusions in the vein, by thrombosis or insufficient blood flow to the peripheral limbs. This has significant health impact on sufferers of this disease, often resulting in lower limb amputation and has great economic impact.
1.2.3. **Angiogenesis is critical for tumour growth and development**

Tumour growth depends implicitly on neovascularisation, as tumour cells become distant from nearby vessels (Carmeliet 2000). Furthermore, tumour neovessels accelerate tumour growth by allowing the passage of inflammatory cells that secrete pro-angiogenic factors (Mantovani et al. 2008). Rapid tumour expansion, through neovascularisation, increases the likelihood of metastasis as tumours have poorly developed lymphatics so secreted tumour cells are shunted to the peripheral lymph nodes (Carmeliet & Jain 2000b). Hypoxia is a common characteristic of locally advanced solid tumours and has been linked with a diminished therapeutic response and malignant progression (Vaupel 2004). As the tumours grow, it rapidly outgrows the available blood supply, resulting in portions of the tumour with hypoxic regions. Cancer cells in these hypoxic microenvironments in solid tumours have been reported to alter their metabolism in order to maintain continuous growth and proliferation.

1.2.4. **Angiogenesis is critical for postnatal wound healing**

Wound healing is a complex multistep process comprised of 3 overlapping but distinct phases: (1) inflammation, (2) proliferation, (3) remodelling as outlined in Figure 1.1. The inflammation phase occurs early (24 – 48 hours) after wound injury. A fibrin clot forms to prevent blood loss, and debris removal occurs through the recruitment of neutrophils and macrophages to the wound site. Macrophages appear in the later part of this stage to transition to the second wound healing phase, proliferation. Proliferation occurs between 2 – 10 days after wound injury. It is characterised by re-epithelialisation to bring the wound to a close. This is aided by angiogenesis which helps recruit macrophages to stimulate healing, and EPCs, keratinocytes, and fibroblasts to form new tissue at the wound site. During this proliferation stage granulation tissue begins scar formation. Remodelling is the final step of wound healing and can last for up to one year. In this phase, inflammation and angiogenesis ceases and new blood vessels disintegrate allowing the fibroblasts to reorganise the collagen matrix, forming a closed wound (Gurtner et al. 2008). Angiogenesis is important during both the inflammation and proliferation phases. Primarily angiogenesis initiates the formation of angiogenic capillary sprouts that allow for recruitment of inflammatory cells to the wound for debris removal. Additionally angiogenesis allows for the invasion of the fibrin/fibronectin-rich wound clot, and
reorganisation of a microvascular network to maintain the newly formed granulation tissue (Tonnesen, Feng & Clark 2000). Wounds that exhibit impaired healing frequently enter a state of pathological inflammation, with most chronic wounds developing into ulcers and the incidence of these are associated with ischaemia, diabetes mellitus, venous stasis disease or pressure (Guo & DiPietro 2010). Chronic, non-healing wounds greatly impact a person’s quality of life and when left untreated sometimes lead to amputation resulting in an enormous health care burden (Mathieu, Linke & Wattel 2006; Menke et al. 2007).

Figure 1.1. The three main stages of wound healing.

Wound healing is composed of three main stages: inflammation (early), proliferation (mid) and remodelling (late). The main objective of the inflammation stage is clot formation to prevent bleeding and removal of debris to prevent infection in the wound. This stage is dominated by neutrophils and angiogenesis begins to occur. The proliferation stage is directed by macrophages which maintain angiogenesis and aid in the re-epithelialisation of the wound area. The last stage, remodelling, is characterised by the formation of granulation tissue, lack of inflammation, and angiogenesis ceases at this stage.
1.3. Intracellular mechanisms for the regulation of angiogenesis in different pathophysiological conditions

Endothelial cells (ECs) are one of the major cell types involved in angiogenesis. As ECs are mainly quiescent, angiogenesis needs to be triggered by a stimulating cytokine, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). These cytokines bind to their cognate receptors on ECs present in pre-existing blood vessels (Carmeliet 2000; Karamysheva 2008). Once activated ECs then release extracellular proteinases such as matrix metalloproteinases (MMPs) that degrade the basement membrane of the existing capillary wall, forming a branch point. Surrounding ECs then proliferate into the adjacent matrix and form solid sprouts connecting neighbouring vessels (Folkman & Shing 1992). As sprouts extend toward the source of the angiogenic stimulus, VEGF facilitates endothelial cell detachment from the parent vessel and directs migration into the neighbouring stroma, using adhesion molecules called integrins. These sprouts then reorganise and form loops to become a full-fledged vessel lumen as cells migrate to the site of angiogenesis. Finally, the endothelial cells secrete platelet derived growth factor (PDGF), which recruits pericytes to the site in order to stabilise the newly formed tubules that are interconnected, forming a network.

Angiogenesis is a multi-step process that occurs in several well defined stages and involves a complex orchestration of growth factors, cytokines, signalling cascades and cellular events. Depending on the pathophysiological context, angiogenesis is driven by distinctly different, although partially overlapping, pathways. The pivotal transcription factor for inflammatory-driven angiogenesis is nuclear factor-κB (NF-κB) while ischaemia-mediated angiogenesis is primarily modulated by the main hypoxic transcription factor, hypoxia-inducible factor-1α (HIF-1α).
1.3.1. Inflammatory-driven angiogenesis is modulated by NF-κB signalling pathway

Prolonged chronic inflammation has been implicated in the pathogenesis of atherosclerosis and cancer. The transcription factor NF-κB is a pivotal promoter of pathological inflammatory-driven neovascularisation (Ng et al. 2007; Ware & Simons 1997). While there are two established NF-κB signalling pathways, namely the (a) canonical or classical (mediated by IκB degradation) and (b) non-canonical or alternative (p100 mediated) (Pomerantz & Baltimore 2002), inflammatory-driven angiogenesis is primarily regulated by the canonical pathway.

A brief schematic diagram of the canonical/classical NF-κB pathway is shown in Figure 1.2. Under normal conditions, the NF-κB complex (p50/p65) is sequestered within the cytosol by the inhibitor of κB (IκB) proteins, specifically IκBα. The canonical pathway is activated by inflammatory stimuli, such as tumour necrosis factor-α (TNF-α), interleukins or lipopolysaccharides, triggering the activation of the IκB kinase (IKK) enzyme complex, resulting in IκBα phosphorylation (Mercurio et al. 1997). Upon phosphorylation, the IκB complex is targeted for ubiquitination and subsequent proteasomal degradation. This releases the NF-κB complex, allowing it to translocate into the nucleus where it binds to the NF-κB response element to initiate the transcription of pro-angiogenic mediators including VEGF, MMPs and cell adhesion molecules (CAMs) (Chen et al. 1995; Chen 2005; Tak & Firestein 2001).
Figure 1.2. Canonical/Classical NF-κB signalling pathway (adapted from Viennois et al., 2013).

Pro-inflammatory stimuli such as TNF-α, IFN-γ, IL-8 or LPS triggers the phosphorylation of IκBα by the IKK complex, causing ubiquitination and degradation of IκBα. The p50/p65 NF-κB complex is released and translocates into the nucleus, binding to the NF-κB response element to activate the production of NF-κB target genes to further drive the inflammatory response. These targets also include pro-angiogenic mediators such as VEGF and MMPs.
Inflammatory-driven pathological angiogenesis may be triggered by direct or indirect stimulation. Direct stimulation involves an increase in inflammatory cytokines and growth factors which in turn regulate endothelial cell migration and proliferation leading to angiogenesis. Conversely, indirect stimulation requires the initial recruitment of macrophages to the inflamed site. Macrophages are potent promoters of pathological angiogenesis as they secrete a host of growth factors and cytokines (VEGF, bFGF, PDGF, TNF-α and IFN-γ) known to promote angiogenesis (Jackson et al. 1997; Sunderkotter et al. 1994), which subsequently accelerate plaque growth and increase plaque instability/rupture (Moulton et al. 1999; Moulton et al. 2003). Key inflammatory mediators including TNF-α and chemokines such as monocyte chemoattractant protein-1 (MCP-1) have all been shown to directly induce angiogenesis in vitro and in vivo (Charo & Ransohoff 2006; Galvez et al. 2005; Jackson et al. 1997; Salcedo et al. 2001; Strasly et al. 2004; Werts et al. 2007; Weyrich et al. 1995).

Atherosclerosis is a chronic inflammatory disease associated with the recruitment of circulating leukocytes into the vascular wall. Atherosclerosis develops over several decades and encompasses a host of cellular processes involved at various stages of disease from the initiation of plaque formation to plaque rupture, with NF-κB shown to be the key transcription factor involved throughout the entire disease progression. The initial stage of atherosclerosis involves damage to the endothelium and the activation of endothelial cells via the NF-κB signalling pathway. This results in the expression of various chemokines and adhesion molecules such as CCL2, CCL5, MIF, and CX₃Cl1 (Liu & Jiang 2011; Morand, Leech & Bernhagen 2006; Zernecke & Weber 2010) that promote the recruitment of leukocytes to the vascular intima. Intimal neovascularisation is triggered by pro-angiogenic mediators including VEGF and TNF-α. The newly formed capillaries often localise in areas rich with cell types that can activate angiogenesis, such as monocytes and macrophages (Polverini et al. 1977). Pro-angiogenic factors expressed by monocytes promote the proliferation of endothelial cells, and mobilisation and homing of bone marrow-derived endothelial progenitor cells (EPCs) (Lambert, Lopez & Lindsey 2008). In addition, activated macrophages generate pro-angiogenic factors including VEGF and FGF-2, further augmenting intimal neovascularisation (Murdoch et al. 2007), with NF-κB being the critical promoter of angiogenesis which induces VEGF expression (Morais et al. 2009). Due to the close proximity of plaque neovessels to inflammatory infiltrates and elevated endothelial expression of cell adhesion molecules such as vascular cell adhesion
molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1) and E-selectin, there is additional recruitment of inflammatory cells to the lesion site (O'Brien et al. 1996). This creates a positive and deleterious feed-forward loop whereby inflammatory cells stimulate angiogenesis in the plaque and the newly formed vessels promoting further inflammatory cell recruitment.

1.3.2. Ischaemia-mediated angiogenesis is driven by HIF-1α signalling pathway

Physiological angiogenesis is stimulated in response to ischaemia, and is primarily driven by the transcription factor HIF-1α which in turn upregulates a range of pro-angiogenic factors in particular VEGF with a 30 fold increase (Carmeliet 2003). Other hypoxia-responsive genes are also upregulated including nitric oxide synthase (NOS) and CXCL12/CXCR4 that promote angiogenesis by increasing EPC migration and mobilisation, and promoting cell differentiation (Cooke & Losordo 2002; Liekens, Schols & Hatse 2010; Zheng et al. 2007). HIF-1α is essential in physiological angiogenesis where inactivation of HIF-1α in mice results in abnormal vascular development and embryonic lethality (Iyer et al. 1998; Kotch et al. 1999; Licht et al. 2006; Ryan, Lo & Johnson 1998).

The hypoxia-inducible factors (HIFs) are heterodimeric nuclear transcription factors comprised of two subunits that regulate transcription of genes mediating cellular homeostatic responses to changes in cellular oxygen levels. The HIF subunits (HIF-1α and HIF-2α) is oxygen-regulated, whereas HIF-β is constitutively expressed in the nucleus (Semenza 2012). The stability and transcriptional activity of HIF-1α is regulated post-translationally within the cytosol by the prolyl hydroxylase domain protein family (PHD1-3), which require molecular oxygen as a co-substrate (Appelhoff et al. 2004; Bruick 2003; Huang & Bunn 2003; Nakayama et al. 2004; Nakayama, Qi & Ronai 2009; Semenza 2004).

A brief schematic diagram of the post-translational modulation of HIF-1α is shown in Figure 1.3. Under aerobic conditions, the PHDs hydroxylate proline residues, causing ubiquitination and proteasomal degradation of HIF-1α subunits (Bruick & McKnight 2001; Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001). Conversely, a decrease in intracellular [O₂] triggers the activation of the PI3K/Akt signalling pathway resulting in the induction of gene transcription of the ubiquitin ligases Siah1 and Siah2.
Siahs promote the degradation of PHD enzymes, protecting HIF-1α from degradation and leading to HIF-1α stabilisation and accumulation (Nakayama et al. 2004). This in turn allows HIF-1α to translocate into the nucleus, where it complexes with the HIF-1β subunit, facilitating its binding to the hypoxia response element (HRE), and driving the expression of angiogenic mediators including VEGF, angiopoietin, bFGF, MMPs and tissue inhibitors of MMPs (TIMPs). In turn, these angiogenic factors drive angiogenic cellular processes including endothelial cell proliferation and migration, smooth muscle cell recruitment, extracellular matrix degradation and stabilisation and maturation of the vessel walls.

Figure 1.3. HIF-1α signalling pathway in normoxia and hypoxia (adapted from Maes et al., 2012).

Under normoxia, PHD1-3 hydroxylates HIF-1α allowing von Hippel-Lindau proteins to bind. This causes the ubiquitination and degradation of HIF-1α. In hypoxia, low oxygen levels stimulate the increase of Siahs. Siahs inhibit PHDs, preventing HIF-1α from ubiquitination and degradation, allowing it to translocate into the nucleus. In the nucleus HIF-1α complexes with HIF-1β and Cbp to bind to the hypoxia response element (HRE) driving angiogenic target genes such as VEGF.
While the role of VEGF is critical in driving ischaemia-mediated angiogenesis, HIF-1α also regulates angiogenesis at every step of the process through multiple signalling pathways and cellular processes. Firstly, activated migrating ECs degrade the extracellular matrix via the upregulation of MMPs including MMP2, which is a direct target of HIF-1α and mediates EC migration in response to hypoxia (Ben-Yosef et al. 2005). Hypoxia also regulates vessel branching via the modulation of Notch signalling as HIF-1α directly binds to the Notch intracellular domain to augment its transcriptional activity, driving vascular branching and morphogenesis (Gustafsson et al. 2005). Endothelial cells then begin to form tubes in order to extend the existing vascular network and hypoxic stimulation has been shown to enhance endothelial tubule formation in vitro, with these effects being dependent on HIF-1α levels (Tang et al. 2004).

Another critical regulator of ischaemia-mediated angiogenesis is endothelial nitric oxide synthase (eNOS). In response to hypoxia eNOS stimulates nitric oxide (NO) production. NO controls growth factors such as VEGF, angiopoietins, fibroblast growth factors and genes involved in matrix metabolism, including MMPs (Munk et al. 2006). Many functional processes of angiogenesis such as cell migration, proliferation and tubulogenesis can also be induced by hypoxia (Krishnamachary et al. 2003; Phillips, Birnby & Narendran 1995). Bone marrow-derived EPCs also make significant contributions to ischaemia-mediated neovascularisation by paracrine release of angiogenic factors or by direct participation in neovessel formation (Sieveking et al. 2008). Taken together, these studies highlight the importance of HIF-1α as a pivotal regulator of ischaemia-mediated angiogenesis, making it a target for therapeutic regulation.
1.3.3. Tumour neovascularisation is driven by both inflammation and hypoxia

Tumour neovascularisation is critical for tumour growth as the formation of tumour neovessels facilitate the recruitment of inflammatory cells that will express pro-angiogenic mediators. This promotes the positive angiogenic feed-forward loop, similar to that of plaque neovessels. Tumour angiogenesis is regarded as a negative prognostic variable for several malignancies including breast and prostate cancer (Cavallaro & Christofori 2000; van Hinsbergh, Collen & Koolwijk 1999; Zachary 1998). It is well established that many different types of human tumours have uncontrolled regulation of NF-κB, in which NF-κB is constitutively active. The link between NF-κB and tumour angiogenesis was highlighted in an in vivo study where inhibition of the NF-κB signalling pathway suppressed the expression of VEGF in cultured human ovarian cancer cells implanted into an orthotropic nude mouse model (Huang et al. 2000). Furthermore, the suppression of VEGF directly correlated with decreased tumorigenicity, reduced neovascularisation of lesions and prolonged survival (Huang et al. 2000). The link between NF-κB, VEGF and angiogenesis was also confirmed in an in vitro study utilising high and low metastatic adenoid cystic carcinoma cell lines (Zhang & Peng 2007).

Furthermore, as tumours expand to become solid tumours, the microenvironment becomes progressively hypoxic, forcing cancer cells to adapt to the environment in order to survive. While hypoxic effects can negatively impact tumour cell growth (Vaupel & Harrison 2004), they may, conversely, lead to hypoxia-mediated responses that enhance malignant progression and aggressiveness, ultimately resulting in increased resistance to therapy and poor long-term prognosis. This is induced by a series of hypoxia-mediated proteomic and genomic changes that facilitate cell survival (Vaupel 2004). In this instance, HIF-1α activation is detrimental as it results in the induction of downstream targets that facilitate key cellular processes that promote tumour growth such as angiogenesis (VEGF and inducible nitric oxide synthase, iNOS), cell proliferation and survival (EGF, IGF-2 and TGF-β) and metabolic adaptation (glucose transporters including glucose transporter-1, GLUT-1 and glycolytic enzymes) (Vaupel 2004).
1.3.4. Wound neovascularisation

Angiogenesis plays a critical role in the early stages of wound healing, particularly during the inflammation and proliferation phases. With this in mind, both the NF-κB and HIF-1α signalling pathways play a role in mediating wound neovascularisation. Following wounding, the wound microenvironment undergoes a range of changes, triggering the activation of cellular responses. The NF-κB signalling pathway is triggered to promote the recruitment of macrophages to the site of injury in order to minimise infection. However, continued chronic inflammatory state in the wounds in turn impairs wound healing, often resulting in delayed wound remodelling. This is a common complication in the increasing population of diabetic patients as well as a frequent occurrence in an aging populace. Additionally, following acute injury, the microenvironment of the skin wound can become hypoxic due to vascular disruption and high oxygen consumption by cells at the wound edge (Hunt, Niinikoski & Zederfeldt 1972; Niinikoski, Grislis & Hunt 1972; Varghese et al. 1986). The importance of HIF-1α in improving wound healing is highlighted in a study on aged mice where impaired wound healing was concomitant to decreased HIF-1α levels (Loh et al. 2009). This acute hypoxic environment has a positive role in early skin wound healing, triggering the expression of HIF-1α (Albina et al. 2001; Elson et al. 2001) and as neovascularisation occurs and the wound recovers, the wound microenvironment returns to normoxic levels (Tandara & Mustoe 2004). Key angiogenic mediators including VEGF, bFGF and MMPs have also been shown to be important in mediating the wound healing process (Eming, Krieg & Davidson 2007; Gurtner et al. 2008; Singer & Clark 1999). After wounding bFGF is released from macrophages, whilst VEGF is released from epidermal cells. The MMPs degrade extracellular matrix proteins allowing recruitment of activated monocytes to the injury site, to subsequently release pro-angiogenic factors (Singer & Clark 1999).
1.3.5. Vascular endothelial growth factor – a crucial angiogenic target

The VEGF family of genes comprises of seven members: VEGF-A, -B, -C, -D, -E, -F and placental growth factor (PlGF) (Ferrara 2004; Ferrara, Gerber & LeCouter 2003). All VEGF family members stimulate cellular responses by binding to tyrosine kinase receptors (termed VEGFRs: VEGFR1, VEGFR2 and VEGFR3) on the cell surface, causing them to dimerise and become activated through transphosphorylation (Shibuya & Claesson-Welsh 2006). While the VEGF receptors are structurally highly related, each displays interesting differences with regard to their abilities to respond to ligand binding and the spectrum of transduced biological responses (Shibuya & Claesson-Welsh 2006). VEGFR1 and VEGFR2 are expressed on the cell surface of most blood ECs while VEGFR3 is restricted to lymphatic ECs. VEGFR2 is the earliest marker for endothelial cell development (Kabrun et al. 1997) and is considered a critical signal transducer in angiogenesis (Shibuya & Claesson-Welsh 2006).

Activation of VEGFR2 stimulates vascular endothelial cell survival, growth and migration resulting in the augmentation of angiogenesis. VEGF-A (also known as VEGF) is the key growth factor involved in physiological and pathological angiogenesis and while it is shown to bind to both VEGFR1 and VEGFR2, its interaction with VEGFR2 has been reported to directly regulate tumour angiogenesis. This has therefore made VEGFR2 the focus of extensive research and the VEGF/VEGFR2 interaction the best characterised signalling pathway in angiogenesis (Ferrara, Gerber & LeCouter 2003; Shibuya 2011). In ischaemia-mediated angiogenesis, hypoxia is the principal regulator of VEGF expression, as it is a direct transcriptional target of both HIF-1α and HIF-2α (Compernolle et al. 2002; Forsythe et al. 1996; Liu et al. 1995). Conversely in inflammatory-driven angiogenesis, VEGF expression is predominantly modulated via NF-κB, and has a well-established role in tumour and plaque angiogenesis (Celletti et al. 2001; Ferrara 2004; Ferrara, Gerber & LeCouter 2003). This is possible as VEGF has both HRE and NF-κB response elements in its promoter region as illustrated in Figure 1.4. Additionally, VEGF has 2 response elements of NF-κB suggesting that it is more affected by inflammation than hypoxia.
Figure 1.4. VEGF has response elements in its promoter region for NF-κB and HIF-1α (adapted from Ramanathan et al., 2007).

Under inflammation, the NF-κB complex (p50/p65) enters the nucleus and binds to the response elements in the promoter region of the VEGF. Under hypoxia, the HIF complex (HIF-1α./HIF-1β/Cbp) enters the nucleus and binds to the HRE in the promoter region of VEGF.
1.4. Current anti-angiogenic therapies

The development of anti-angiogenic agents are of great interest due to the high prevalence of diseases associated with angiogenesis. However, current anti-angiogenic agents target both pathological and physiological angiogenesis. This often results in several side effects such as vomiting, nausea, gastrointestinal perforations, and reduced wound healing. These side effects can be quite severe, thus an agent able to specifically target inflammatory-driven angiogenesis whilst preserving hypoxia-mediated angiogenesis is needed. Anti-angiogenic agents such as VEGF inhibitors or VEGF receptor inhibitors are found to reduce plaque size and tumour growth in a variety of malignancies including breast, lung and prostate (Borgstrom et al. 1998; Chan 2009; Di Costanzo et al. 2008; Kerr 2004; Moulton et al. 1999). However, the reported side effects from these agents include: hypertension, bleeding, severe weight loss, diarrhoea and nausea in a large proportion of patients (Cabebe & Wakelee 2007; Mourad et al. 2008; Satchi-Fainaro et al. 2004). Furthermore, the clinically available bevacizumab (an anti-vascular endothelial growth factor, VEGF, antibody) and multi-targeted tyrosine kinase inhibitors (TKIs, target the VEGF receptor), cause endothelial dysfunction and capillary rarefaction (Cabebe & Wakelee 2007; Mourad et al. 2008; Satchi-Fainaro et al. 2004). Both classes of drug also cause clinically significant elevations in blood pressure (12 – 15%), bleeding and haemorrhage – all of which are associated with impaired regenerative angiogenic responses (Cabebe & Wakelee 2007; Mourad et al. 2008; Satchi-Fainaro et al. 2004). These problems are due to the complete inhibition of angiogenesis in all pathophysiological contexts. This highlights the need for the development of novel therapies that have more specific targeting of pathological angiogenesis whilst preserving physiological angiogenesis.
1.5. Chemokines

Chemokines are chemotactic cytokines that direct the migration of cells to sites of inflammation and injury and are secreted by a wide variety of cells. There are currently 50 known chemokines (ligands) with 20 chemokine receptors (highlighted in Table 1.1). Chemokines are divided into four groups based on the placement and number of cysteine residue at the N-terminal (Figure 1.5). The CC-chemokine group has 2 cysteine residues adjacent to each other, the CXC-chemokine group has 2 cysteine residues separated by an amino acid. This group can be further separated based on the presence of ELR (Glu-Leu-Arg) motif, into ELR⁻ (angiostatic) and ELR⁺ (angiogenic). The CX₃C-chemokine group has 3 amino acids between 2 cysteine residues and the XC-chemokine group has only one cysteine residue at the N terminal (Chan 2009; Fernandez & Lolis 2002; Rossi & Zlotnik 2000). Of the four chemokine groups the largest are the CC-chemokines (28 members), followed by the CXC-chemokines (17 members), with the CX₃C- and XC-chemokines having 1 and 2 members respectively. Table 1 illustrates the ability of chemokines to bind to multiple receptors and also for receptors to bind to multiple chemokines, indicating some redundancy in chemokine signalling. However, chemokine/chemokine receptors interactions are specific within each chemokine group. The complexity and specificity of the chemokine network may allow for a more enhanced and specific immune response (Devalaraja & Richmond 1999; Mantovani 1999).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>CCL3, CCL5, CCL7, CCL13, CCL14, CCL15, CCL16, CCL23</td>
</tr>
<tr>
<td>CCR2</td>
<td>CCL2, CCL7, CCL8, CCL13, CCL16</td>
</tr>
<tr>
<td>CCR3</td>
<td>CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28</td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL17, CCL22</td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16, CCR6, CCL20, CCR7, CCL19</td>
</tr>
<tr>
<td>CCR6</td>
<td>CCL20</td>
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<tr>
<td>CCR7</td>
<td>CCL19, CCL21</td>
</tr>
<tr>
<td>CCR8</td>
<td>CCCL1</td>
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<tr>
<td>CCR9</td>
<td>CCL25</td>
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<tr>
<td>CCR10</td>
<td>CCL27, CCL28</td>
</tr>
<tr>
<td>CXCR1</td>
<td>CXCL6, CXCL7, CXCL8</td>
</tr>
<tr>
<td>CXCR2</td>
<td>CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8</td>
</tr>
<tr>
<td>CXCR3-A</td>
<td>CXCL9, CXCL10, CXCL11</td>
</tr>
<tr>
<td>CXCR3-B</td>
<td>CXCL4, CXCL9, CXCL10, CXCL11</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12</td>
</tr>
<tr>
<td>CXCR5</td>
<td>CXCL13</td>
</tr>
<tr>
<td>CXCR6</td>
<td>CXCL16</td>
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<tr>
<td>CXCR7</td>
<td>CXCL12</td>
</tr>
<tr>
<td>XCR1</td>
<td>XCL1, XCL2</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3CL1</td>
</tr>
<tr>
<td>CCXCKR</td>
<td>CCL19, CCL21, CCL25</td>
</tr>
<tr>
<td>D6</td>
<td>CCL2, CCL3L1, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22</td>
</tr>
<tr>
<td>DARC/Duffy</td>
<td>CCL2, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16, CCL17, CXCL1, CCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL11, CXCL13</td>
</tr>
</tbody>
</table>
Figure 1.5. Chemokine classification is based on the number and placement of cysteine residues at the N-terminal. CC-chemokines have 2 cysteine residues, CXC-chemokines have 2 cysteine residues separated by an amino acid (X), XC-chemokines have one cysteine residue and CX₃C-chemokines have 2 cysteine residues separated by 3 amino acids (XXX).

CC-chemokines primarily stimulate inflammatory-driven angiogenesis by first binding to glycosaminoglycans (GAGs) as shown in Figure 1.6. In response to an inflammatory stimulus, circulating CC-chemokines arrive at the site of inflammation/injury (Figure 1.6A) and then bind GAGs (Figure 1.6B) such as heparin and heparin sulphate, on the surface of endothelial cells. This is essential, as many chemokines are not activated unless they are bound to GAGs (Proudfoot et al. 2003b). GAGs are highly negatively charged and form an electrostatic interaction with the basic chemokine (Kuschert et al. 1999), tethering the chemokines to the cell surface ready for receptor interaction. Chemokine retention on the cell surface configures a concentration gradient which attracts leukocytes and macrophages to the area of inflammation or injury. GAG binding also causes oligomerisation which is thought to be important in establishing chemokine activity but not receptor interaction (Allen, Crown & Handel 2007; Proudfoot et al. 2003a). Chemokines then bind to the chemokine receptors that are G Protein seven transmembrane Coupled Receptors (GPCR) expressed on the surface of the inflammatory cells (Figure 1.6C). Receptor binding causes a conformational change in the chemokine leading to
activation of the cell and attraction of more inflammatory cells as shown in Figure 1.6D (Allen, Crown & Handel 2007). Alternatively CC-chemokines can bind directly to endothelial cells to activate angiogenesis by binding the GPCRs on the surface of endothelial cells as shown in Figure 1.7. This activates signalling pathways such as the PI3K/Akt pathway to increase NO production, and endothelial cell proliferation and migration leading to increased angiogenesis.

Figure 1.6. CC-chemokine activity on endothelial cells.
(A) CC-chemokines bind to glycosaminoglycans (GAGs) on the surface of endothelial cells at the injury site. (B) This produces a chemokine gradient which attracts monocytes to the site of inflammation or injury. (C) The monocytes bind, causing activation and differentiation into macrophages and migration into the injury site, (D) where macrophages release pro-angiogenic and pro-inflammatory cytokines and growth factors.
Figure 1.7. Alternate pathway of angiogenesis stimulation by CC-chemokines.
Chemokines are recruited to the injury site, where they attach to chemokine receptors on the surface on endothelial cells. This activates the PI3K/Akt pathway, the release of NO, and stimulates endothelial cell migration and proliferation, causing the increase of angiogenesis.
1.6. Chemokines in health and disease

The major role of chemokines is to act as a chemoattractant guide for migrating immune cells that are attracted to the signal of increased chemokine concentration. The body maintains a homeostatic environment of immune cells that are ready to respond to injury or inflammatory stimulation. This fine balance is regulated by chemokines. Chemokines are functionally divided into two groups based on the pathophysiological conditions: (1) homeostatic chemokines that are constitutively expressed and are involved in physiological basal leukocyte migration; and (2) inflammatory chemokines that are stimulated under pathological conditions and actively recruit immune cells to the site of inflammation/injury. The CXC-chemokine ligands (CXCL) and receptors (CXCR) are the main group of chemokines involved in physiological homeostasis, while the CC-chemokine ligands (CCL) and receptors (CCR) regulate the migration of cells under inflammatory pathological conditions.

1.6.1. Homeostatic immune cell trafficking under physiological conditions

The control of chemokine immune cell trafficking begins in the bone marrow. In the bone marrow, hematopoietic stem cells (HSC) are in close proximity to CXCL12 abundant reticular (CAR) cells. The homeostatic regulation of immune cells such as neutrophils, B-cells, and monocytes under physiological conditions is dependent on CXCR4/CXCL12 chemokine interaction. Although not activated, immune cells are needed to patrol the peripheral blood and tissue until they are needed. As neutrophils mature, the expression of CXCR4 is reduced to allow for the release of neutrophils into the bloodstream. However, neutrophils are unable to survive for more than a few hours in circulation, undergoing senescence and increasing the expression of CXCR4 to facilitate their return to the bone marrow for apoptosis (Martin et al. 2003). Circulating neutrophils do not enter the peripheral tissue site unless stimulated in an inflammatory response. Eosinophils and basophils, stimulated under allergic reactions are predominantly found in the gastrointestinal tract during physiological conditions. Their migration into peripheral tissue is dependent on the interaction between CCR3 and CCL11 (Mishra et al. 1999). CCL11 also promotes the release of mature eosinophils and basophils from the bone marrow. Mature monocytes released from the bone marrow is dependent on CCR2 interaction and reduced CXCR4 interaction. Under homeostasis, monocytes can
differentiate into either pro-inflammatory \( \text{CCR}^\text{high} \) or anti-inflammatory \( \text{CX}^3 \text{CR}^1 \text{high} \) states. The majority of monocytes in physiological conditions are \( \text{CX}^3 \text{CR}^1 \text{high} \) and are found throughout peripheral blood and tissue (Foussat et al. 2000; Geissmann, Jung & Littman 2003). They function to monitor the bloodstream for infection and promote tissue homeostasis. The \( \text{CCR}^2 \text{high} \) monocytes are only found in the bloodstream and have minimal expression under physiological conditions (Geissmann, Jung & Littman 2003).

1.6.2. **Inflammatory immune cell trafficking under pathological conditions**

Under inflammatory conditions there is a reduction of systemic granulocyte-colony stimulating factor (G-CSF) levels which leads to the decrease of CXCL12 production in the bone marrow (Kawakami et al. 1990). This causes the release of various immune cells including neutrophils and monocytes. For neutrophils, the reduction of CXCL12 coincides with the increase of CXCL2 on the endothelium. This combination of events causes the release of neutrophils into the bloodstream under inflammation (Kawakami et al. 1990; Kim et al. 2006; Semerad et al. 2002). Once in the bloodstream, neutrophils are attracted to chemokine gradients of CCL3, CCL5, CXCL1, and CXCL2 through the CCR1 and CXCR2 receptors, to promote the migration of neutrophils to the inflammatory site of the tissue (Chou et al. 2010). Neutrophils can release various chemokines including CCL3, CCL4, CCL5, CCL20 CXCL1, CXCL8, CXCL9 and CXCL10 to further promote the inflammatory response and recruit more immune cells (Bennouna et al. 2003). Monocytes released from the bone marrow are promoted upon reduction of CXCL12, and is stimulated by CCL2 through the CCR2 receptor. Detection of CCL2 in the serum during infection and inflammation indicates the release of monocytes from the bone marrow to the bloodstream (Shi & Pamer 2011). In the bloodstream, monocytes primarily interact with CCL2, but also have receptors to allow for interaction with CCL3, CCL4, CCL5, and CCL7, highlighting the importance of the CC-chemokine class in inflammation. CCL2 attaches to tissue by GAG binding, creating a chemokine gradient to attract \( \text{CCR}^2 \text{high} \) monocytes to the inflamed tissue site, and differentiation to macrophages (Kuziel et al. 1997). Monocytes also express CXCR2, a receptor for CXCL8 and macrophage migration inhibitory factor (MIF). These chemokines have been found to promote adhesion and migration across the endothelium, but are not responsible for migration to the inflammatory site (Bernhagen et al. 2007; Gerszten et al. 1999). \emph{In vitro} studies have also found that CCR1 and CCR5 are essential.
for monocyte migration across the endothelium (Weber et al. 2001). Other than neutrophils and monocytes, eosinophils migrate from the bone marrow, through CCR3 signalling with CCL11. Eosinophils also express CCR1 receptors to respond to CCL24 and CCL26 stimulation. CCL11, CCL24 and CCL26 promote the accumulation and migration of eosinophils in the tissue.
1.7. Chemokines in angiogenesis associated diseases

In addition to their role in maintaining immune cell homeostasis, some chemokines have also been shown to have roles in the development of and, in particular, the promotion of angiogenesis. Each chemokine class has been shown to elicit angiogenic activity by distinct but overlapping mechanisms. The CC-chemokine class is primarily involved in stimulating pathological inflammatory-driven angiogenesis, while a number of the CXC-chemokine family members direct physiological ischaemia-mediated angiogenesis.

1.7.1. Chemokines in pathological inflammatory-driven angiogenesis

CC-chemokines are increasingly implicated in disease pathologies in which inflammatory-driven angiogenesis plays a key role. They are largely known to promote angiogenesis indirectly by recruiting macrophages to the site of inflammation or injury where they release pro-inflammatory cytokines and growth factors that are required for neovessel formation. For example in liver fibrosis, angiogenesis is due to macrophage recruitment stimulated by CCL2 (Ehling et al. 2014). Under inflammation, chemokines are secreted by a wide variety of cells including endothelial cells, smooth muscle cells and inflammatory cells. Chemokines are located away from the luminal surface of the endothelium. It is believed that chemokines bind to the outer surface of the endothelium and are then internalised into caveolae to be transported to the luminal surface as observed with CCL5 (Ebnet et al. 1996; Middleton et al. 1997). However, transportation of CCL19 is thought to occur via high endothelial venules rather than through caveolae (Baekkevold et al. 2001). Once at the luminal surface, chemokines attach to GAGs on the endothelium causing a chemokine gradient (Middleton et al. 2002). Chemokines are displayed to the inflammatory cells, such as monocytes, which attracts them to the site of inflammation or injury (Hub & Rot 1998). CC-chemokines bind to and activate monocytes through binding of the chemokine receptor GPCRs presented on the surface of the cell. Attachment through the N-terminal of the CC-chemokine is the most important as it causes a conformational change to activate the monocyte (Fernandez & Lolis 2002). Activation of the monocyte causes migration into the tissue and differentiation into macrophages. It is well-established that this process is facilitated by CC-chemokines (Yamamoto, Nagata & Sakamoto 2005) and the interaction of vascular cell adhesion molecule-1 (VCAM-1).
Recent evidence demonstrates that CC-chemokines can also directly regulate inflammatory-driven angiogenesis. For example, CC-chemokines have been shown to promote signalling pathways which induce inflammatory-driven angiogenesis including the signalling proteins phosphatidylinositol 3-kinase (PI3K), Akt and mitogen-activated protein kinase (MAPK) and ERK1/2 (Galvez et al. 2005; Stamatovic et al. 2006). Stimulation of these pathways subsequently increases the production of VEGF to further augment angiogenesis (Hong, Ryu & Han 2004). Additionally, CCL2 is associated with the increase of MMP14, essential for endothelial cell migration and neovessel formation (Galvez et al. 2005). CCL2 has also been shown to accelerate endothelialisation through the recruitment of EPCs (Fujiyama et al. 2003). Endothelial tubule formation in vitro was seen following incubation with CCL1, CCL2, CCL11, CCL15 and CCL16 (Bernardini et al. 2000; Hwang et al. 2004; Salcedo et al. 2001; Stamatovic et al. 2006; Strasly et al. 2004). Furthermore, all CC-chemokines contain NF-κB binding motifs and their expression is dramatically increased under inflammatory conditions (Charo & Ransohoff 2006; Werts et al. 2007; Weyrich et al. 1995). A summary of the direct and in-direct effects of chemokines on pathological inflammatory-driven angiogenesis is depicted in Figure 1.8.
Figure 1.8. The direct and in-direct effects of chemokines on pathological inflammatory-driven angiogenesis.

Taken together, CC-chemokines play a critical role in the development of inflammatory-driven diseases reliant on macrophage infiltration, such as in atherosclerosis. In support of this a number of CC-chemokines have been identified in human atherosclerotic plaques, including: CCL2, CCL3, CCL5, CCL11, CCL13, CCL18, CCL19 (Berkhout et al. 1997; Bursill et al. 2003; Reape et al. 1999; Weber, Schober & Zernecke 2004). Furthermore the double knockout CCR2−/−/ApoE−/− mice exhibit diminished lesion development and in targeted CC-chemokine receptor or CC-chemokine deletion studies ApoE−/− mice have less plaque development (Boring et al. 1998; Gosling et al. 1999; Gu et al. 1998). Met-RANTES (an antagonist to CCL5) also reduced the size of atherosclerotic lesions in hypercholesterolaemic LDLR−/− mice (Veillard et al. 2004).

Studies have shown that in rheumatoid arthritis (RA), another inflammatory-driven pathology, new blood vessels are abundant in the synovial tissue. Neovessels promote the infiltration of leukocytes that increase the progression of RA (Szekanecz et al. 2009). Previous studies have found that production of CCL2, CCL3, CCL5, and CCL20 are associated with increased leukocyte infiltration and angiogenesis in models of RA (Schutyser, Struyf & Van Damme 2003; Szekanecz & Koch 2001; Szekanecz et al. 2009).
While CC-chemokines have been shown to be the primary family involved in inflammatory-driven pathologies, studies have shown that members from other chemokine classes also play a role. For example, it has been previously reported that a member of the CXC-chemokine family, CXCL8 (IL-8) is important in the development of atherosclerosis, particularly in monocyte adhesion on endothelial monolayers expressing E-selectin under flow conditions (Kraaijeveld et al. 2007). Additionally, the expression of CX3CL1 (fractalkine) and its receptor CX3CR1 is upregulated in early and late atherosclerotic plaques (Stolla et al. 2012). The CX3CR1 receptor is also found on endothelial cell and smooth muscles in the plaque (Lucas et al. 2003; Yang et al. 2007).

1.7.2. Chemokines in physiological ischaemia-mediated angiogenesis

In contrast to their critical role in inflammatory-driven angiogenesis, the CC-chemokines have little involvement in ischaemia-mediated angiogenesis. It is the CXC-chemokines that are involved in important developmental processes including haematopoiesis, organogenesis and tissue repair. Extensive studies have shown that the critical hypoxia transcription factor HIF-1α augments the increased expression of the CXC-chemokine CXCL12 (also known as stromal-cell derived factor-1α, SDF-1α). CXCL12 is the single natural ligand for the chemokine receptor CXCR4 (Liekens, Schols & Hatse 2010) and the CXCL12/CXCR4 axis is important in the mobilisation, migration and recruitment of progenitor stem cells to sites of ischaemia (Dar, Kollet & Lapidot 2006; van Weel et al. 2007) with CXCL12 expression in ischaemic sites directly correlating with the degree of hypoxia (Ceradini et al. 2004). Furthermore, increased CXCL12 levels have been reported in infarcted myocardium in both human and rodent studies (Askari et al. 2003; Ma et al. 2005; Pillarisetti & Gupta 2001; Wang et al. 2006), with increased CXCL12 levels detected as early as 1 hour following ischaemic induction in the myocardium or in hindlimbs, indicative of a role in the initiation of tissue repair and revascularisation (De Falco et al. 2004; Lee et al. 2006; Yang et al. 2004). The importance of CXCL12 is highlighted in CXCL12 knockout mice that are not viable due to the absence of vessel development (Schober et al. 2006).

In parallel with CXCL12, HIF-1α also increases the production of VEGF. VEGF stimulates the production of endothelial nitric oxide synthase (eNOS) to stimulate nitric oxide production, which is essential for endothelial cell migration. Simultaneously,
CXCL12 mobilises bone marrow-derived EPCs, critical in the regenerative response to ischaemia and injury, through PI3K/Akt/eNOS activation (Zheng et al. 2007). The upregulation of eNOS mobilises EPCs and further increases the production of VEGF (Aicher et al. 2003). A summary of the role of chemokines in physiological ischaemia-mediated angiogenesis is depicted in Figure 1.9.

Figure 1.9. The role of chemokines in physiological ischaemia-mediated angiogenesis.
1.7.3. Chemokines in tumour neoangiogenesis

CC-chemokines have been associated with a number of malignancies. Studies reveal that CCL2 and CCL5 expression is elevated in breast and prostate cancer (Soria & Ben-Baruch 2008; Zhang, Lu & Pienta 2010b), CCL1 and CCL3 are shown to promote development of leukaemia and CCL19 and CCL21 direct tumour metastasis (Tanaka et al. 2005). Murine cancer models have shown that CC-chemokines are important in tumour development in vivo. Inhibition of CCL2 reduced haemangioma size in nude mice inoculated with cancer cells, while infusion of Met-RANTES inhibited tumour development in murine models of breast cancer (Robinson et al. 2003). The CXC-chemokine class has also been shown to play a role in tumour neoangiogenesis, with IL-8 (CXCL8) shown to play an important role in tumour growth, angiogenesis and metastasis (Li et al. 2003). Furthermore, CXCL12 has been implicated in the promotion of tumour growth in mouse prostate tumours following overexpression of its receptor CXCR4 (Darash-Yahana et al. 2004). Consistent with this, CXCL12 production in tumour cells is triggered under hypoxic environments (Kryczek et al. 2005).

1.7.4. Chemokines in wound healing

CC-chemokines have been shown to regulate inflammation and angiogenesis, two key processes of wound healing. In the wound healing process, macrophage infiltration is highly regulated by CC-chemokine gradients released by hyper-proliferating keratinocytes, fibroblasts and other macrophages (Eming, Krieg & Davidson 2007; Gillitzer & Goebeler 2001). In human wounds, a host of CC-chemokines including CCL1, CCL2, CCL3, CCL4, CCL5 and CCL7 are expressed in the wound during the first week after injury and high levels of CCL2 have been found in human burns (Engelhardt et al. 1998; Gibran et al. 1997). Additionally studies in excisional wounds showed localised expression of CCL2 and CCL3 in the epidermis, while CCL3 was also expressed in follicular epithelium, and sebaceous glands (Jackman et al. 2000). CC-chemokines have also been shown to regulate key processes involved in wound angiogenesis (Folkman & Shing 1992), such as the recruitment of inflammatory cells to the wound to provide support for proliferating and migrating cells, and the formation of granulation tissue (Gillitzer & Goebeler 2001). Furthermore, CC-chemokines were found to have direct effects on wound angiogenesis, for example stimulating tubulogenesis and endothelial cell migration through stimulation
of Ets-1 by CCL2 (Stamatovic et al. 2006), and through interactions with CCL16 (Strasly et al. 2004), and the CCL11/CCR3 complex (Salcedo et al. 2001). Additionally, CCL2 is involved in the regulation of MMP1 to breakdown the extracellular matrix for cell migration (Galvez et al. 2005), and CCL5/CCR5 interaction is critical for recruitment of endothelial progenitor cells to the wound site (Ishida et al. 2012).

In summary, the CC-chemokine class plays an important role in the regulation of inflammatory-driven pathological angiogenesis, with little to no role in ischaemia-mediated physiological angiogenesis. This difference in angiogenic regulation raises the possibility that specifically inhibiting the CC-chemokine class will inhibit pathological inflammatory-driven angiogenesis, while preserving physiological ischaemia-mediated angiogenesis. Furthermore, there is redundancy in CC-chemokine class signalling, where chemokines in one class can bind to several receptors from the same class. Conversely, receptors can have multiple ligands within a chemokine class. Therefore, due to this redundancy, targeting just a single chemokine may not be completely effective (Devalaraja & Richmond 1999; Mantovani 1999). A broad-spectrum CC-chemokine blockade approach may therefore be a more successful therapeutic approach. Chemokine inhibitors of this nature have already evolved from viruses allowing the virus to bypass the inflammatory pathway preventing detection. They are expressed following host invasion to propagate their own infection. The clear utility of these ‘chemokine-binding proteins’ is a growing area of medical research.
1.8. **Viral mimicry**

Viruses have evolved mechanisms to evade detection and destruction of host immune systems. One such mechanism used by large DNA viruses, such as Poxviruses and Herpes viruses, is to target the chemokine network by encoding homologues of chemokines and chemokine receptors to block chemokine activity. Viruses have 3 mechanisms to interfere with the chemokine network. Viruses may produce viral chemokines (vCKs) which assist in viral spreading and growth, viral chemokine receptors (vCKRs) which influence the endothelial cells ability to respond to chemotactic signals, or they produce viral chemokine binding proteins (vCKBPs) which disrupt chemokine-GAG binding, chemokine-GPCR interaction or both (Alcami 2003).

There are 3 types of vCKBPs produced by large viruses, firstly those that block GAG binding such as M-T7, which is expressed from the myxoma virus. M-T7 interferes with the IFN-γ receptor binding to CC-, CXC- and XC-chemokines (Lalani et al. 1997; Mossman et al. 1996). Secondly, there are vCKBPs that bind to chemokines with high affinity and therefore compete for binding to GPCRs, such as 35K from the Vaccinia virus (Alcami et al. 1998; Beck et al. 2001; Graham et al. 1997; Smith et al. 1997; Zhang et al. 2006). Thirdly, viruses produce vCKBPs that bind both GAGs and GPCRs, such as M3. M3 is expressed from the mouse herpes virus and uniquely binds to a number of key inflammatory chemokines from all 4 chemokine classes (Alexander-Brett & Fremont 2007; Parry et al. 2000; Webb, Smith & Alcami 2004). This thesis is focused on the vCKBP ‘35K’ that binds with high affinity to nearly all of the CC-chemokine class.
1.9. **35K – broad-spectrum CC-chemokine inhibitor**

35K is a soluble 35 kDa protein (previously known as vCCI, vCKBP2, VV-35kDa) from the Vaccinia virus (Figure 1.10). It is a globular protein, composed primarily of two parallel β-sheets, two short α-helices and large loops connecting the secondary structure elements (Carfì et al. 1999). This β-sandwich topology is unique and does not resemble any other chemokine receptor. It is mainly composed of non-polar residues and is highly acidic. 35K has been shown to have high affinity binding to a broad range of CC-chemokines but has low or no affinity for chemokines belonging to other chemokine classes (Zhang et al. 2006). This is due to the fast association and slow dissociation rate with CC-chemokines, where in some cases 35K has 10 times greater affinity to the CC-chemokine than its cognate receptor (Seet et al. 2001; Smith et al. 1997). Despite this high binding affinity to CC-chemokines, 35K has no sequence homology to host CC-chemokine receptors, instead it is thought to recognise common structural features shared by most CC-chemokines (Carfì et al. 1999).

![Figure 1.10. Structure of 35K. A and B are related by 90° rotation. Green arrows represent β-strands, blue ribbons, α-helices (Carfì et al. 1999).](image-url)
35K has no resemblance to any known chemokine receptor, however it is able to bind to all CC-chemokines with high affinity. It has been identified that 35K binds to residues conserved throughout all CC-chemokines. In doing this, 35K binds over the receptor sites inevitably blocking chemokine receptor interaction. Previous binding studies with 35K show that point mutations on CCL2 at residues Tyr13, Arg18 and Arg24 greatly affected the binding affinity of 35K (Beck et al. 2001; Lau et al. 2004). These residues are highly conserved within the entire CC-chemokine class and were found to be critical for binding with 35K (Beck et al. 2001; Seet et al. 2001). Additionally, Arg18 is critical for binding to 35K, as CCL1 which contains glutamine at position 18 has low binding affinity with 35K (Seet et al. 2001). Furthermore, replacing Arg18 with alanine drastically reduced CC-chemokine binding affinities with 35K (Seet et al. 2001).

Binding tests on the most widely characterised CC-chemokine, CCL2 revealed that 35K has no sequence homology to CCL2 or CCR2, however it recognises similar residues that are important for CCR2 receptor binding as illustrated in Figure 1.11 (Seet et al. 2001). Most importantly these residues span the area in which CCR2 binds, suggesting that residues bound to 35K overlap with the binding site of CC-chemokine receptors thereby causing direct inhibition of CCR2 binding to CCL2 indicative of competitive binding between 35K and CC-chemokine receptors (Bahar et al. 2008).
Figure 1.11. Binding sites on CCL2 for 35K and CCR2 (adapted from Beck et al, 2001). CCR2 and 35K high affinity (red) binding sites overlap at Arg-24 and Tyr-13 (yellow circles).

More recent binding studies looking at the interaction between 35K and CCL11 (eotaxin-1) show that the basic residues (Arg16, Arg22 and Lys44) are important in 35K-CCL11 binding. These positions are equivalent to the residues found in CCL2 (Kuo et al. 2014). When bound to 35K, these residues bind to highly acidic negatively charged residues within 35K’s β-sheet. Sequence alignment analysis also revealed that CC-chemokines with high affinity binding to 35K all have basic positively charged residues at this equivalent position. The equivalent residue to Arg16, in CCL4 (Arg18), is found within the GAG binding region suggesting that 35K may affect the production of a concentration gradient in relation to some CC-chemokines (McCornack, Cassidy & LiWang 2003; Zhang et al. 2006). Given that GAG binding is important to chemokine function, studies also looked at whether this impacts on 35K binding. This was done by either overexpressing or inhibiting GAGs. Studies overexpressing heparin and heparin sulphate (the main GAG group) in pre-incubation studies with CCL3 did not affect binding of 35K to CCL3 (Alcami et al. 1998). Similarly, 35K binding is unaffected when GAG binding to CCL2 was lost through substitution of Lys58 and His66 by alanine (Beck et al. 2001). These findings suggest that whilst a chemokine gradient is still achieved through
GAG binding, 35K prevents the activation of receptors on the inflammatory cells, suggesting that 35K can bind both free and unbound CC-chemokines.

When 35K binds, the release of intracellular calcium which normally occurs with CC-chemokine and CC-chemokine receptor binding is suppressed, causing a reduction in CC-chemokine activity (Lalani et al. 1998). Despite this, 35K does not prevent receptor activation. This is because CC-chemokine receptor and ligand activation is facilitated through N-terminal binding (Carfì et al. 1999; Salanga & Handel 2011) and 35K does not bind over the N-terminal or bind to residues within this site. Instead, activity is suppressed when 35K binds due to the loss of the essential connection of the CC-chemokine and CC-chemokine receptor thereby preventing activation through the N-terminal. To further confirm the specificity of 35K binding to only the CC-chemokine class, it was found that incubation of 35K with CXCL8 did not affect the release of calcium (Alcami et al. 1998; Lalani et al. 1998; Smith et al. 1997).

1.9.1. 35K in inflammatory disease

The effect of broad-spectrum CC-chemokine inhibition by 35K has been investigated in a number of inflammatory-driven diseases. In the atherosclerosis-prone ApoE knockout mouse model, systemic delivery of an adenovirus to overexpress 35K protein (Ad35K) inhibited CC-chemokine activity in the plasma and aortas (Ali et al. 2005; Bursill et al. 2009). Ad35K infused mice also had reduced plaque macrophage recruitment and atherosclerotic plaque size in both short and long term 35K treatment studies as shown in Figure 1.12 (Bursill et al. 2004; Bursill et al. 2009). In short term studies, ApoE+/− mice fed on a western diet for 6 weeks developed large atherosclerotic lesions in the aortic root (Figure 1.12A). However, intervention with adenovirus overexpressing 35K (Ad35K), 2 weeks prior to sacrifice decreased atherosclerotic lesions by more than 50% compared to PBS and adenoviral controls (AdGFP). Additionally, macrophage infiltration was significantly reduced in the Ad35K treated mice compared to PBS and AdGFP. Similar results were seen, in a long term (12 week) study, in which ApoE+/− mice fed a regular diet, were injected with a lentivirus to overexpress 35K (Lenti35K) (Figure 1.12B). Mice were found to have significantly reduced atherosclerotic plaques and macrophages in the aortic sinus of the mice injected with Lenti35K compared to PBS or LentiGFP. The beneficial effects of 35K have also been reported in other inflammatory-driven diseases, such as
acute peritonitis, where mice injected with an adenovirus overexpressing membrane-bound 35K had less inflammatory cells in peritoneal exudates (Bursill et al. 2006). Studies in mouse livers that received Ad35K infusions exhibited reduced hepatitis and liver fibrosis in mice (Bursill et al. 2006; Seki et al. 2009). Additionally, 35K attached to a Fc fusion protein (35K-Fc) improved pulmonary function and reduced inflammation in the lung (Dabbagh et al. 2000)

In summary, broad-spectrum CC-chemokine inhibition by 35K has potent anti-inflammatory effects in disease pathologies associated with inflammation. However, its effects have not as yet been investigated or compared in pathological and physiological angiogenesis.
Figure 1.12. Short and long term expression studies with 35K in ApoE−/− mice. (Bursill et al. 2004; Bursill et al. 2009).

(A) Short term expression with adenovirus overexpressing 35K, and (B) long term expression with lentivirus overexpressing 35K, reduced lesion size detected by determining area of the lesion, and macrophage infiltration detected by staining for Mac-3, in ApoE−/− mice compared to PBS and GFP controls.
1.10. Hypothesis and aims

Angiogenesis associated diseases are widespread globally, with the World Health Organisation reporting that cardiovascular disease (CVD) and cancer are the leading causes of mortality worldwide (WHO 2013). Current anti-angiogenic agents cause severe side effects due to the inhibition of angiogenesis in both pathological and physiological conditions. Thus, there is a need to find an alternate therapeutic approach to overcome the shortcomings of current treatments.

Angiogenesis is regulated via different, yet partially overlapping, pathways in inflammation and hypoxia. There is increasing evidence that chemokines regulate angiogenesis, with the CC-chemokine class playing an important role in pathological inflammatory-driven neovascularisation, while having little to no effect on physiological ischaemia-mediated angiogenesis. With this in mind, broad-spectrum inhibition of the CC-chemokine class may be an alternate approach to overcome the often severe side effects caused by the global inhibition of angiogenesis by current anti-angiogenic therapies. This strategy will also have advantages over single CC-chemokine inhibition due to redundancies in chemokine signalling and the multifaceted role of CC-chemokines in inflammatory diseases. The broad-spectrum CC-chemokine inhibitor 35K has been shown to have positive outcomes in pre-clinical models of inflammatory-driven diseases including atherosclerosis, liver fibrosis and acute peritonitis. However, its role in the regulation of angiogenesis, and a comparison between inflammatory and hypoxic conditions remains to be explored.

Given the above rationale, the central hypothesis of this thesis is that broad-spectrum inhibition of the CC-chemokine class may suppress unwanted pathological inflammatory-driven angiogenesis whilst preserving physiological ischaemia-mediated angiogenesis. Broad-spectrum inhibition of the CC-chemokine class by 35K may be an alternate therapeutic strategy to reduce neovascularisation in pathological angiogenesis-associated diseases such as atherosclerosis and cancer, without the severe side effects of current non-selective angiogenic inhibitors that block angiogenesis in all pathophysiological contexts.
The main aims of this work are to determine the importance of chemokines, specifically the CC-chemokine class, in the regulation of angiogenesis in both pathological and physiological contexts. Specifically, using the broad-spectrum CC-chemokine inhibitor 35K, we seek to:

1. Elucidate the role of broad-spectrum CC-chemokine inhibition on functional angiogenic assays and key angiogenic mediators *in vitro* in inflammatory and hypoxic conditions.
2. Investigate the effect of broad-spectrum CC-chemokine inhibition in two animal models that represent pathological and physiological angiogenesis: (a) the peri-arterial femoral cuff model and (b) the hindlimb ischaemia model.
3. Explore the effect of broad-spectrum CC-chemokine inhibition in a tumour neovascularisation model that is driven by both inflammation and ischaemia.
4. Determine the effect of broad-spectrum CC-chemokine inhibition in a mouse model of wound healing, where angiogenesis is critical in the early-mid phases of healing.

To achieve these aims we tested purified 35K protein in *in vitro* angiogenesis studies outlined in Chapters 3 and 4. We next investigated in Chapter 5 the effect of 35K in *in vivo* angiogenesis models using an adenoviral gene transfer approach in which mice were injected with an adenovirus overexpressing 35K (Ad35K) protein systemically. Chapter 5 compares and contrasts the effects of 35K on two murine surgical models of angiogenesis, namely the peri-arterial cuff model (inflammatory-driven) and the hindlimb ischaemia model (ischaemia-mediated). Chapter 6 explores the effect of 35K on a Lewis Lung cancer model and Chapter 7 describes the effect of CC-chemokine inhibition in a murine model of wound healing with topical application of purified 35K protein.
CHAPTER 2.
GENERAL METHODS
This Chapter will outline the general materials and methods used throughout the thesis.

2.1. Solutions

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2.2.  *In vitro* methodology

2.2.1.  *Cell Culture*

Cell culture experiments were conducted under 3 different conditions: 1) unstimulated controls, 2) inflammation or 3) hypoxia. To mimic the inflammatory environment, human coronary artery endothelial cells (HCAECs) were incubated with macrophage-conditioned media (MCM), while the hypoxic environment was achieved by incubation in a hypoxic incubator with conditions of 1% O₂/5% CO₂.

2.2.1.1  *Human monocyte derived macrophages (HMDM)*

Human monocyte derived macrophages (HMDMs) were used to produce macrophage-conditioned media (MCM). MCM was used instead of TNF-α or IFN-γ as the release of pro-inflammatory cytokines and growth factors, such as VEGF, bFGF, PDGF, TNF-α, and IFN-γ, from stimulated HMDMs, is more physiologically relevant to the endothelial cells. Human monocytes were isolated by elutriation and seeded at 1×10⁶ cells/well in 6 well plates, and grown for 12 days in RPMI 1640 supplemented with 10% (v/v) FBS. To stimulate the release of pro-inflammatory cytokines and growth factors into the media, HMDMs were stimulated with 10 ng/mL of interferon-γ at day 10 for 48 hours. After stimulation, the media was collected and centrifuged at 2,500 rpm for 5 minutes to remove cellular debris, and the supernatant was stored in 1 mL aliquots at -80°C until further use.

2.2.1.2  *Human coronary artery endothelial cells (HCAEC)*

Human coronary artery endothelial cells (sourced from ATCC) were used from passage 3 up to passage 6 and maintained in MesoEndo endothelial media.
2.2.1.3 AD293 cells

Frozen AD293 cells were re-suspended in DMEM 293 media containing 10% (v/v) FBS. For viral amplification, cells were grown to 70 – 80% confluency in 20 T175 cm² flasks.

2.2.2. Generation of Adenovirus expressing 35K

A recombinant adenovirus expressing 35K (Ad35K) was generated by cloning the 35K open reading frame which also contains a HA epitope tag upstream from its promoter region (Figure 2.1) into a pShuttle-CMV (ps) plasmid (Figure 2.2), creating the ps35K plasmid. The ps35K plasmid was then transfected into AD293 cells to create recombinant Ad35K virus. Adenovirus overexpressing GFP (AdGFP) was generated in the same way to use as an adenoviral control.
Figure 2.1. Ad35K construct (Bursill et al. 2003)

Figure 2.2. Cloning map of pShuttle-CMV (He et al. 1998)
2.2.3. Large scale Adenovirus production

To generate large quantities of Ad35K and AdGFP, $5 - 6 \times 10^{11}$ virus particles (vp) of the recombinant Ad35K or AdGFP virus were added to 400 mL DMEM 293 cell culture media (D5546, Sigma-Aldrich, Missouri, USA) supplemented with 10% (v/v) foetal bovine serum (SFBS, Bovogen Biologicals, Victoria, Australia) and L-Glutamine (17-605C, Lonza, Basel, Switzerland). This media containing the virus was divided equally over 20 T175cm$^2$ flasks (20 mL/flask) of AD293 cells at 70 – 80% confluency in the incubator at 37°C. After 24 – 48 hours cells were then microscopically examined for evidence of complete cytopathic effect (CPE). This is indicated by the cells in the monolayer starting to round up and they then start to detach from the flask. A change in media colour was also observed from pink to orange. Flasks were forcefully tapped to detach remaining cells. Pooled media and cells were centrifuged at 4,500 rpm for 30 minutes. The media, known to contain high amounts of secreted 35K protein following Ad35K infection, was removed from the cell pellet that contains the viral particles, collected in 50 mL Falcon tubes and stored at -20°C to be used for in vitro studies. The cell pellet was resuspended in 10 mL hypotonic VSB (26 mM Tris-HCl, 5 mM KCl, 2 mM MgCl$_2$, pH 7.4) and stored at -80°C overnight. The following day the cell suspension was freeze thawed twice, before centrifuging at 2,200 rpm for 10 minutes. The supernatant was removed and kept on ice, while the pellet was resuspended in a further 10 mL of hypotonic VSB and re-spun at 2,200 rpm for 10 minutes. The supernatant was pooled and density adjusted to 1.1 – 1.2 g/mL by adding caesium chloride (CsCl) before filtering with 0.22 μm filter.
2.2.4. Purification of Adenovirus

To purify adenovirus from the pooled supernatant, 2.5 mL hypertonic VSB (26 mM Tris-HCl, 5 mM KCl, 2 mM MgCl$_2$, 139 mM NaCl, pH 7.4) solution with a density of 1.4 g/mL was carefully underlayed under 2.5 mL hypertonic VSB solution with a density of 1.3 g/mL in a 12 mL polyallomer centrifuge tube (331372, Beckman Coulter, California, USA). The viral supernatant was overlayed above this solution to fill the remainder of the tube (approximately 7 mL) leaving 5 mm without solution. Tubes were transferred to centrifuge holders from the SW 41 Ti rotor (Beckman Coulter). Three tubes were made per viral construct. Tubes were centrifuged at 30,000 rpm at 4°C for 2 hours in the ultracentrifuge. After 2 hours the resolving virus band was collected by piercing the tube approximately 1 cm below the white virus band (Figure 2.3) with an 18 gauge needle (SG2-1838, Terumo Medical Corporation, New Jersey, USA) and drawn out with a 1 mL syringe (SS+01T, Terumo [Philippines] Corporation, Laguna, Philippines), extra care was taken to avoid collection of any cellular debris present above the virus band. The virus was further purified in a caesium chloride density gradient via a second ultracentrifugation at 30,000 rpm at 4°C for 15 hours. After 15 hours the virus was collected and desalted by centrifuge filtration in columns of 50% (v/v) CL-4B sepharose (CL4B200, Sigma-Aldrich) at 1,000 rpm for 3 minutes. The sepharose column was initially washed twice with hypotonic VSB and the virus was run over two separate columns as part of the de-salting process.

Total virus particles of the purified Ad35K or AdGFP were measured using a spectrophotometer (SmartSpec Plus, BioRad, California, USA) by measuring the absorbance at 260 nm ($A_{260} = 1 \times 10^{12}$ vp). Purified virus was diluted 1:1 in a mix of 80% foetal bovine serum (FBS) and 20% glycerol, and aliquots of $1 \times 10^{11}$ vp were frozen at -80°C.
Figure 2.3. Virus band seen following 2 hour CsCl gradient ultracentrifugation with cellular debris above

2.2.5. Protein 35K isolation

Column purification was used to isolate HA-tagged 35K protein from the viral media. Anti-HA tagged bead conjugate (A2095, Sigma-Aldrich) was added to an empty chromatography column and allowed to settle by gravity flow, the beads were packed into the column with 3 washes of PBS. The column was further washed with 15 mL 3 M sodium thiocyanate, to lower the pH, and then washed once more with 15 mL PBS. To bind HA-tagged 35K protein to the anti-HA beads, 40 mL of the 35K adenoviral media, collected during virus production, was passed through the column by gravity flow. The 35K protein was eluted from the beads in 1 mL aliquots of 3 M sodium thiocyanate and collected in Eppendorf tubes containing 50 μL 1 M Tris-HCl (pH 8) to neutralise the solution. A total of 10 fractions were collected and tested via Western blot to confirm the presence of 35K.
2.2.6. Western blot to determine 35K in fraction

To confirm the presence of 35K isolated protein, eluted column fractions were run on a Western blot. From each fraction 10 µL was taken and combined with 5 µL Novex® LDS sample buffer (B0007, Life Technologies, California, USA) and 1 µL Novex® reducing agent (NP0009, Life Technologies) and heated at 95°C for 5 minutes, before placing the tubes on ice for 2 minutes. Reduced samples and SeeBlue® Plus2 Pre-stained Protein Standard (LC5925, Life Technologies) were loaded onto wells of a 12-well Bolt® 4% Bis-tris plus gel (NW04122BOX, Life Technologies) and ran at 100 V for 90 minutes in 1X Bolt® MES SDS Running Buffer (B0002, Life Technologies). Proteins from the gel were then transferred onto nitrocellulose membranes using the iBlot® Transfer Stack (IB3010-31, Life Technologies) and iBlot Gel Transfer system (IB21001, Life Technologies). Membranes were blocked in 10% (w/v) skim milk in TBST (20 mM Tris, 136 mM NaCl, and 0.1% [w/v] Tween 20, pH 7.4) for 1 hour at room temperature on a rocking platform. Following this, membranes were washed 3 times in TBST for 5 minutes at room temperature before incubating with rabbit anti-35K primary antibody (1:1000, Peter Turner, University of Florida, Gainesville, FL, USA) overnight at 4°C. The membranes were washed before incubating with goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase (HRP) (sc-2030, Santa Cruz Biotechnologies, Texas, USA) (1:1000) in 2% (w/v) skim milk in TBST for 2 hours at room temperature.

Membranes were developed by chemiluminescence on the Chemidoc MP Imaging System (170-8280, BioRad with ECL reagents in a 1:1 ratio of Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent (170-5061, Biorad). Images were recorded every 1 second using Image Lab Software (v.5.0, 170-9692, BioRad) with a total exposure time of 10 seconds.
2.2.7. Functional studies

Three functional angiogenic assays were used to assess the effect of 35K on the functional angiogenic properties of HCAECs. All experiments were performed in triplicate and repeated at least 3 times under inflammatory conditions stimulated by MCM or hypoxic conditions stimulated by 1% O2/5% CO2 and N2 balance in a hypoxic incubator.

2.2.7.1 Proliferation assay

Human coronary artery endothelial cells (HCAECs) were plated on 6-well plates at 3.8 x 10^4 cells/well in EBM-2 (Lonza) supplemented with 5% (v/v) FBS to serum starve cells overnight.

Cells were then incubated for 4 hours with 50% (v/v) PBS (control) in EBM-2 with FBS or 50% (v/v) MCM in EBM-2 with FBS and 35K (0 – 400 nM) or subjected to hypoxia (1% O2/5% CO2) in EBM-2 with FBS. After incubation, 0.1% (v/v) EdU in MesoEndo media or RPMI media was added overnight. Proliferation was determined the following day using the Click-IT® EdU assay (C10425, Life Technologies) and proliferating cells were detected by flow cytometry (FC-500, Beckman and Coulter, CA, USA). Briefly, cells were harvested in PBS with 10% (v/v) FBS and centrifuged at 3.4 rcf for 4 minutes. The pellet was resuspended in 50 µL PBS with 10% (v/v) FBS. Cells were fixed in 50 µL Click-iT fixative D (4% paraformaldehyde) and incubated for 15 minutes.
protected from light at room temperature before washing in PBS with 10% FBS and centrifuged for 4 minutes at 3.4 rcf. Samples were vortexed with 50 μL 1X saponin permeabilisation buffer (E) and 50 μL Click-iT reaction mix (285 μL Click-iT reaction buffer [G], 6.5 μL CuSO₄ [H], 1.6 μL fluorescent dye azide – Alex Fluor 488 [B] and 32.5 μL 1xClick-iT-EdU buffer [I]). Samples were incubated for 30 minutes at room temperature protected from light. Following incubation cells were washed in 1 mL 1X buffer E and centrifuged at 3.4 rcf for 4 minutes. Supernatant was discarded and cells resuspended in 500 μL buffer E. Cells were transferred to FACs tubes and measured by flow cytometry.

2.2.7.2 Migration assay

Lab-Tek chamber slides (177437, Thermo Fisher Scientific, Massachusetts, USA) were coated in 670 μL/well rat tail collagen coating solution (122-20, Cell Applications Inc.) and incubated at 37°C for 1 hour. During this time 5 mL of HCAECs at a cell density of 8x10⁴ cells/mL were prepared by resuspending in EBM-2 media with 5% FBS. Following the 1 hour incubation, the remaining collagen was removed and each well washed with HT-PBS at least 3 times. Finally, wells were rinsed with EBM-2, 5% FBS and L-glutamate before 1 mL/well of cell suspension at a density of 8 x 10⁴ cells/mL was added. Cells were allowed to adhere for 6 hours before proceeding with scratch assay.

A P200 pipette tip was used to scratch a basic grid pattern through the cell layer. The media was removed and replaced with treatments in MesoEndo media. Cells were then incubated in PBS (control) or MCM and 35K (0 – 50 nM) and monitored over at least 10 hours by live cell imaging (Zeiss Axiovert 200M, Germany) at 37°C/ 5% CO₂. Images were measured from three sites along the scratch per well. For hypoxic conditions, cells were plated out and allowed to adhere overnight. Media was changed in the morning to MesoEndo media and cells were subjected to hypoxia (1% O₂/ 5% CO₂) or normoxia for 6 hours. A grid pattern scratch was then made through the cell layer and cells were incubated in PBS and 35K (0 – 50 nM) then subjected to live cell imaging as described above. The average distance between cell fronts of the scratch was measured at 2 hour intervals for up to 10 hours; this was used to determine the speed of migration.
2.2.7.3 Tubulogenesis assay

Thawed growth factor reduced matrigel (356231, BD Bioscience, Massachusetts, USA) were carefully plated at 40 µL/well into a 96 well plate, ensuring no bubbles were present, and left to polymerise at 37°C for 30 minutes. HCAECs were plated at 9x10⁴ cells/mL in 100 µL MesoEndo Endothelial Cell Media (212-500, Cell Applications Inc., California, USA) and 35K (0 – 50 nM) under inflammation, stimulated with 50% MCM (v/v), or hypoxic (1% O₂/ 5% CO₂) conditions at 37°C for 4 – 6 hours. HCAEC tubule formation was imaged at 10X magnification and counted in a minimum of 3 fields of view per well using Image J (National Institutes of Health, Maryland, USA).

2.2.8. Mechanistic studies

2.2.8.1 Protein extraction

To elucidate the mechanisms of 35K action, key angiogenic markers were detected by Western blot. To measure protein expression, HCAECs were plated in 6 well plates at a cell density of 1.5x10⁵ cells/mL for inflammation and 8x10⁴ cells/mL for hypoxia.

For the experiments with an inflammatory stimulus, cells were incubated for 4 hours in either control conditions of MesoEndo endothelial media only or the inflammatory stimulus comprised of 50% (v/v) MCM + 50% (v/v) MesoEndo endothelial media and 35K (0 – 200 nM). Likewise in hypoxia, cells were incubated for 4 hours in MesoEndo endothelial media with or without 35K (50 – 200 nM) in hypoxia (1% O₂/5% CO₂) or normoxia.

To collect treated cells, media was removed and cells washed once in cold PBS. Cells were scraped in 100 µL radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM sodium orthovanadate, and 0.2% Triton X-100; pH 7.4), protease inhibitor cocktail (1:100, P8340, Sigma-Aldrich), and phenylmethanesulfonyl fluoride solution (93482, Sigma-Aldrich), and transferred to Eppendorf tubes. Each sample was sonicated for 2 seconds to further lyse the sample. Samples were centrifuged for 10 minutes at 14,000 rpm at 4°C and stored at -80°C.
2.2.8.2 Nuclear extraction

To extract the nuclear fraction of incubated cells the NE-PER Nuclear and Cytoplasmic Extraction kit (PIE78833, Thermo Fisher Scientific) was used. In 6 well plates, cells were washed in cold PBS prior to adding 100 µL ice cold CER I reagent to each well. Cells from each well were scraped on ice and transferred to Eppendorf tubes kept on ice. Tubes were vortexed for 15 seconds before incubation on ice for 10 minutes. Following this, 5 µL CER II reagent was added, tubes were vortexed for 5 seconds and allowed to incubate on ice for 1 minute. Tubes were vortexed once more prior to centrifugation at 16,000 g for 5 minutes at 4°C. The supernatant containing the cytoplasmic fraction was transferred to a new tube and stored at -80°C. The pellet containing the nuclear fraction was resuspended in 50 µL ice cold NER reagent by vortexing for 15 seconds at 10 minute intervals for a total of 40 minutes. Samples were kept on ice between vortexing. Finally samples were centrifuged at 16,000 g for 10 minutes and the supernatant containing the nuclear fraction was transferred to new Eppendorf tubes and stored at -80°C.

2.2.8.3 Protein estimation

The protein concentration for each sample was determined using the Bicinchoninic (BCA) Protein Assay (Thermo Fisher Scientific). A standard curve using bovine serum albumin (BSA, A7906-100G, Sigma-Aldrich) concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 mg/mL was constructed from 2 mg/mL BSA stock as prepared in Table 2.1.
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<th>BSA (mg/mL)</th>
<th>BSA stock (μL)</th>
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The assay was conducted in a 96 well plate. Each well contained 10 μL of standard or sample in duplicate, and 190 μL of reagent mix consisting of 50 parts reagent A (23223, Thermo Fisher Scientific) and 1 part reagent B (copper (II) sulphate) (23224, Thermo Fisher Scientific). The assay was incubated at 37°C for 30 minutes and absorbance of each well measured at 595 nm using microplate reader (Flex Station 3, Molecular Devices, California, USA) using the SoftMax Pro program (v5.3, Molecular Devices, California, USA) for microplate data acquisition and analysis. The protein concentration of unknown samples were interpolated from the standard curve.

2.2.8.4 Western blot

Extracted whole cell protein lysate was used to determine changes in VEGF, HIF-1α, PHD1-3. The nuclear fraction was used to detect protein levels of the active subunit of NF-κB (p65).

Equal protein (20 μg for VEGF, HIF-1α, PHD1, PHD2, PHD, 3 μg for p65) was loaded on each gel. To prepare samples, protein was added to 5 μL Novex® LDS sample buffer (Life Technologies) with 1 μL Novex® reducing agent (Life Technologies) and made up to 40 μL with distilled H₂O. Samples were denatured by heating at 95°C for 5 minutes before placing on ice for 2 minutes. Samples and SeeBlue® Plus2 Pre-stained Protein Standard (Life Technologies) were loaded into 12 well Bolt® 4% Bis-tris plus gel (Life Technologies) and run at 100 V for 90 minutes in Bolt® MES SDS Running Buffer
(Life Technologies) diluted in distilled H₂O. Proteins from the gel were then transferred onto nitrocellulose membranes (Life Technologies) using the iBlot Gel Transfer system (Life Technologies) run at 20 V for 7 minutes. Membranes were blocked in 10% (w/v) skim milk in TBST (20 mM Tris, 136 mM NaCl, and 0.1% [w/v] tween 20, pH 7.4) for 1 hour at room temperature on a rocking platform. Following this, membranes were washed 3 times in TBST for 5 minutes at room temperature before probing with primary antibodies for either rabbit anti-VEGF (1:1000, Ab46154, Abcam, Cambridge, UK), mouse anti- HIF-1α (1:1000, NB100-105, Novus Biologicals, Colorado, USA), mouse anti-PHD1 (NB100-310, Novus Biological), mouse anti-PHD2 (NB100-2219, Novus Biological), mouse anti-PHD3 (NB100-303, Novus Biological), or rabbit anti-NF-κB p65 (1:1000, Ab32536, Abcam) in 2% (w/v) skim milk overnight at 4°C. The membranes were washed before incubating with goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase (HRP) (1:1000, sc-2030, Santa Cruz Biotechnologies) or goat anti-mouse secondary antibody conjugated with HRP (1:1000, sc-2005, Santa Cruz Biotechnologies) in 2% (w/v) skim milk in TBST for 2 hours at room temperature. To account for protein loading, membranes were probed for either anti-α-tubulin (AB40742, Abcam) in the whole cell lysate samples or anti-TATA-binding protein TBP (AB818, Abcam) for the nuclear fractions.

Membranes were developed by chemiluminescence on the Chemidoc MP Imaging System (170-8280, BioRad) with ECL reagents in a 1:1 ratio of Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent (170-5061, Biorad). Images were recorded using Image Lab Software (v.5.0, 170-9692, BioRad).
2.3.  *In vivo* methodology

2.3.1. Animal studies

All experimental procedures and protocols were conducted with approval from the Central Sydney Area Health Service Animal Welfare Committee (#2013/027A), and conformed to the Guide for the Care and Use of Laboratory Animals (USA National Institute of Health). For all animal studies male C57BL/6J mice were used at 6 – 8 weeks old. Mice were housed for up to one week in quarantine prior to experimentation. Four *in vivo* studies were completed; these included the peri-arterial cuff model, the hindlimb ischaemia model, the tumour neovascularisation model, and the wound healing model.

The peri-arterial cuff model, is a model of inflammatory-driven angiogenesis. A non-occlusive polyethylene cuff is placed around the femoral artery. This induces inflammation, which triggers macrophage recruitment. The macrophages secrete growth factors to the site of injury. The opposite leg is sham operated. The hindlimb ischaemia model is a model of hypoxia/ischæmia-mediated angiogenesis. The femoral artery and vein is removed from the hindlimb to induce ischaemia. The opposite leg is sham operated. The tumour neovascularisation model, involves injecting Lewis Lung Carcinoma (LLC) cells into the right flank of the treatment mice. The tumour grows over a time period of 12 days and angiogenesis in this model is driven by both hypoxia and inflammation. For the wound healing model, where angiogenesis is critical in the early phases of the process, subcutaneous wounds are created on the back of the mice and silicone splints were secured to mimic the human process of wound healing. Expanded details of these animal models will be discussed in the relevant chapters.

For the peri-arterial cuff, hindlimb ischaemia, and tumour neovascularisation model mice were injected intravenously with adenovirus overexpressing either 35K (Ad35K) or GFP (AdGFP) or PBS three days prior to surgery. While for the wound healing model, topical delivery of treatments (purified 35K protein or PBS vehicle control) were used.

2.3.2. Tail-vein injection

For systemic delivery of adenovirus, intravenous injections were performed via the tail vein using 27 gauge x ½” Terumo insulin needle (SS10M2713, Terumo Medical
Corporation, New Jersey, USA). The mouse was warmed prior to injection and the animal was restrained during the procedure. Frozen virus was diluted in HT-PBS to a final injection volume of 200 µL.

2.3.3. **Sacrifice**

Sacrifice was performed by an overdose of methoxyflurane anaesthetic followed by right ventricular puncture. For peri-arterial cuff model this was followed by circulatory perfusion with 10 mL of saline via the left ventricle.

Mice were dissected and tissue, liver and blood samples were collected for histology, lipid profile, mRNA expression, and protein expression analyses.

2.3.4. **Blood collection**

At sacrifice, blood was collected by right ventricular puncture with a heparinised 1 cc/mL syringe. Blood was kept on ice until centrifugation at 5,000 rpm for 5 minutes. Plasma was stored in 50 µL aliquots at -80°C.

2.3.5. **Laser Doppler**

The Laser Doppler imaging system (moorLDI2-IR, Moor instruments, Devon, UK) was used to determine blood flow perfusion to the site of injury. This was measured in the hindlimb ischaemia and wound healing models. Mice were anaesthetised by inhalation of methoxyflurane. After imaging mice were allowed to recover before returning to housing.

For the hindlimb ischaemia study, mice were placed in the dorsal recumbent position to image the left and right hindlimbs. Images were taken pre- and post-surgery and at 2, 4, 7, 10, 12 and 14 days post-surgery, with three scans taken per mouse at each time point.

For the wound healing study, mice were placed flat in the prone position to image the dorsum. Images were taken at either 1, 2, 3, and 4 days post-surgery for the early stage
cohort or at 2, 4, 7, 10 days post-surgery for the late stage cohort, with three scans taken per mouse at each time point.

2.3.6. **Histology**

2.3.6.1 **Sample preparation**

Samples from the peri-arterial cuff and wound healing models, harvested for paraffin processing were placed in 4% (v/v) paraformaldehyde in PBS solution for 2 hours at room temperature before storing in 70% ethanol for processing. Samples were infused with paraffin using automated overnight processing with graded ethanol concentrations and then paraffin in a 7-hour run, before being embedded in blocks and stored at room temperature.

Samples taken from the hindlimb ischaemia and tumour neovascularisation models, for fresh frozen processing were immediately placed in OCT, snap frozen in liquid nitrogen and stored at -80°C.

2.3.6.2 **Immunofluorescence**

Samples from the hindlimb ischaemia and tumour neovascularisation models, were sectioned on the CryoStar™ NX70 Cryostat (Thermo Fisher Scientific) at 7 μm using the MX35 Premier Disposable Low-Profile Microtome Blades (3052835, Thermo Fisher Scientific) and mounted on to StarFrost® advanced adhesive slides (G313, Proscitech) before drying at room temperature for at least 15 minutes. To stain, slides were fixed in cold acetone for 10 minutes and washed in PBS before staining in the DAKO Autostainer Plus (Dako, Glostrup, Denmark). The Autostainer Plus was programmed to rinse slides in 1X Envision™ FLEX wash buffer (K8007, Dako), before proceeding with a 1 hour incubation in primary antibody solution comprised of antibodies for CD31 (1:500, AB25644, Abcam) to assess neovessels, smooth muscle α-actin (1:1000, F3777, Sigma-Aldrich) to assess arterioles, and laminin (1:300, AB79057, Abcam) to assess myocytes, in antibody diluent with background reducing agent (S080983-2, Dako). Following incubation with primary antibody, the autostainer was programmed to rinse slides in wash
buffer two times before a 1 hour incubation with Alexa Fluor 350 secondary antibody (1:500, A21093, Invitrogen, California, USA) in antibody diluent. Coverslips were mounted onto stained slides in fluorescent mounting media. Slides were allowed to dry for 24 hours and kept in the dark before imaging. Samples were imaged at 10X magnification with 3 fields of view per section.

2.3.6.3 Immunohistochemistry

Paraffin samples were sectioned at 5 µm using the microtome (Microm HM 200 Ergostar, Walidorf, Germany) with MX35 Premier Disposable Low-Profile Microtome blades and mounted to StarFrost® advanced adhesive slides, drying overnight at 42°C before staining in Milligan’s Trichrome or staining to detect CD31, smooth muscle α-actin, or CD68. To stain, slides were deparaffinised in 2 x 10 minute incubations of xylene and rehydrated in decreasing ethanol concentrations comprised of 2 x 2 minute washes in absolute ethanol, 90% ethanol and 70% ethanol to water.

2.3.6.3.1 Milligan’s Trichrome

Milligan’s Trichrome stain was used to visualise: muscle (pink), collagen (green), red blood cells (orange/red) and nuclei (purple) as seen in Figure 2.5. This stain was performed only in samples from the peri-arterial cuff and wound healing study. For the trichrome stain, slides were fixed in Bouin’s solution (25088A, Polyscience Inc., Pennsylvania, USA) for 1 hour at 60°C before thoroughly rinsing in running water for 5 minutes. Slides were incubated in solution A (2.25% [w/v] potassium dichromate, 2.25 mL HCl, 25% absolute ethanol [v/v] in distilled H₂O) for 5 minutes. The potassium dichromate acts as a secondary fixative to preserve fine cell structures. Following a quick rinse by immersion in water, slides were stained for muscle by incubating in solution B (0.1% [w/v] acid fuchs in in distilled H₂O) for 5 minutes. This produces a dark pink stain. After rinsing in water a differentiation step to differentiate between muscle and collagen occurs, where slides were incubated in solution C (1% [w/v] phosphomolydbic acid in distilled H₂O) for 5 minutes and rinsed in water. Red blood cells were stained by incubating slides in solution D (2% orange G [w/v], 1% [w/w] phosphomolydbic acid in distilled H₂O) for 10 minutes and rinsed in water. Finally to stain collagen, slides were incubated in solution E (1% [v/v]
acetic acid in distilled H$_2$O) for 2 minutes, before a 10 minute incubation in solution F (0.1% [w/v] Fast green FCF, 0.2% [v/v] acetic acid in distilled H$_2$O) and an additional 3 minute incubation in solution E. Acetic acid was used to further differentiate collagen from muscle and to maximise the intensity of the dye. Slides were dehydrated in increasing gradients of ethanol and cleared in xylene before mounting in dibutyl-phthalate in xylene (DPX).

![Image](image.png)

*Figure 2.5. A Milligan’s trichrome stained femoral artery subjected to peri-arterial cuffing.*

2.3.6.3.2 CD31 staining to detect presence of capillaries

To determine capillaries, CD31 immunohistochemical staining was performed on paraffin embedded tissue sections from the peri-arterial cuff and wound healing models. Antigen retrieval was performed by microwaving sections on a high power setting for 10 minutes in citrate buffer. Slides were cooled in ice water before rinsing in PBST (PBS with 0.1% [v/v] Tween-20) for 5 minutes. Endogenous peroxidase was blocked by incubation in hydrogen peroxide solution (3% H$_2$O$_2$ in methanol) for 30 minutes at room temperature followed by a 5 minute wash in PBST. Non-specific binding was prevented by blocking in goat serum (10% [v/v] normal goat serum [Ab156046, Abcam] in PBS) for 4 hours at room temperature, which was followed by an overnight incubation of primary antibody
CD31 (1:100, Ab28364, Abcam) in antibody diluent at 4°C. Excess primary antibody was removed with two 5 minute washes in PBST, before a 30 minute incubation of pre-diluted HRP secondary antibody α-rabbit (K4011, Dako) for 30 minutes at room temperature. Excess secondary antibody was removed by two 5 minutes washes in PBST, prior to detection of horse radish peroxidase (HRP) enzyme by incubation in 3,3’-Diaminobenzidine (DAB) substrate (K4007, Dako) as per the manufacturer’s instructions. Slides were counterstained with haematoxylin, dehydrated in increasing gradients of ethanol and cleared in xylene before mounting in DPX.

2.3.6.3.3 Smooth muscle α-actin staining to detect presence of arterioles

To determine arterioles, smooth muscle α-actin staining was performed on sample sections. Antigen retrieval was performed by microwaving on high for 10 minutes in citrate buffer (10 mM sodium citrate tribasic dehydrate in 0.05% [w/v] Tween-20 at pH 6.0). Slides were cooled in ice water before rinsing in PBST for 5 minutes. Endogenous peroxidase was blocked by incubation in hydrogen peroxide solution (3% H2O2 in methanol) for 30 minutes at room temperature followed by a 5 minute wash in PBST. Non-specific binding was prevented by blocking in goat serum (10% [v/v] normal goat serum in PBS) for 2 hours at room temperature (ab156046, Abcam). This was followed by overnight incubation with a mouse α-smooth muscle cell-actin primary antibody conjugated to alkaline phosphatase (AP) at 1:100 (A5691, mouse monoclonal, Sigma-Aldrich) at 4°C. Excess primary antibody was removed by two 5 minute washes in PBST, followed by a 5 minute incubation with 200 mM Tris-HCl. Vector Red alkaline phosphatase substrate (SK-5100, Vector Laboratories, California, USA) was then incubated with the sections for 10 minutes and colour change was halted in H2O. Slides were counterstained in haematoxylin, dehydrated in increasing gradients of ethanol and cleared in xylene before mounting in DPX.

2.3.6.3.4 CD68 staining to detect presence of macrophages

To determine macrophage infiltration, CD68 immunohistochemical staining was performed on paraffin embedded tissue sections. Antigen retrieval was performed by microwaving on high for 10 minutes in citrate buffer. Slides were cooled in ice water before rinsing in PBST for 5 minutes. Endogenous peroxidase was blocked by incubation
in hydrogen peroxide solution (3% H₂O₂ in methanol) for 30 minutes at room temperature followed by 5 minute wash in PBST. Non-specific binding was prevented by blocking in goat serum (10% [v/v] normal goat serum in PBS) for 4 hours at room temperature, which was followed by an overnight incubation of primary antibody CD68 (1:100, Ab31630, Abcam) in antibody diluent at 4°C. Excess primary antibody was removed with two 5 minute washes in PBST, before 30 minute incubation of pre-diluted HRP secondary antibody α-rabbit (Dako) for 30 minutes at room temperature. Excess secondary antibody was removed by two 5 minutes washes in PBST, prior to detection of horse radish peroxidase (HRP) enzyme by incubation with DAB substrate as per the manufacturer’s instructions. Slides were counterstained in haematoxylin, dehydrated in increasing gradients of ethanol and cleared in xylene before mounting in DPX.

2.3.6.4 Image capture and processing

Histology samples were photographed using a Zeiss microscope, attached to a digital camera (Axio Imager.Z2, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Images were recorded digitally using a computer-assisted video-imaging system (v1.1.2.0, ZEN 2012, Carl Zeiss Microscopy, Göttingen, Germany). On the first image the megapixel count, aperture, shutter speed, white balance and ISO were set and used for all subsequent images.

Images of arteries from the peri-arterial cuff model were taken at 20X magnification in one field of view. Images of skin samples from the wound healing model were taken at 10X using the tile function to image the whole sample by stitching multiple fields of view. Gastrocnemius muscle was imaged under fluorescent channels for FITC, alexa fluor 350 and phycoerythrin (R-PE) at 10X magnification in 3 fields of view per section. Similarly, tumour tissue was imaged under fluorescent channels for FITC and alexa fluor 350 at 10X magnification in 3 fields of view.
2.3.6.5 **Image quantification**

All images were analysed digitally while being blinded to the treatment allocation. Image-Pro Premier software (v9.0.4, Media Cybernetics, Maryland, USA) was used for all analyses.

For images taken under bright field (artery, from peri-arterial cuff model and wounds, from wound healing model), neovessels, arterioles, macrophages and collagen were quantified by setting the colour threshold of CD31, α-actin, CD68 and collagen (green) staining and optimised visually on the first image respectively, and the same parameters were used for all subsequent images.

For images taken under fluorescence (muscle, from hindlimb ischaemia model and tumour, from tumour neovascularisation model), neovessels, arterioles and myocytes were quantified by setting the light threshold for CD31 and α-actin, and the dark threshold for laminin, and optimised visually on the first image respectively with the same parameters then used for all subsequent images.
2.3.7. *Real-time Polymerase Chain Reaction (RT-PCR)*

Real-time PCR was used to detect changes in mRNA expression from samples obtained from femoral arteries, gastrocnemius muscle, tumours and wounds.

2.3.7.1 *RNA extraction*

Total RNA was isolated from femoral arteries following peri-arterial cuff surgery, gastrocnemius muscle following hindlimb surgery, tumours following the Lewis Lung carcinoma studies and healed wounds following wound healing studies.

To homogenise artery samples from the peri-arterial cuff model, samples were sonicated in frozen 100 μL TRI reagent (Sigma-Aldrich) for 10 seconds at 30% amplitude. To ensure complete homogenisation, samples were sonicated 3 times allowing TRI reagent to harden on dry ice after each sonication.

For hindlimb muscle and wound samples, tissues were homogenised with 1.4 mm zirconium oxide beads (03961-1-103, Bertin Technologies) in 500 μL TRI reagent, using the Precellys 24 homogeniser (Bertin Technologies) at 6,000 rpm for 30 seconds, 4 times. Between homogenisation, samples were centrifuged at 14,000 rpm for 1 minute at 4°C and allowed to rest on ice. Homogenised lysates were transferred to clean Eppendorf tubes.

Following homogenisation of samples, 1/10 volume 1-Bromo-3-chloropropane (BCP) was added to the lysates. Volume was adjusted based on initial TRI reagent volume i.e. 10 μL BCP to artery samples and 50 μL BCP to muscle and skin samples. Samples were vortexed for 15 seconds, ensuring both phases were completely mixed, before centrifugation at 14,000 rpm for 15 minutes at 4°C. The aqueous (top) layer was transferred to a new Eppendorf tube containing 50 μL (for artery) or 250 μL (for muscle and skin) isopropanol and kept at -20°C overnight.

The following day, samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. Supernatant was removed, leaving a small RNA pellet at the bottom of the tube. The pellet was washed by adding 250 μL of ice cold 75% (v/v) ethanol, vortexed and centrifuged again at 14,000 rpm for 10 minutes at 4°C. Ethanol was removed and the sample was air-dried for 10 minutes in the hood. 20 μL pre-warmed nuclease free water was added to the
RNA pellet and resuspended by pipetting. To aid in complete dissolution, samples were agitated in an Eppendorf thermomixer at 300 rpm for 5 minute intervals at 60°C for up to 15 minutes.

For the tumour samples the RNeasy fibrous tissue mini kit (74701, QIAGEN, Hilden, Germany) was used. Tissues were homogenised with 1.4 mm zirconium oxide beads (Bertin Technologies) in 300 µL buffer RTL, using the Precellys 24 homogeniser (Bertin Technologies) spun at 6,000 rpm for 30 seconds, 2 times. Between homogenisation, samples were centrifuged at 14,000 rpm for 1 minute at 4°C and allowed to rest on ice. Homogenised lysates were transferred to clean Eppendorf tubes and 590 µL RNase-free water and 10 µL warmed (to 55°C on heating block) proteinase K was added. Eppendorf tubes were incubated at 55°C for 10 minutes before centrifuging for 3 minutes at 10,000 x g at room temperature. The supernatant was then transferred to a new eppendorf tube, being careful not to remove pellet. To the supernatant, 450 µL ethanol is added and mixed thoroughly. 700 µL of mixture was transferred to an RNeasy Mini spin column and place in a 2 ml collection tube. The column and tube was spun at 10,000 rpm for 15 seconds. After centrifuge, the flow through was discarded and the remainder of the sample mixture was centrifuged and flow through discarded. To the spin column, 350 µL buffer RW1 was added, column was centrifuged for 15 seconds at 10,000 rpm. DNase 1 mix (10 µL DNase1 and 70 µL buffer RDD) was added to the spin column and allowed to incubate for 15 minutes at room temperature. After incubation, 350 µL RW1 was added to the spin column and centrifuged at 10,000 rpm for 15 seconds. Flow through was discarded before adding 500 µL buffer RPE and centrifuged at 10,000 rpm for 15 seconds. Flow through was discarded before centrifuging again in 500 µL RPE at 10,000 rpm for 2 minutes. Finally a new collection tube is used to elute RNA by centrifuging with 50 µL RNase-free water at 10,000 rpm for 1 minute.

The amount of RNA was quantitated using a spectrophotometer (Nanodrop 2000/2000c, Thermo Scientific, Massachusetts, USA). Absorbance was measured at 260 and 280 nm, and the relative purity of the isolated RNA was calculated from the absorbance ratio \((A_{260}/A_{280})\). The RNA concentration was calculated from the absorbance reading at 260 nm, corrected for the conversion factor as follows, 
\[ [RNA] = A_{260} \times 40 \]
For all samples, the ratio was within the range of 1.7 – 2.0. RNA samples were normalised to 100±3 ng/mL and stored at -80°C until further use.
2.3.7.2 Complementary DNA synthesis – reverse transcriptase PCR

To synthesise complementary DNA (cDNA), 300 ng RNA was reverse transcribed using the iScript cDNA synthesis kit (170-8890, Bio-Rad), performed in triplicate.

The PCR cycle was run on the T100 Thermal Cycler (BioRad) using the pre-set protocol for first-strand cDNA synthesis: annealing 25°C for 5 minutes, extension 42°C for 30 minutes, inactivation 95°C for 5 minutes. Triplicate samples were pooled, diluted 1:5 in water and stored at -20°C until ready to use for real-time PCR.

Table 2.2. Reverse transcription reaction mix

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iScript Reverse Transcriptase</td>
<td>0.5</td>
</tr>
<tr>
<td>5X iScript Reaction Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>4.5</td>
</tr>
<tr>
<td>RNA sample</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>
2.3.7.3 **Quantitative real-time PCR**

The mRNA expression of our genes of interest, including CC-chemokines; CCL2, CCL5, inflammatory markers; CD68, p65, hypoxic markers; Siah1/2, PHD1-3, angiogenic markers; VEGF, MIF, HIF-1α CXCL12, tumour markers; p53, BCL-2, caspase 3, caspase 8, caspase 9, Ki67, c-Jun, MMP2, MMP9, were measured quantitatively by real-time PCR.

Primers for our genes of interest were designed in house using the Primer-BLAST program (National Centre for Biotechnology Information, NCBI). Primer sets used were manufactured by Geneworks, Australia and sequences shown in Table 2.3 -
Table 2.8. Primers were optimised by generating standard curves using tissue from the tumour neovascularisation or hindlimb ischaemia model as seen in Figure 2.6. Mouse 36B4 was used as housekeeping gene to correct for variations between samples in RT-PCR efficiency and to control for small differences in sample quantification. Relative changes in mRNA levels of the genes of interest were normalised using the $^{\Delta\Delta}C_t$ method to 36B4.

![Standard Curve](image)

**Figure 2.6.** Example of standard curve generated using tissue from hindlimb ischaemia model for mouse MIF primers.
Table 2.3. Primer sequences for housekeeping gene 36B4 and CC-chemokine

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse 36B4 F'</td>
<td>5'-CAACGGCAGCATTTTATAACC-3'</td>
</tr>
<tr>
<td>mouse 36B4 R'</td>
<td>5'-CCCATTGATGGGAGTGTG-3'</td>
</tr>
<tr>
<td>mouse CCL2 F'</td>
<td>5'-GCTGGAGCATCCACGTT-3'</td>
</tr>
<tr>
<td>mouse CCL2 R'</td>
<td>5'-ATCTTTGGTGGAATGAGTAGCA-3'</td>
</tr>
<tr>
<td>mouse CCL5 F'</td>
<td>5'-GCAAGTGCTCCAATCTTGCA-3'</td>
</tr>
<tr>
<td>mouse CCL5 R'</td>
<td>5'-CTTCTCTGGGACACACA-3'</td>
</tr>
</tbody>
</table>

Table 2.4. Primer sequences for markers of inflammation

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse p65 F'</td>
<td>5'-AGTATCCATAGCCATCCAGAACC-3'</td>
</tr>
<tr>
<td>mouse p65 R'</td>
<td>5'-ACTGCAATTCAAGTCATAGCC-3'</td>
</tr>
<tr>
<td>mouse CD68 F'</td>
<td>5'-GGGCTCTTGGGAACATACAC-3'</td>
</tr>
<tr>
<td>mouse CD68 R'</td>
<td>5'-GTACCGTCACAACCTCC-3'</td>
</tr>
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</table>

Table 2.5. Primer sequences for markers of hypoxia

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse HIF-1α F'</td>
<td>5'-TCCCTGGCTCTTGGGTTGGG-3'</td>
</tr>
<tr>
<td>mouse HIF-1α R'</td>
<td>5'-AACGTAAGCCGCTGACCAG-3'</td>
</tr>
<tr>
<td>mouse Siah1 F'</td>
<td>5'-GACTGCTACAGCATCCACT-3'</td>
</tr>
<tr>
<td>mouse Siah1 R'</td>
<td>5'-GTTCGATGCGGTCGCG-3'</td>
</tr>
<tr>
<td>mouse Siah2 F'</td>
<td>5'-CTAACGCCAGCATCAGGAA-3'</td>
</tr>
<tr>
<td>mouse Siah2 R'</td>
<td>5'-GAACAGCCCTGAGTAGCATA-3'</td>
</tr>
<tr>
<td>mouse PHD1 F'</td>
<td>5'-TAAGGTGCATGGCCTGC-3'</td>
</tr>
<tr>
<td>mouse PHD1 R'</td>
<td>5'-TGGCTGCTGCCGCTTTG-3'</td>
</tr>
<tr>
<td>mouse PHD2 F'</td>
<td>5'-ATCACCTGGATCGAGGCAA-3'</td>
</tr>
<tr>
<td>mouse PHD2 R'</td>
<td>5'-CGTTCGGGCTTTATCTGT-3'</td>
</tr>
<tr>
<td>mouse PHD3 F'</td>
<td>5'-GAGGCGCGCTGGGCAATACT-3'</td>
</tr>
<tr>
<td>mouse PHD3 R'</td>
<td>5'-GGGTTGTCCATGGGCGAA-3'</td>
</tr>
</tbody>
</table>
**Table 2.6. Primer sequences for markers of angiogenesis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse HIF-1α F'</td>
<td>5'-TCCCTTGCTCTTTGTGGTGGGT-3'</td>
</tr>
<tr>
<td>mouse HIF-1α R'</td>
<td>5'-AACGTAAGCGCTGACCCAGG-3'</td>
</tr>
<tr>
<td>mouse VEGF F'</td>
<td>5'-GGCTGGCTGTAACGATGAAG-3'</td>
</tr>
<tr>
<td>mouse VEGF R'</td>
<td>5'-CTCTCTATGTGCTGGCTTTTG-3'</td>
</tr>
<tr>
<td>mouse MIF F'</td>
<td>5'-AGAACACGCGGTGCAGTAAG-3'</td>
</tr>
<tr>
<td>Mouse MIF R'</td>
<td>5'-ATTACGACATGAACCGCTG-3'</td>
</tr>
<tr>
<td>mouse CXCL12 F'</td>
<td>5'-CACCTCGGTGTCTCTTTG-3'</td>
</tr>
<tr>
<td>mouse CXCL12 R'</td>
<td>5'-GGTGAATGCACACAGCTTG-3'</td>
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</table>

**Table 2.7. Primer sequences for markers of proliferation and metastasis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse Ki67 F'</td>
<td>5'-GAACAGACTTTGCTCTGGCCT-3'</td>
</tr>
<tr>
<td>mouse Ki67 R'</td>
<td>5'-GCTGAGTGCTGGCAAGACT-3'</td>
</tr>
<tr>
<td>mouse c-Jun F'</td>
<td>5'-TGGGCACATCACCACTACAC-3'</td>
</tr>
<tr>
<td>mouse c-Jun R'</td>
<td>5'-TCTGAGCTATGCAAGCTACG-3'</td>
</tr>
<tr>
<td>mouse MMP2 F'</td>
<td>5'-CCCATGTGTCTCTGGCCTTC-3'</td>
</tr>
<tr>
<td>mouse MMP2 R'</td>
<td>5'-GTCTAGAGTCGCGGACATCAG-3'</td>
</tr>
<tr>
<td>mouse MMP9 F'</td>
<td>5'-GTCCAGACCAAGGGTACAGC-3'</td>
</tr>
<tr>
<td>mouse MMP9 R'</td>
<td>5'-ATACAGCAGTGACATGAGCG-3'</td>
</tr>
</tbody>
</table>
**Table 2.8. Primer sequences for markers of apoptosis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse p53 F</td>
<td>5'-GGCAACTATGGCTTCCACCT-3'</td>
</tr>
<tr>
<td>mouse p53 R'</td>
<td>5'-CTCCGTCATGTGCTGTGACT-3'</td>
</tr>
<tr>
<td>mouse BCL-2 F'</td>
<td>5'-TACGAGTGATGGCTGGAGA-3'</td>
</tr>
<tr>
<td>mouse BCL-2 R'</td>
<td>5'-CGGAGCGAGAGAATGTC-3'</td>
</tr>
<tr>
<td>mouse Caspase-3 F'</td>
<td>5'-GGGAGCAAGTCAGTGGACTC-3'</td>
</tr>
<tr>
<td>mouse Caspase-3 R'</td>
<td>5'-CCGTACGAGCGAGATGAC-3'</td>
</tr>
<tr>
<td>mouse Caspase-8 F'</td>
<td>5'-CAGGAGACCATCGAGGATGC-3'</td>
</tr>
<tr>
<td>mouse Caspase-8 R'</td>
<td>5'-CCCACCGACTGATGGAAAA-3'</td>
</tr>
<tr>
<td>mouse Caspase-9 F'</td>
<td>5'-AGAACGACCTGACTGCCAAG-3'</td>
</tr>
<tr>
<td>mouse Caspase-9 R'</td>
<td>5'-TGCCCTTGAGAGGAATGCGAG-3'</td>
</tr>
</tbody>
</table>
Table 2.9. Real-time PCR reaction mix

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iQ SYBR Green Supermix</td>
<td>7.5</td>
</tr>
<tr>
<td>Primer F’</td>
<td>0.6</td>
</tr>
<tr>
<td>Primer R’</td>
<td>0.6</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>1.3</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

PCR reactions were performed in triplicate. Primers were used at 20 pM using the iQ SYBR green Supermix (Bio-Rad) in a Bio-Rad Cfx96 thermocycler with the pre-set protocol for real-time PCR. This includes: initial denaturation and enzyme activation 95°C for 3 minutes; 40 cycles of denaturation 95°C for 30 seconds, annealing 60°C for 30 seconds, followed by extension 72°C for 30 seconds. A non-template control (NTC) was included to monitor contamination and primer-dimer formation that could produce false positive results.

2.3.8. Protein analysis

2.3.8.1 Tissue homogenisation

Protein expression was also measured in the samples from the hindlimb ischaemia and wound healing studies. Tissue lysates for ELISA or Western blot were obtained by homogenising gastrocnemius muscles from the hindlimb ischaemia model and the wounds from the wound healing model. Homogenisation was done by lysing in 750 µL cell lysis buffer (0.1 M Tris HCl, 0.1 M NaCl, 0.05 M NaF, 0.005 M Na₄P₂O₇, 1 mL Triton-X 100, pH 7.4), protease inhibitor cocktail (Sigma-Aldrich), and phenylmethanesulfonyl fluoride solution (Sigma-Aldrich) for up to 5 times with a quick spin in between homogenisations. Tubes were kept on ice at all times. After tissue homogenisation, cell debris was pelleted by centrifugation at 1,000 rpm for 1 minute, before cell lysate was collected into a new Eppendorf tube.
2.3.8.2 ELISA

ELISA kits were used to determine the concentration of VEGF (MMV00, R&D Scientific, Minnesota, USA), CCL2 (MJE00, R&D Scientific), CCL5 (MMR00, R&D Scientific) and MIF (SEA698Mu, Cloud-Clone Corp, Texas, USA) protein. For circulating protein levels, blood from mice injected with AdGFP or Ad35K was spun at 2,500 rpm for 5 minutes to collect mouse plasma. For detection of CCL2 and CCL5, 75 µL of plasma was used while 100 µL of plasma was used to measure VEGF levels. For tissue protein levels, from the hindlimb ischaemia model, 200 µg of tissue lysate was used to measure VEGF and MIF, 500 µg of tissue lysate was used to measure CCL5 and 1000 µg of tissue lysate was required for the detection of CCL2 expression. For tissue from the wound healing model, 100 µg of tissue lysate was used to measure VEGF, CCL2, and CCL5.

2.3.8.3 Western blot

Western blot was performed to determine protein levels of PHD1, PHD2, PHD3 as in Section 2.2.8.4 with 50 µg of hindlimb tissue lysate, with even protein loading measured by probing for α-tubulin. Following transfer, membranes were blocked in 3% (w/v) BSA and then probed with antibody diluted in 3% (w/v) BSA.

2.3.9. Boyden chamber assay

The Boyden Chamber assay was used to determine chemokine activity in mouse plasma. Cells were placed in an insert and a solution containing a chemoattractant placed in the bottom chamber to encourage cell migration across the membrane. Human monocyte derived macrophages (HMDMs) in RPMI media were incubated with 5 µM Calcein AM (C34852, Invitrogen) at 37°C for 30 minutes. Cells were spun at 2,500 rpm for 5 minutes and 5x10^5 cells/mL was resuspended in filtered chemotaxis media (25 mM HEPES, 0.1% [w/v] BSA in RPMI media). In a 24 well, 6.5 mm transwells with 3.0 µm pore polycarbonate membrane insert (CLS3415-48EA, Sigma-Aldrich), 2 µL of mouse plasma in 600 µL chemotaxis media was aliquoted into the bottom chamber. HMDMs, as a 100
μL cell suspension, were distributed into each insert. The inserts were then placed inside each well containing the mouse plasma solution.

The plate was incubated for 1 hour at 37°C. For analysis of cell migration on the underside of the membrane, wells and inserts were emptied and washed with PBS. A cotton tip was used to scrape excess cells from the inside of the insert, ensuring that the outer bottom of the insert was not touched. To remove the membrane, a scalpel blade was carefully inserted into the side wall of the membrane and the membrane carved out. The membrane was mounted to slides cell side up with fluorescent mounting media. Five images per membrane were taken at 10X magnification. Images were analysed using Image Pro software.

2.3.10. Total cholesterol

Total cholesterol concentration was determined using the Cholesterol E kit (439-17501, Wako Diagnostics, Virginia, USA). The kit is an enzymatic colorimetric method for the quantitative determination of total cholesterol in serum. Standards were provided in the kit. To a 96 well plate, 10 μL of mouse plasma or standard was added to 190 μL colour reagent and incubated at 37°C for 5 minutes. Plates were read at an absorbance of 505 nm on a microplate reader (Flex Station 3, Molecular Devices, California, USA) using the SoftMax Pro program (v5.3, Molecular Devices, California, USA) for microplate data acquisition and analysis. Total cholesterol for unknown samples were interpolated from the standard curve.

2.3.10.1 HDL and LDL Cholesterol

Plasma (30 μL) was mixed 1:1 with 20% (w/v) polyethylene glycol (PEG) (81260, Sigma-Aldrich) in H₂O and incubated at room temperature for 5 minutes. Subsequently, samples were centrifuged at 13,000 rpm for 5 minutes to pellet the non-HDL precipitate. Total cholesterol was then determined using 10 μL of the HDL-containing supernatant, as per the same protocol as described in 2.3.10. LDL levels were determined by subtracting total HDL from total cholesterol.
2.3.11. Triglycerides

Triglyceride levels were determined using the Triglyceride E kit (432-40201, Wako Diagnostics). Standards were provided in the kit. To a 96-well plate, 10 μL mouse plasma or standard was added to 190 μL colour reagent and incubated at 37°C for 5 minutes. Plates were read at an absorbance of 600 nm on a microplate reader (Flex Station 3, Molecular Devices, California, USA) using the SoftMax Pro program (v5.3, Molecular Devices, California, USA) for microplate data acquisition and analysis. Total triglycerides for unknown samples were interpolated from the standard curve.

2.3.12. Statistics

Data was analysed on GraphPad Prism software (v6.0, Software MacKiev, California, USA). All results are expressed as mean±SEM. All data were compared using either an unpaired t-test or a One-way ANOVA, followed by post hoc comparison using Tukey’s multiple comparison tests, with p<0.05 considered statistically significant.
CHAPTER 3. 
CONDITIONAL 
REGULATION OF 
ANGIOGENESIS BY 35K 
IN KEY FUNCTIONAL 
ASSAYS IN VITRO
3.1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is induced by both hypoxia and inflammation. Hypoxia-mediated angiogenesis is essential during embryo development, but more importantly plays a critical role in postnatal physiological processes such as wound healing and tissue regeneration in response to tissue ischaemia. Uncontrolled inflammatory-driven angiogenesis occurs in diseases such as cancer and atherosclerosis and causes the exacerbation of the disease. Upon activation of angiogenesis, endothelial cells proliferate and migrate towards the site of angiogenic stimulation where they then assemble into tubular structures to form the basis of new blood vessels (Carmeliet 2000). Current anti-angiogenic therapies inhibit angiogenesis in all pathophysiological contexts, resulting in severe side effects. This highlights the need for alternate therapeutic strategies that can specifically target inflammatory-driven angiogenesis whilst having minimal or no effects in hypoxia-mediated angiogenesis.

CC-chemokines have been show to play a dominant role in diseases associated with inflammation, but their effects in angiogenesis across all pathophysiological contexts are not well established. Previous in vitro studies have found that a host of CC-chemokines including CCL1 (Bernardini et al. 2000), CCL2 (Stamatovic et al. 2006), CCL5 (Liu et al. 2014), CCL11 (Salcedo et al. 2001), CCL15 (Hwang et al. 2004), and CCL16 (Strasly et al. 2004) augment endothelial cell migration and tubule formation, highlighting the critical role of the CC-chemokine family in two key cellular angiogenic processes. Interestingly, CC-chemokines were unable to directly promote endothelial cell proliferation (Bernardini et al. 2000; Hwang et al. 2004; Salcedo et al. 2001; Strasly et al. 2004). Instead it has been shown that CC-chemokines facilitate endothelial cell proliferation by promoting the production of the key pro-angiogenic mediator VEGF through the PI3K/Akt signalling pathway (Liu et al. 2014; Strasly et al. 2004). Taken together, these studies suggest that CC-chemokines are critical to angiogenesis, however whether they are involved in both inflammatory-driven and hypoxia-mediated angiogenesis has not been fully elucidated in vitro.

The current study aims to elucidate the importance of CC-chemokines in angiogenesis upon either inflammatory or hypoxic stimulation using in vitro functional assays representing the 3 key cellular angiogenic processes. These assays were conducted in human coronary artery endothelial cells (HCAEC) to measure: (1) proliferation assessed
by incorporation of 5-ethynyl-2′-deoxyuridine (EdU), (2) migration assessed by scratch assay and (3) tubulogenesis assessed by matrigel assay. For these studies, HCAECs were incubated with purified 35K protein, in inflammatory (stimulated with 50% [v/v] macrophage conditioned media, MCM) or hypoxic conditions (1% O₂). The use of MCM from IFN-γ stimulated human monocyte derive macrophages was chosen due to its physiological relevance in inflammation-stimulated angiogenesis (Prosser et al. 2014). Stimulated macrophages secrete every pro-inflammatory cytokine and growth factor required to stimulate angiogenesis.
3.2. Methods

3.2.1. Endothelial cell proliferation under inflammation and hypoxia

In this study, the Click-iT® EdU Alexa Fluor 488 assay was used, where EdU is taken up by newly proliferating cells during DNA synthesis and the percentage of cells in S-phase was detected by fluorescence using flow cytometry. Cells were serum starved before stimulation to make sure cells were in an unstimulated state and all in a similar cell cycle phase before treatments. Proliferation was tested in both inflammation and hypoxia with 35K treatment at concentrations between 50 nM – 400 nM, to observe any differential effects of 35K.

3.2.2. Endothelial cell migration under inflammation and hypoxia

A scratch assay on collagen coated chamber slides were used to assess HCAEC migration speed in response to 50 nM 35K under inflammatory or hypoxic conditions. This was monitored by live cell imaging over 10 hours.

3.2.3. Endothelial cell tubulogenesis under inflammation and hypoxia

In vitro tubule formation was assessed using growth factor reduced matrigel as the matrix to allow for HCAEC attachment and tubule formation in vitro. Cells were plated on to the growth factor reduced matrigel and stimulated with 30 nM – 50 nM 35K under both inflammation and hypoxia.
3.3. Results

3.3.1. The effect of broad-spectrum CC-chemokine inhibitor 35K on endothelial cell proliferation

As CC-chemokines promote inflammatory-driven angiogenic processes, it was therefore anticipated that broad-spectrum inhibition of CC-chemokines would suppress cell proliferation in response to the inflammatory stimulus of macrophage conditioned media (MCM) but have no effects in response to hypoxia. When cells were incubated with the broad-spectrum CC-chemokine inhibitor 35K, there was a concentration dependant reduction of endothelial cell proliferation in both the unstimulated and inflammatory-stimulated (MCM) conditions (Figure 3.1). Comparative analysis of the effects of 35K in both the unstimulated and inflammatory-stimulated conditions showed that the ability of 35K to suppress proliferation was more pronounced in the inflammatory-stimulated cells, where at the lowest concentration of 50 nM, the ability of 35K to suppress proliferation was only seen in the inflammatory-stimulated cells, with a 23.7% (p<0.05) reduction. As the concentration of 35K increases, the effects of 35K were more prominent in the MCM-stimulated cells; although 35K was also able to significantly suppress proliferation in the unstimulated cells. When unstimulated cells were exposed to 100 nM of 35K, there was a 48.5% (p<0.001) decrease in proliferation. However, at the same concentration, 35K suppressed inflammatory-stimulated proliferation by 60.8% (p<0.001). At the higher concentration range of 200 – 400 nM, the effect of broad-spectrum CC-chemokine inhibition was more striking in the inflammatory-stimulated cells. In the unstimulated control cells, 35K inhibited cell proliferation at the three highest concentrations with a 69.0%, 72.8% and 80.7% decrease seen at 200 nM, 300 nM and 400 nM respectively (p<0.001 for all). 35K also retained its inhibitory effects in cells stimulated by MCM, where a 59.7% decrease was seen at 200 nM of 35K (p<0.001). Finally, at the highest concentrations of 300 nM and 400 nM, 35K attenuated inflammatory-induced proliferation by 85.4% and 88.3% respectively (p<0.001 for all).
Figure 3.1. 35K inhibits endothelial cell proliferation in unstimulated and inflammatory-stimulated endothelial cells.

HCAECs were incubated with PBS or 35K (0 – 400 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. Cell proliferation was assessed by the Click-iT EdU assay. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. **p<0.001 compared to unstimulated control. ^p<0.05, ^^^p<0.001 compared to inflammatory MCM-stimulated control.
Compared to the striking inhibitory effect of 35K in inflammation, broad-spectrum CC-chemokine inhibition by 35K showed more modest inhibitory effects on hypoxia-stimulated proliferation concentration (Figure 3.2). While the inhibitory effects of 35K on proliferation were retained at both the higher concentrations of 300 nM and 400 nM in unstimulated normoxic conditions (25.7%, p<0.05 and 50.0%, p<0.01 decrease respectively), only the highest concentration of 400 nM was effective in inhibiting hypoxia-mediated proliferation compared to its hypoxia-stimulated control (43.9% decrease, p<0.001). These effects were significantly more modest compared to inflammatory-driven proliferation, particularly at the highest concentration of 400 nM, where a near complete inhibition (88.3%) was seen.

**Figure 3.2.** High concentrations of 35K inhibits endothelial cell proliferation in unstimulated and hypoxia-stimulated endothelial cells.

HCAECs were incubated with PBS or 35K (0 – 400 nM) in unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. Cell proliferation was assessed by the Click-iT EdU assay. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05, **p<0.01 compared to unstimulated normoxic control. ^^^p<0.001 compared to hypoxia-stimulated control.
3.3.2. The effect of broad-spectrum CC-chemokine inhibitor 35K on endothelial cell migration

A single concentration of 50 nM was chosen to elucidate the effects of broad-spectrum CC-chemokine inhibition in inflammation- and hypoxia-induced cell migration. This medium-level concentration was chosen rather than a higher concentration of 35K as they were strongly inhibitory and may not allow for the detection of conditional regulation by 35K. Only a single concentration of 35K could be assessed in the chamber slide set up of the live cell imager that only allowed for the assessment of four treatments/experiment, including the controls.

In the presence of 50 nM of 35K, migration speed significantly decreased by 22.3% (p<0.05) compared to the inflammatory-stimulated control (Figure 3.3). The ability of 35K to hamper cell migration was not seen in the unstimulated cells.

Similar to the observations seen with the proliferation assay broad-spectrum CC-chemokine inhibition by 35K at a 50 nM concentration did not affect migration speed in hypoxia-stimulated cells (Figure 3.4).
Figure 3.3. 35K reduces endothelial cell migration speed in inflammatory conditions. HCAECs were incubated with PBS or 35K (50 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. Endothelial cell migration was assessed by live cell imaging. Representative images in the upper panel indicate distance between cell fronts 10 hours after the initial scratch. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to inflammatory MCM-stimulated control.
Figure 3.4. 35K does not effect hypoxia-stimulated endothelial cell migration speed. HCAECs were incubated with PBS or 35K (50 nM) in unstimulated normoxic or hypoxia-stimulated (1% O$_2$/5% CO$_2$) conditions. Endothelial cell migration was assessed by live cell imaging. Representative images in the upper panel indicate distance between cell fronts 10 hours after the initial scratch. Data is expressed as mean±SEM.
3.3.3. The effect of broad-spectrum CC-chemokine inhibitor 35K on endothelial cell tubulogenesis

The final in vitro assay used to elucidate the effects of 35K on angiogenic processes is the matrigel assay that allows cells to attach and form tubules within a cellular matrix, thereby resembling in vivo tubule formation. Three concentrations of 35K within the lower range (30 – 50 nM) were used for this study. Firstly, the inflammatory stimulus (50% [v/v] MCM) significantly increased (55.0% p<0.01) endothelial cell tubule formation compared to unstimulated cells (Figure 3.5). Similar to our observations in the proliferation and migration assays, we found that 35K was able to significantly inhibit inflammatory-driven tubule formation, even at the lower concentration range of 30 – 50 nM, with 58.1% (p<0.01), 43.9% (p < 0.05) and a near complete 95.8% (p<0.001) inhibition seen at 30, 40 and 50 nM respectively. Furthermore, the ability of 35K to inhibit tubule formation was also observed in the unstimulated cells, with 41.2% (p<0.05) and 100% (p<0.001) reductions seen at 30 nM and 50 nM respectively.

As was seen in the earlier angiogenic assays, the inhibitory effects of 35K were substantially more modest under hypoxic stimulation compared to inflammation. When compared to the hypoxia-stimulated control, 35K attenuated tubule formation in a concentration dependent manner with decreases of 25.4%, 30.9% and 45.3% seen at 30, 40 and 50 nM of 35K respectively (all p<0.01, Figure 3.6). 35K was more potent in suppressing tubule formation in the unstimulated control cells with decreases of 40.4% (p<0.05), 72.6% (p<0.001), and 72.0% (p<0.001) at 30, 40 and 50 nM respectively.
**Figure 3.5.** 35K inhibits endothelial cell tubule formation in unstimulated and inflammatory-stimulated cells.

HCAECs were incubated with PBS or 35K (0 – 50 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. Endothelial cell tube formation was assessed by matrigel assay. Representative images in the upper panel indicate tubule formation. Scale bars represent 100 µm. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 compared to unstimulated control. ^p<0.05, ^^p<0.01, ^^^p<0.001 compared to inflammatory MCM-stimulated control.
Figure 3.6. 35K inhibits endothelial cell tubule formation in unstimulated and hypoxia-stimulated cells.

HCAECs were incubated with PBS or 35K (0 – 400 nM) under unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. Endothelial cell tube formation was assessed by matrigel assay. Representative images in the upper panel indicate tubule formation. Scale bars represent 100 μm. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 compared to unstimulated normoxic control. ^^p<0.01 compared to hypoxia-stimulated control.
3.4. Discussion

*In vitro* functional angiogenic assays are an important tool to determine the key steps modulated in the process of angiogenesis by a treatment. To elucidate the effects of broad-spectrum CC-chemokine inhibition, three key functional assays that reflected the key cellular processes involved in angiogenesis, namely endothelial cell proliferation, migration and tubule formation, were used. We have shown for the first time that broad-spectrum CC-chemokine inhibition by 35K conditionally regulates functional angiogenic assays *in vitro*. In this study we found that 35K significantly reduced inflammatory-driven angiogenesis across all three key functional assays in a concentration dependant manner. However, the effects of 35K on these assays in response to hypoxia were more modest by comparison.

The first assay, proliferation, assessed the ability of endothelial cells to divide from a quiescent state when treated with broad-spectrum CC-chemokine inhibitor, 35K, under inflammation or hypoxia. We found that 35K significantly attenuated inflammatory-driven proliferation at higher concentrations (200 – 400 nM), whilst at these same concentrations, these effects were more modest in response to hypoxic stimulation. The requirement for high concentrations of 35K suggests a less significant role for CC-chemokines in the stimulation of proliferation. This aligns with previous studies in which increased CC-chemokine levels had no effect on proliferation compared to bFGF or VEGF stimulation (Hwang et al. 2004; Strasly et al. 2004), although we were able to induce inhibition at the higher concentrations. Previous studies have shown that CCL2 is highly expressed during proliferation of smooth muscle cells (Salcedo et al. 2000). However it appears that CCL2 has different effects depending on the cell type as it was shown to have no effect on proliferation in endothelial cells (Weber et al. 1999). Our broad-spectrum CC-chemokine inhibition findings suggest that other members of the CC-chemokine family may play a more important role in endothelial proliferation than CCL2, and therefore our broad-spectrum approach is more effective. Furthermore, given that the effects of 35K protein were only seen at the higher concentrations, this suggests that the CC-chemokines involved in endothelial cell proliferation may not be highly expressed in endothelial cells.

The second functional assay performed was the migration assay to replicate endothelial cell migration *in vitro*. The assay was performed in 4 well chamber slides, limiting the number of concentrations tested to one per experiment. The 50 nM
concentration was chosen as higher concentrations showed complete inhibition of migration (data not shown). This study found that broad-spectrum CC-chemokine inhibition, even at a small concentration of 35K, reduced the speed of migration in inflammation but had no effect in hypoxia. This verifies the important role of CC-chemokines in endothelial cell migration as a medium-level concentration caused significant inhibition of migration. This suggests that several CC-chemokines may be involved in the migration of endothelial cells. Alternatively, there may be a high abundance of a few specific CC-chemokines that play an important role in stimulating the migration of endothelial cells. Previous studies show that the CC-chemokine CCL2 directly phosphorylates cytoskeletal proteins essential for endothelial cell migration (Galvez et al. 2005). Additionally, CCL1 was shown to stimulate chemotaxis and invasion of endothelial cells in vitro (Bernardini et al. 2000).

The final assay to assess angiogenesis in vitro was the matrigel tubulogenesis assay. Tubule formation was assessed using growth factor reduced matrigel where endothelial cells, plated on matrigel, form a network of tube-like structures over 4 – 6 hours. Inhibition of the CC-chemokine class caused near-complete attenuation of endothelial cell tubule formation in inflammatory conditions, particularly at the 50 nM concentration. Interestingly, the effects of 35K on hypoxia-stimulated tubule formation were far more modest at these same concentrations. In previous studies, tubulogenesis was induced with the incubation of CC-chemokines CCL2, CCL11, and CCL16 in vitro (Salcedo et al. 2001; Stamatovic et al. 2006; Strasly et al. 2004). Endothelial neovessel formation was also directly stimulated by CCL2 (Galvez et al. 2005). Our findings suggest that CC-chemokines might be more important in mediating inflammatory-driven tubule formation, whilst exerting only minimal regulatory effects on hypoxia-mediated tubulogenesis.

Overall, our observations from this study found that the effects of broad-spectrum CC-chemokine inhibition by 35K were more modest in the three in vitro functional angiogenic assays in hypoxia, suggesting that broad-spectrum CC-chemokines may play a minimal role in promoting physiological hypoxia-driven angiogenesis. Previous studies have shown that the expression of CCR5 and the production of CCL2 are inhibited following hypoxic stimulation, resulting in an inhibition of macrophage recruitment to hypoxic sites (Bosco, Puppo, et al. 2004; Bosco, Reffo, et al. 2004; Turner et al. 1999). Furthermore in hypoxic conditions, the CXC chemokines such as CXCL1, CXCL2,
CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, and CXCL12 are the dominant chemokines and are expressed to regulate vascular repair (Frangogiannis 2007; Schober 2008). CXC-chemokines with the ELR (Glu-Leu-Arg) motive such as CXCL1, CXCL2, CXCL5 and CXCL8 are also involved in the recruitment of neutrophils to the ischaemic site (Bizzarri et al. 2006; Clark-Lewis et al. 1995; Clark-Lewis et al. 1991).

In conclusion, we have shown that broad-spectrum CC-chemokine inhibition by 35K conditionally regulated angiogenesis-related *in vitro* processes. 35K has striking inhibitory effects in inflammation and more modest or no effects in hypoxia. Our findings show that concentrations of 35K as low as 50 nM were sufficient to see significant reductions in both inflammatory-driven endothelial cell migration and tubule formation, whilst upon hypoxic stimulation, this concentration had no effect. Similarly endothelial cell proliferation was significantly reduced with 35K in inflammation but these effects were again more modest in response to hypoxia at the same concentration. Furthermore, 35K required higher concentrations of up to 400 nM to exhibit its inhibitory effects in endothelial cell proliferation. These findings correlate with previous studies in which CC-chemokines are reported to be highly involved in migration and tubulogenesis of endothelial cells but appear to play a lesser role in proliferation. The mechanism by which 35K exerts the conditional regulation of angiogenesis remains to be explored. Accordingly, the aim of our next chapter was to examine the cellular mechanisms by which 35K conditionally regulates angiogenesis.
CHAPTER 4.
ELUCIDATING THE MECHANISMS OF CC-CHEMOKINE INHIBITION BY 35K IN VITRO
4.1. Introduction

Our functional in vitro angiogenic assay studies in Chapter 3 showed that broad-spectrum CC-chemokine inhibition with 35K conditionally regulates angiogenesis, suppressing inflammatory-driven angiogenesis with more modest or minimal effects on hypoxia-mediated angiogenesis. This suggests that the CC-chemokine class play a more important role in inflammatory-driven angiogenesis and little to no role in hypoxia-mediated angiogenesis. However, the mechanism by which 35K exerts these differential effects remains to be elucidated. The studies outlined in this chapter sought to elucidate the role of 35K on two critical signalling pathways involved in angiogenesis.

Angiogenesis is driven by two distinct yet overlapping pathways, depending on the angiogenic stimulation. Inflammatory-driven angiogenesis is driven by the key inflammatory transcription factor nuclear factor-κB (NF-κB). A subunit of NF-κB, p65 also known as RelA, is critical in activating the canonical pathway of NF-κB for innate immunity and cell survival (Beg & Baltimore 1996). Conversely, hypoxia-inducible factor-1α (HIF-1α) is the main transcription factor for hypoxia-mediated angiogenesis. HIF-1α can also be post-translationally modulated by prolyl hydroxylases (PHD 1-3). Under normoxic conditions the PHDs hydroxylate HIF-1α allowing von Hippel-Lindau proteins (pVHL) to bind, targeting it for proteasomal degradation (Berra, Ginouvès & Pouysségur 2006). However under hypoxic conditions, the action of PHDs are inhibited by the activation of the E3 ubiquitin ligases Siah1 and Siah2 (Nakayama et al. 2004). Both NF-κB and HIF-1α augment the expression of the key pro-angiogenic mediator VEGF. In angiogenesis VEGF promotes growth of endothelial cells derived from arteries, veins and lymphatics (Ferrara & Davis-Smyth 1997). Additionally, VEGF promotes monocyte/macrophage migration from the bone marrow (Clauss et al. 1990; Shibuya 2011). In inflammatory-driven angiogenesis, the expression of VEGF expression is modulated by NF-κB, as seen in tumour and plaque angiogenesis studies (Celletti et al. 2001; Ferrara 2004; Ferrara, Gerber & LeCouter 2003). Conversely in hypoxia-mediated angiogenesis, VEGF is regulated by HIF-1α (Forsythe et al. 1996; Liu et al. 1995). This multi-conditional regulation of VEGF is possible, as VEGF has response elements for both NF-κB and HIF-1α in its promoter region that allows for activation of both signalling pathways (Ramanathan et al. 2007).
Previous studies show that CC-chemokine expression can be triggered by both the NF-κB and HIF-1α signalling pathways. NF-κB binding motifs have been found on CC-chemokines including CCL2 and CCL5 (Nelson et al. 1993; Ueda et al. 1994) possibly complementing their role in inflammatory-driven angiogenesis. Furthermore, CC-chemokines have indirect effects in inflammatory-driven angiogenesis, whereby CC-chemokines recruit monocytes/macrophages to the inflammatory site that secrete pro-angiogenic growth factors and cytokines. Interestingly, despite having no reported role in hypoxia-mediated angiogenesis, CCL2, CCL5, and CCL12 were found to contain hypoxic response elements in its promoter region (Mojsilovic-Petrovic et al. 2007; Yeligar et al. 2009). These were studied in pathologies where ischaemia is critical, such as in allergic airway inflammation or ischaemic brain damage (Baay-Guzman et al. 2012; Mojsilovic-Petrovic et al. 2007). Additionally, HIF-1α was induced by CCL5 in the liver under chronic alcohol consumption (Yeligar et al. 2009).

Based on our findings in Chapter 3, we hypothesise that 35K through its inhibition of CC-chemokines reduces inflammation and subsequently prevents further activation of NF-κB. This, in turn, prevents the activation of endothelial cell migration, proliferation and tubule formation. However under hypoxia we propose that 35K will have no effect on HIF-1α, thereby allowing for the preservation of hypoxia-mediated angiogenesis.
4.2. Methods

4.2.1. Cell culture treatments

To determine the mechanism of action of 35K, HCAECs were treated with 35K (0 – 200 nM) and subjected to either inflammatory or hypoxic stimulation as detailed in Chapter 2 (2.2.7). Protein was isolated from whole cell lysates or nuclear fraction from treated cells (Section 2.2.8) and run on a Western blot to determine the expression of the key angiogenic markers including p65 (active subunit of NF-κB), HIF-1α, VEGF, and PHD1-3. Even protein loading was confirmed by probing for either α-tubulin for whole cell lysates or TATA-binding protein (TBP) for nuclear fractions.
4.3. Results

4.3.1. The effect of broad-spectrum CC-chemokine inhibitor 35K on p65 in inflammation

Western blot analysis revealed a non-significant 107.7% (~2 fold) increase in nuclear p65 protein levels in response to the inflammatory stimulus (MCM) compared to unstimulated control (Figure 4.1). However, despite no significant changes, 35K appeared to suppress this increase, although these effects were not significant. The addition of 50 nM of 35K reduced p65 nuclear levels by 35.9% and 200 nM of 35K resulted in a 37.3% reduction when compared to inflammatory MCM-stimulated controls. Unexpectedly, in the unstimulated cells, non-significant increases of p65 levels were detected in the cells treated with 35K when compared to the unstimulated control, where incubation with 35K increased p65 protein levels by 256.6% at the lower 50 nM dose, but only a 91.1% increase was seen at the higher dose of 200 nM.
Figure 4.1. 35K did not affect p65 protein levels in inflammatory-stimulated endothelial cells.

HCAECs were incubated with PBS or 35K (0 – 200 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. Nuclear p65 protein levels were assessed by Western blot analysis. Even protein loading was confirmed by TATA-binding protein (TBP). Data is expressed as mean±SEM.
4.3.2. The effect of broad-spectrum CC-chemokine inhibitor 35K on HIF-1α in inflammation and hypoxia.

HIF-1α has an NF-κB response element upstream of its promoter region. As CC-chemokines mediate inflammatory cellular responses, the effects of 35K on HIF-1α protein levels were measured in both inflammation and hypoxia. Upon inflammatory stimulation with MCM, HIF-1α protein levels increased by 45.7% (p<0.05), compared to the unstimulated control (Figure 4.2). More importantly, HIF-1α protein levels were significantly decreased in cells treated with 35K at both the 50 nM (68.8%, p<0.01) and 200 nM (73.5%, p<0.001) concentrations. However, the inhibition of HIF-1α protein by 35K was more modest in the unstimulated cells with non-significant decreases seen at both 50 nM (26.5%) and 200 nM (58.8%) doses.

In contrast to the inflammatory environment, there was only a non-significant 23.1% increase in HIF-1α protein levels in response to hypoxia compared to the unstimulated controls (Figure 4.3). Compared to the hypoxia-stimulated control, 35K was only able to significantly suppress HIF-1α protein levels at the highest concentration of 200 nM (68.8% decrease, p<0.001), with a non-significant 26.3% decrease seen at the lower concentration of 50 nM. These effects were also observed in the normoxic unstimulated cells, with 35K significantly inhibiting HIF-1α protein levels at the 200 nM concentration (54.9% decrease, p<0.05).
Figure 4.2. 35K reduced HIF-1α protein levels in inflammatory-stimulated endothelial cells.

HCAECs were incubated with PBS or 35K (0 – 200 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. HIF-1α protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to unstimulated control. ^^p<0.01, ^^^p<0.001 compared to inflammatory MCM-stimulated control.
Figure 4.3. 35K reduced HIF-1α protein levels in hypoxia-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0 – 200 nM) in unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. HIF-1α protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to unstimulated normoxic control. ^^^p<0.001 compared to hypoxia-stimulated control.
4.3.3. The effect of broad-spectrum CC-chemokine inhibitor 35K on VEGF in inflammation and hypoxia.

The effects of broad-spectrum CC-chemokine inhibition on the critical pro-angiogenic mediator VEGF was measured next. Upon inflammatory MCM-mediated stimulation, there was a slight, non-significant 23.1% increase in VEGF protein levels compared to the unstimulated controls (Figure 4.4). In inflammation, 35K suppressed VEGF protein levels in a dose dependent fashion, with a non-significant 24.9% decrease seen at the lower concentration of 50 nM, while at the highest concentration of 200 nM, VEGF levels were significantly inhibited by 42.2% (p<0.05). In contrast to the effects seen in inflammation, 35K had no effect on VEGF protein levels in hypoxic conditions (Figure 4.5).
Figure 4.4. 35K reduced VEGF protein levels in inflammatory-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0–200 nM) in the presence of unstimulated control media or macrophage conditioned media (MCM) to stimulate inflammation. VEGF protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to inflammatory MCM-stimulated control.
Figure 4.5. 35K did not affect VEGF protein levels in hypoxia-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0 – 200 nM) in unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. VEGF protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM.
4.3.4. The effect of broad-spectrum CC-chemokine inhibitor 35K on post-translational HIF-1α modulators, the prolyl hydroxylases (PHDs) in hypoxia.

It is well established that HIF-1α can be post-translationally modulated by the prolyl hydroxylase family, of which there are three members (PHD1-3). 35K suppressed HIF-1α protein levels in hypoxia (although not as strikingly as in inflammation) but had no effect on VEGF expression. We therefore sought to determine the effect of 35K on prolyl hydroxylase expression. Following hypoxic stimulation, endothelial cell PHD1 protein levels decreased by 20.6% compared to normoxic controls, although these changes were not significant (Figure 4.6). Overall, 35K had no effects on PHD1 expression, whether in normoxic or hypoxic conditions. A similar pattern was also observed when measuring PHD2 protein levels. Again, there was a non-significant 30.4% decrease in response to hypoxic stimulation compared to unstimulated normoxic controls (Figure 4.7), but no significant effects with 35K were found. These observations were also seen in PHD3 protein levels, where a non-significant 16.2% reduction in PHD3 protein levels was detected in response to hypoxic stimulation (Figure 4.8). However, 35K treatment had no effect on PHD3 expression levels in either normoxic or hypoxic conditions.
Figure 4.6. 35K did not affect PHD1 protein levels in hypoxia-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0 – 200 nM) in unstimulated normoxic or hypoxia-stimulated (1% O$_2$/5% CO$_2$) conditions. PHD1 protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM.
Figure 4.7. 35K did not affect PHD2 protein levels in hypoxia-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0 – 200 nM) in unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. PHD2 protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM.
Figure 4.8. 35K did not affect PHD3 protein levels in hypoxia-stimulated endothelial cells. HCAECs were incubated with PBS or 35K (0 – 200 nM) in unstimulated normoxic or hypoxia-stimulated (1% O₂/5% CO₂) conditions. PHD3 protein levels were assessed by Western blot analysis. Even protein loading was confirmed by α-tubulin. Data is expressed as mean±SEM.
4.4. Discussion

To elucidate the mechanism for the conditional regulation of in vitro angiogenesis by broad-spectrum CC-chemokine inhibition, the effect of 35K on two key signalling pathways that mediate angiogenesis were assessed in this chapter. We found that 35K conditionally regulated VEGF protein levels, such that VEGF was inhibited by 35K in inflammation but had no effect on VEGF in hypoxia. Interestingly, we found that 35K significantly inhibited HIF-1α levels under both stimulated conditions, although these inhibitions were more striking in inflammation and the effects were more modest in hypoxia. This could not be explained by changes in the post-translational regulation of HIF-1α, as 35K had no effect on the expression of the prolyl hydroxylase family (PHD1-3). Finally, 35K had no effect on NF-κB in inflammation.

The current study found that, despite a 37.3% reduction in the protein expression of the active subunit of NF-κB (p65) in inflammation, broad-spectrum inhibition of the CC-chemokine class had no significant effect on p65. Previous studies have demonstrated that specific inhibition of NF-κB in lung epithelial cells reduces inflammation by suppressing CC-chemokine expression of CCL2, CCL5, CCL11, CCL20 (Poynter et al. 2004). The current data showing a lack of change in NF-κB by 35K indicate that the anti-inflammatory/angiogenic effects of 35K are influenced by alternate inflammatory mediators in the promoter region of chemokines such as the TPA response element (TRE) which target AP-1, also known to modulate inflammation (Schonthaler, Guinea-Viniegra & Wagner 2011; Ueda et al. 1994). Interestingly, in unstimulated conditions, 35K increased p65 levels. This was not significant however, it raises the possibility that in these conditions, the inhibition of CC-chemokines causes NF-κB to exhibit cell survival mode characteristics, whereby NF-κB prevents TNF-induced apoptosis in macrophages, T-cells, hepatocytes, and the inhibition of NF-κB increases cell susceptibility to apoptosis (Cardoso & Oliveira 2003; Shih et al. 2011).

While HIF-1α is the primary transcription factor involved in hypoxia-mediated angiogenesis, it can also be driven by the NF-κB signalling pathway as HIF-1α has response elements for both HIF and NF-κB in its promoter region. In hypoxic conditions, HIF-1α is the critical mediator of the cellular angiogenic response. The current study found that HIF-1α was inhibited by 35K in both inflammation and hypoxia, although these effects were more modest in hypoxia. The ability of 35K to suppress HIF-1α significantly
in hypoxia was surprising but these observations could explain why 35K had mild inhibitory effects in the functional angiogenic assays in response to hypoxia. HIF-1α is known to be post-translationally modulated by prolyl hydroxylases (PHDs 1-3), which trigger HIF-1α degradation (Berra, Ginouvès & Pouysségur 2006). Here we find that 35K did not affect PHDs 1-3 under hypoxic conditions. This suggests that 35K may be affecting HIF-1α through a pathway separate to the post translational modification of HIF-1α. In hypoxia, the normal inhibition of PHDs increases NF-κB activation through phosphorylation of its upstream enzyme IKKβ (Cummins et al. 2006), however when IKKβ is inhibited it impairs HIF-1α in hypoxia (Rius et al. 2008), suggesting that NF-κB plays a role in the regulation of HIF-1α in hypoxia. Thus, HIF-1α regulation may be indirectly linked to the regulation of inflammatory-driven angiogenesis by CC-chemokines.

Our studies show that broad-spectrum CC-chemokine inhibition by 35K had differential effects on VEGF expression in response to inflammation and hypoxia, where 35K was able to strikingly suppress inflammatory-driven VEGF expression while having no effect on hypoxia-mediated VEGF augmentation. Conditional regulation of VEGF by 35K is possible as VEGF has response elements for both NF-κB and HIF-1α in its promoter region (Ema et al. 1997; Forsythe et al. 1996; Levy et al. 1995; Shi et al. 2001). It was interesting to note that in hypoxia, VEGF levels in 35K-treated cells were maintained in spite of a decrease in HIF-1α expression. Previous studies have shown that while HIF-1α is the main transcription factor driving VEGF expression in hypoxia-mediated angiogenesis, the NF-κB signalling pathway may also be activated in response to hypoxia. Given that NF-κB can be triggered in response to hypoxia, activation of NF-κB may help to maintain VEGF levels in spite of the suppression of HIF-1α by CC-chemokine inhibition. Furthermore, studies show that CCL2 induces VEGF production through p42/44 MAPK (Hong, Ryu & Han 2004), which has also been shown to induce expression of HIF-1α through non-hypoxic signalling and in turn increased VEGF expression (Bardos & Ashcroft 2004).

In conclusion, the studies of this chapter have found that the conditional regulation of angiogenesis by 35K is mediated via the critical pro-angiogenic mediator VEGF. Moreover, 35K has no effect on post-translational HIF-1α modulation. Overall, the in vitro findings from Chapter 3 and the present chapter have demonstrated that broad-spectrum
CC-chemokine inhibition by 35K suppresses inflammatory-driven angiogenesis whilst having minimal to no effects on hypoxia-mediated angiogenesis in vitro. Whether these differential angiogenic effects of 35K can be translated in vivo was investigated in the next chapter using two murine surgical models of angiogenesis.
CHAPTER 5.
THE EFFECT OF
BROAD-SPECTRUM
CC-CHEMOKINE
INHIBITION IN MODELS
OF ANGIOGENESIS
5.1. Introduction

The studies in Chapters 3 and 4 found that broad-spectrum CC-chemokine inhibition using 35K strikingly suppressed inflammatory-driven functional angiogenesis *in vitro* (Chapter 3) and inhibited key mediators of the inflammatory angiogenic pathway specifically HIF-1α and VEGF (Chapter 4). Conversely, in the context of hypoxia, broad-spectrum CC-chemokine inhibition had minimal to no effects *in vitro*, where 35K had no effect on proliferation, migration, and VEGF expression. The aim of this study was to determine if the observed *in vitro* effects of 35K could be translated *in vivo* in murine models of angiogenesis. To compare inflammatory-driven and hypoxia-mediated models of angiogenesis *in vivo*, the peri-arterial femoral cuff model and hindlimb ischaemia model were utilised.

Inflammatory-driven angiogenesis is regulated primarily by the NF-κB signalling pathway which creates an inflammatory response that then promotes the recruitment of monocytes/macrophages to the inflamed sites. Recruited macrophages, in turn, secrete pro-angiogenic proteins such as VEGF, bFGF, TNF-α, and IFN-γ (Mantovani et al. 2008; Sunderkotter et al. 1994) that promote neovascularisation. In hypoxia, HIF-1α is the key transcription factor that drives the angiogenic response. HIF-1α promotes the recruitment of endothelial progenitor cells (EPC) to the ischaemic site, by upregulation of CXCL12. The pro-angiogenic mediator VEGF is a critical stimulator of angiogenesis in both inflammation and hypoxia. In inflammation, VEGF expression is released by macrophages at the injury site. Macrophages are recruited by CC-chemokines, however MIF (macrophage migration inhibitory factor) also plays a role in the recruitment of macrophages particularly in inflammation.

Previous *in vivo* studies with 35K found that CC-chemokine activity was inhibited in plasma and aortas (Ali et al. 2005; Bursill et al. 2009). Additionally, in ApoE<sup>−/−</sup> mice, atherosclerotic plaque size and macrophage infiltration was reduced in mice treated with 35K (Ali et al. 2005; Bursill et al. 2004). Furthermore, 35K has been shown to inhibit a host of inflammatory-driven diseases including acute peritonitis, in which mice injected with adenovirus overexpressing 35K (Ad35K) had reduced inflammatory cells in peritoneal exudates (Bursill et al. 2006). Additionally, mice treated with Ad35K had reduced liver fibrosis and reduced hepatitis compared to the adenoviral controls (Bursill et al. 2006; Seki et al. 2009). Currently, 35K has only been investigated in *in vivo* models...
associated with inflammatory pathologies, but its effects in pathological and physiological angiogenic *in vivo* models have not yet been investigated.
5.2. Method

5.2.1. Peri-arterial cuff surgery

Three days prior to surgery mice were injected with either 200 μL PBS, 1x10^{11} vp AdGFP in PBS (total volume 200 μL), or 1x10^{11} vp Ad35K (total volume 200 μL) (n = 12 per treatment group). To induce inflammatory-driven neovascularisation, the peri-arterical cuff model was used as described previously (Moroi et al. 1998; Prosser et al. 2014). Prior to surgery, mice were anaesthetised by inhalation of methoxyflurane. A 1 cm incision was made along the length of the femoral artery, above the knee towards the medial thigh. The femoral artery was isolated from the neuromuscular bundle by carefully placing fine tip forceps between the vessels and under the artery. Once the artery was isolated, a 2 mm non-constricting polyethylene cuff made of PE50 tubing (427410 BD Bioscience), with inner diameter 0.56 mm and outer diameter 0.965 mm, was placed around the artery (Figure 5.1). To do this, the cuff was slit along the length of one side, opened with forceps and closed around the artery before tying with 6-0 PERMA HAND silk suture (639G, ETHICON LLC, San Lorenzo, Puerto Rico). The surgical site was sutured closed with 6-0 PROLENE™ polypropylene suture (8805H, ETHICON LLC). A sham procedure was performed on the opposite hindlimb. After surgery mice were injected subcutaneously with carprofen to prevent pain during recovery. For this study two cohorts were completed, one for histology and another for RNA processing of the cuffed femoral artery.

![Figure 5.1. Polyethylene cuff placed around the mouse femoral artery](image-url)
5.2.2. Hindlimb ischaemia surgery

As with the peri-arterial cuff model, the mice were injected with PBS, AdGFP or Ad35K three days prior to surgery (n =12 per treatment group). To induce hypoxia-mediated angiogenesis, the hindlimb ischaemia model was used as described previously (Niiyama et al. 2009), where the femoral artery and vein was removed from the left hindlimb. This procedure involved the separation of the vessels from the nerve in the neuromuscular bundle. Once separated, the femoral artery and vein was ligated above the epigastrica and profunda femoris, using 6-0 PROLENE™ polypropylene suture (8805H, ETHICON LLC) (Figure 5.2). The vessels were severed distal to the ligation and excised as distal to the popliteal from the hindlimb of the mice. A sham procedure was performed on the opposite hindlimb. After surgery mice were injected subcutaneously with carprofen to prevent pain during recovery. Laser Doppler images (moorLDI2-IR, Moor instruments, Devon, UK) were taken pre- and immediately post-surgery and then at days 2, 4, 7, 10, 12 and 14 post-surgery (Figure 5.3).
Figure 5.2. Ligation of the mouse femoral artery for the hindlimb ischaemia model (Niiyama et al. 2009).

Figure 5.3. Representative Laser Doppler images taken pre- and post- surgery and at the Day 14 end point
5.2.3. Processing of animal tissues

For the peri-arterial cuff model, 21 days after surgery mice were euthanised by overdose of anaesthesia with methoxyflurane followed by right ventricular puncture. Circulatory perfusion flushing with 10 mL of saline was then performed through the left ventricle. Whole blood was collected during the ventricular puncture for plasma analysis and the femoral artery from each hindlimb was dissected out (cuff and non-cuff). The femoral artery was dissected 1 – 2 mm from each end of the cuff. To remove the cuff, the suture was cut and surrounding connective tissue was removed before sliding the cuff off. For histological analysis, the sample was sandwiched between 2 pieces of biopsy foam in an embedding cassette and placed in 4% (v/v) paraformaldehyde in PBS solution for 2 hours at room temperature, then stored in 70% ethanol at 4°C prior to processing in graded ethanol concentrations before paraffin embedding. For RNA analysis, tissues were snap frozen in Eppendorf tubes and stored at -80°C.

For the hindlimb ischaemia model, 14 days after surgery mice were euthanised by overdose of anaesthesia with methoxyflurane followed by right ventricular puncture. Whole blood was collected during the ventricular puncture for plasma analysis. The gastrocnemius muscle was dissected from the hindlimb, half the muscle was snap frozen at -80°C in Eppendorf tubes for RNA/protein analysis. The other half was placed in a tube containing Tissue Tek® OCT™ compound (IA018, ProSciTec, QLD, Australia). The muscle was snap frozen in liquid nitrogen and stored at -80°C until ready to be processed for histology.

5.2.4. Plasma isolation from whole blood

Whole blood collected by right ventricular puncture was kept on ice until centrifugation at 5000 rpm for 5 minutes. The plasma layer was collected in three 50 μL aliquots with a fourth aliquot of remaining plasma, and stored at -80°C. Plasma was used to determine CC-chemokine concentration by ELISA, CC-chemokine activity by chemotaxis assay and lipid profile (Chapter 2, Section 2.3.8 – 2.3.11).
5.2.5. Tissue processing

After collection of femoral artery or hindlimb muscle tissue, samples were prepared for immunohistochemical or immunofluorescence histological staining as stated in Chapter 2 Section 2.3.6. Histological staining was used to detect CD31, smooth muscle α-actin, CD68, or collagen. A portion of this tissue was set aside for gene analysis by RT-PCR (Chapter 2, Section 2.3.7) to detect CCL2, CCL5, markers of inflammation, hypoxia, and angiogenesis. For analyses in the hindlimb model, a portion of hindlimb tissue was used to detect protein levels of CC-chemokines, MIF, and VEGF, by ELISA (Chapter 2, Section 2.3.8.2) and PHDs and HIF-1α by Western blot (Chapter 2, Section 2.3.8.3).
5.3. Results

5.4. Plasma lipids

Assessment of total cholesterol, HDL, LDL and total triglycerides showed no significant differences between treatment groups for both the peri-arterial cuff (Table 5.1) and hindlimb ischaemia (Table 5.2) models.

Table 5.1. Plasma lipids in the peri-arterial cuff model.

<table>
<thead>
<tr>
<th>PERI-ARTERIAL CUFF</th>
<th>PBS</th>
<th>AdGFP</th>
<th>Ad35K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/mL)</td>
<td>1.53 ± 0.02</td>
<td>1.81 ± 0.03</td>
<td>1.61 ± 0.02</td>
</tr>
<tr>
<td>HDL (mg/mL)</td>
<td>1.21 ± 0.31</td>
<td>1.18 ± 0.93</td>
<td>1.06 ± 0.55</td>
</tr>
<tr>
<td>LDL (mg/mL)</td>
<td>0.31 ± 0.75</td>
<td>0.62 ± 0.89</td>
<td>0.55 ± 0.79</td>
</tr>
<tr>
<td>Triglycerides (mg/mL)</td>
<td>0.74 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.73 ± 0.02</td>
</tr>
</tbody>
</table>

Data is expressed as mean±SEM.

Table 5.2. Plasma lipids in the hindlimb ischaemia model.

<table>
<thead>
<tr>
<th>HINDLIMB ISCHAEMIA</th>
<th>PBS</th>
<th>AdGFP</th>
<th>Ad35K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/mL)</td>
<td>1.82 ± 0.02</td>
<td>1.46 ± 0.01</td>
<td>1.84 ± 0.01</td>
</tr>
<tr>
<td>HDL (mg/mL)</td>
<td>1.58 ± 0.23</td>
<td>1.09 ± 0.38</td>
<td>1.42 ± 0.42</td>
</tr>
<tr>
<td>LDL (mg/mL)</td>
<td>0.23 ± 0.90</td>
<td>0.38 ± 0.73</td>
<td>0.42 ± 0.91</td>
</tr>
<tr>
<td>Triglycerides (mg/mL)</td>
<td>0.45 ± 0.02</td>
<td>0.76 ± 0.06</td>
<td>0.47 ± 0.01</td>
</tr>
</tbody>
</table>

Data is expressed as mean±SEM.
5.5. Confirmation of adenoviral gene transfer of Ad35K and AdGFP

To confirm the successful gene transfer of Ad35K by systemic delivery, plasma from mice of each group were run on a Western blot following immunoprecipitation of 35K with Anti-HA agarose (Chapter 2, Section 2.2.5). Figure 5.4A shows that 35K protein was only detected in the plasma of mice injected with Ad35K, whilst the plasma from mice injected with PBS and AdGFP produced no band. Additionally in Figure 5.4B, gene transfer was confirmed for AdGFP, a cytosolic protein, represented by green fluorescence detected in the livers of the AdGFP mice only. Levels of both circulating 35K protein and green liver fluorescence were higher in the mice from the hindlimb ischaemia study (euthanised 14 days post-surgery) compared to the peri-arterial cuff model (euthanised 21 days post-surgery).
Figure 5.4. Confirmation of adenoviral gene transfer.

(A) Western immunoblotting was used to detect the presence of circulating 35K protein in plasma samples from mice injected with PBS, AdGFP or Ad35K for the peri-arterial cuff and hindlimb ischaemia model. (B) Livers of mice injected with PBS, AdGFP, or Ad35K from both the peri-arterial cuff and hindlimb ischaemia model were sectioned (5 μm) and viewed for green fluorescence using fluorescence microscopy. Scale bars represent 100 μm.
5.6. Histological analysis of *in vivo* angiogenesis models

5.6.1. *The effect of broad-spectrum CC-chemokine inhibition by Ad35K on neovascularisation in the in vivo model of inflammatory-driven angiogenesis*

Neovessels and arterioles were determined by histological staining for CD31 (endothelial cells in neovessels) and smooth muscle α-actin (smooth muscle in arterioles) as seen in **Error! Reference source not found.**A. Neovessels indicate vessels in the early stage of development whilst arterioles represent the more advanced vessels that contain a smooth muscle cell outerlayer. In the peri-arterial cuff model, the Ad35K injected mice displayed a non-significant 30.9% reduction in the number of neovessels compared to PBS injected mice (Figure 5.5B), while the AdGFP injected mice displayed a non-significant 28.4% increase of neovessels compared to PBS injected mice. Comparison of adenoviral cohorts showed that Ad35K injected mice also had reduced neovessels (49.2%, ns) compared to the AdGFP viral mice. Upon analysis of the number of arterioles in the cuffed artery (Figure 5.5C), arterioles in the AdGFP injected mice increased substantially (223.3%, p<0.01) compared to the PBS injected mice. However, there were significantly less arterioles in Ad35K infused mice when compared to AdGFP infused mice (85.7%, p<0.001 vs. AdGFP). Furthermore, there were fewer arterioles (52.7%, ns) formed in the Ad35K cohort compared to the PBS control.
Figure 5.5. Ad35K inhibits neovascularisation and arteriole formation in the peri-arterial cuff model.

(A) Representative images of neovessels as detected by CD31 staining (brown) and arterioles identified by α-actin staining (pink) in the femoral artery adventitia of PBS, AdGFP and Ad35K injected mice following peri-arterial cuffing. (B) Number of neovessels was determined as CD31⁺ cells in the adventitia. (C) Number of arterioles was determined as α-actin⁺ staining in the adventitia. Scale bars represent 50 μm. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis **p<0.01 compared to PBS. ^^^p<0.001 compared to AdGFP.
5.6.2. The effect of broad-spectrum CC-chemokine inhibition by Ad35K on macrophage infiltration in the in vivo model of inflammatory-driven angiogenesis

Macrophage infiltration was assessed in the peri-arterial cuff model as a marker of inflammation (Figure 5.6). In the Ad35K injected mice there was a significant decrease in CD68$^+$ staining compared to the PBS group (69.5%, p<0.05) while no significant differences were seen between AdGFP and Ad35K.

Figure 5.6. Macrophage infiltration is reduced with Ad35K in the peri-arterial cuff model. Representative images of macrophage infiltration as detected by staining for CD68 (brown) staining in the femoral artery adventitia of PBS, AdGFP and Ad35K injected mice following peri-arterial cuffing. Scale bars represent 50 μm. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS.
5.6.3. The effect of broad-spectrum CC-chemokine inhibition by Ad35K on the intima:media ratio in the in vivo model of inflammatory-driven angiogenesis

For the peri-arterial cuff model, the intima:media ratio was assessed by first staining sections of the artery with Milligan’s trichrome to differentiate between the intima, media and adventitia of the vessel (Figure 5.7). When assessing the intima:media ratio we found no differences in the intima:media ratio across all three treatment groups.

![Figure 5.7](image)

*Figure 5.7. Intima:media ratio is unchanged with Ad35K treatment in the peri-arterial cuff model.*

Sections of cuffed femoral arteries were stained with Milligan’s trichrome stain in mice receiving PBS, AdGFP or Ad35K. Solid red line indicates the media, dotted red line indicates the intima. Scale bars represent 50µm. Data is expressed as mean±SEM.
5.6.4. The effect of broad-spectrum CC-chemokine inhibition by Ad35K on blood flow recovery in the in vivo model of ischaemia-mediated angiogenesis

In the hindlimb ischaemia model, laser Doppler imaging (LDI) was used to assess blood perfusion as a marker of angiogenesis (Figure 5.8). The LDI perfusion ratio was determined as the ratio of the ischaemic leg compared to the non-ischaemic, sham leg, for each mouse. Blood flow was equally reduced in hindlimbs of all treatment groups following unilateral femoral artery ligation. Comparison of LDI perfusion ratios confirmed that sufficient blood flow returned to all treatment groups by day 14. No differences in blood flow perfusion were seen between the Ad35K cohort compared to both AdGFP and PBS controls.

![Image of LDI perfusion ratios](image.png)

Figure 5.8. Blood flow recovery was not inhibited in the Ad35K mice of the hindlimb ischaemia model.

Representative Laser Doppler imaging (LDI) images of ischaemic (Isc) and non-ischaemic (Non) hindlimbs from mice injected with PBS, AdGFP, or Ad35K at day 14 end point (left panel). Cool colours represent low blood flow, whilst warm colours represent high blood flow. In these mice, LDI perfusion ratio was calculated as a ratio of ischaemic:non-ischaemic hindlimb (right panel). Data is expressed as mean±SEM.
5.6.5. The effect of broad-spectrum CC-chemokine inhibition by Ad35K on neovascularisation in the in vivo model of ischaemia-mediated angiogenesis

In contrast to the strong inhibitory effects of 35K in the peri-arterial cuff model, broad-spectrum CC-chemokine inhibition in the Ad35K mice had no effect on the neovasculature compared to the AdGFP and PBS controls in the hypoxia-mediated angiogenesis model of hindlimb ischaemia (Figure 5.9). The capillary density, as indicated by the amount of red CD31+ staining relative to blue laminin (myocytes) staining (Figure 5.9A), was not affected by the injection of Ad35K compared to injections with AdGFP or PBS (Figure 5.9B). In contrast to the suppression in arterioles seen in the peri-arterial cuff model, a significant increase in arterioles was seen in the Ad35K injected mice (191.8%, p<0.05, Figure 5.9C).
Figure 5.9. Ad35K increases arterioles in the hindlimb ischaemia model.

(A) Representative images of neovessels detected by CD31 (red), arterioles identified by α-actin (green), and myocytes detected by laminin (blue) in the ischaemic hindlimb tissue of the PBS, AdGFP, or Ad35K injected mice. Arrows indicate presence of neovessels in the tissue. (B) Capillary density was determined as CD31+ cells/myocyte. (C) Arteriole density was determined as α-actin+ cells/myocyte. Scale bars represent 100 μm. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS. ^p<0.05 compared to AdGFP.
5.7. CC-chemokine analysis

5.7.1. Circulating CC-chemokines

In this study, we found that circulating levels of CC-chemokines CCL2 and CCL5 drastically increased in the plasma of Ad35K injected mice in both the peri-arterial cuff and hindlimb ischaemia models compared to both the non-viral (PBS) and adenoviral (AdGFP) controls (Figure 5.10). In the peri-arterial cuff model, injection of Ad35K increased circulating CCL2 when compared to both the non-viral PBS and adenoviral AdGFP control groups (834.1% and 368.5% respectively, p<0.001, Figure 5.10A). Furthermore, Ad35K also increased circulating CCL5 levels above the non-viral PBS (142.1%, p<0.001) control group (Figure 5.10B). Similarly, in the hindlimb ischaemia study, Ad35K increased circulating CCL2 levels by 1324.5% (p<0.001) compared to PBS, whilst a 238.5% increase (p<0.001) was seen when compared to AdGFP control (Figure 5.10C). Ad35K also increased circulating CCL5 levels above both the PBS and AdGFP groups (410.1% and 152.4% respectively, p<0.001, Figure 5.10D).
Figure 5.10. Circulating CC-chemokines are increased in Ad35K mice.
Circulating (A) CCL2 and (B) CCL5 levels was assessed by ELISA in mouse plasma from peri-arterial cuff model. Circulating (C) CCL2 and (D) CCL5 levels were also measured in the hindlimb ischaemia model. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05, ***p<0.001 compared to PBS. ^^^p<0.001 compared to AdGFP.
5.7.2. CC-chemokine activity

To determine the activity of the circulating CC-chemokines, the chemotaxis assay was used. For this assay, fluorescently-labelled monocytes are allowed to migrate across a porous membrane towards a stimulus (i.e. CC-chemokines in plasma).

There was no difference in the number of monocytes migrating towards the plasma samples of Ad35K mice compared to the PBS cohort, however a 27.1% decrease (p<0.05) was seen when compared to the adenoviral AdGFP controls from the peri-arterial cuff model (Figure 5.11A). Similarly when comparing the samples from the hindlimb ischaemia model, the number of monocytes migrating towards the Ad35K plasma stimulus decreased by 21.2% (p<0.05) compared to PBS plasma (Figure 5.11). These results demonstrate that 35K suppresses CC-chemokine activity in the plasma of Ad35K injected mice in both models.

Figure 5.11. Reduction of CC-chemokine activity in Ad35K plasma. Chemokine activity was determined using the Boyden chamber assay to assess monocyte migration towards plasma samples from PBS, AdGFP or Ad35K injected groups from both the (A) peri-arterial cuff model and (B) hindlimb ischaemia model. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS. ^p<0.05 compared to AdGFP.
5.7.3. **CC-chemokine tissue expression**

The expression of CC-chemokines in tissue was determined by measuring mRNA and protein levels in homogenised tissue.

In the peri-arterial cuff model 35K significantly reduced CCL2 mRNA levels by 76.6% (p<0.05) compared to PBS, whilst a non-significant 45.6% decrease was observed when compared to AdGFP (Figure 5.12). Overall, CCL5 mRNA levels were unchanged across all three groups (Figure 5.12B).

Conversely in the hindlimb ischaemia model, Ad35K injected mice had reduced CCL2 mRNA levels compared to the adenoviral AdGFP control group (31.4% decrease, p=0.051, Figure 5.13A). This trend in reduction of CCL2 in the Ad35K injected mice appeared to be consistent with the protein expression, with a non-significant 26.8% decrease seen when compared to AdGFP (Figure 5.13C). Interestingly, CCL5 mRNA (Figure 5.13) and protein (Figure 5.13) levels were significantly higher in the AdGFP group compared to the PBS controls (675.2% and 332.4% respectively, p<0.05). More importantly, 35K was able to suppress this increase, with decreases seen at both the mRNA (66.0%) and protein (70.5%) levels (p<0.001).
Figure 5.12. CCL2 and CCL5 expression in peri-arterial cuff model.

RT-PCR was used to detect mRNA levels of (A) CCL2 and (B) CCL5 in peri-arterial cuff tissue of PBS, AdGFP, or Ad35K injected mice. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS.
Figure 5.13. CCL2 and CCL5 expression in hindlimb ischaemia model.

RT-PCR was used to detect mRNA levels of (A) CCL2 and (B) CCL5 in ischaemic hindlimb tissue from PBS, AdGFP, or Ad35K injected mice. ELISAs were used to determine protein levels of (C) CCL2 and (D) CCL5 in hindlimb tissue. Data is expressed as mean ± SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. ***p<0.001 compared to PBS, ^^^p<0.001 compared to AdGFP.
5.8. Expression of angiogenic markers

5.8.1. Gene expression of markers involved in inflammation

Inflammatory-driven angiogenesis is regulated primarily by the NF-κB signalling pathway which creates an inflammatory response that then promotes the recruitment of monocytes/macrophages to the inflamed sites. Tissue gene expression of the active NF-κB subunit, p65 (Figure 5.14A) and CD68 (Error! Reference source not found.B) showed no significant differences across all three groups.

Figure 5.14. Gene expression of inflammatory markers in the peri-arterial cuff model. RT-PCR was used to determine mRNA levels of (A) p65 and (B) CD68 in peri-arterial cuff tissue of PBS, AdGFP, or Ad35K injected mice. Data is expressed as mean ± SEM.
5.8.2. Gene expression of markers involved in hypoxia

Hypoxia-mediated angiogenesis is driven by the key transcription factor HIF-1α, which is post-translationally modulated by the E3 ubiquitin ligases Siah1 and Siah2 and the prolyl hydroxylase family (PHD1-3). The expression of these angiogenic hypoxic markers were determined in hindlimb tissue following femoral artery ligation.

Overall, the majority of markers involved in the post-translational modulation of HIF-1α saw little to no inhibition in mice injected with Ad35K when compared to the PBS and AdGFP control mice. No significant differences were seen in the mRNA levels of Siah1 (Figure 5.15A) and Siah2 (Figure 5.15B) across all three groups. Interestingly, PHD1 mRNA levels were significantly increased with Ad35K, compared to PBS (200.4%, p<0.001) and AdGFP (78.4%, p<0.05) (Figure 5.15C). These changes were not reflected in the other two members of the prolyl hydroxylase family, with no differences detected in the mRNA levels of PHD2 (Figure 5.15D) and PHD3 (Figure 5.15E). Additionally, protein expression of PHD1 (Figure 5.16A), PHD2 (Figure 5.16B), and PHD3 (Figure 5.16C) were not changed in ischaemic tissue of mice injected with Ad35K compared to PBS and AdGFP injected mice.
Figure 5.15. Gene expression of hypoxic markers in the hindlimb ischaemia model. RT-PCR was used to determine mRNA levels of (A) Siah1, (B) Siah2, (C) PHD1, (D) PHD2, and (E) PHD3 in the ischaemic hindlimb tissue from PBS, AdGFP, or Ad35K injected mice. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. ***, p<0.001 compared to PBS. ^p<0.05 compared to AdGFP.
Figure 5.16. Protein expression of hypoxic markers in the hindlimb ischaemia model. Western blot was used to determine protein levels of (A) PHD1, (B) PHD2, and (C) PHD3 in the ischaemic hindlimb tissue from PBS, AdGFP, or Ad35K injected mice. Data is expressed as mean±SEM.
5.8.3. *Gene expression of key angiogenic markers*

The expression of key angiogenic markers involved in both inflammatory- and hypoxia-driven angiogenesis (HIF-1α, VEGF, CXCL12, and MIF) were determined for both models.

We observed interesting trends in gene expression of the angiogenic markers in the peri-arterial cuff model. Firstly, HIF-1α mRNA levels were not significantly different across all three groups, although there was a trend towards an increase in both adenoviral groups above the vehicle PBS group (Figure 5.17A). While there was no significant differences in VEGF gene expression between PBS and AdGFP groups, there was a significant 47.2% (p<0.05) decrease in Ad35K mice when compared to PBS and a non-significant 22.9% decrease (p=0.053) when compared to AdGFP (Figure 5.17B). Overall, CXCL12 levels appeared lower in the Ad35K group while the expression levels were more variable in the PBS and AdGFP groups, with a 66.1% and 51.3% non-significant reduction seen when compared to PBS and AdGFP respectively (Figure 5.17C). Finally, 35K increased MIF expression by 94.5% (p<0.05) compared to AdGFP while there was a trend to increased levels when compared to PBS (Figure 5.17D).

In contrast, HIF-1α (Figure 5.17B), VEGF (Figure 5.17D), CXCL12 (Figure 5.17F), and MIF (Figure 5.17H) mRNA levels were not significantly different across all three treatment groups in the hindlimb ischaemia model. Furthermore, no significant differences in HIF-1α (*Error! Reference source not found.*A), VEGF (Figure 5.18B) and MIF (Figure 5.18C) protein levels were observed.
Figure 5.17. Gene expression of angiogenic markers in the peri-arterial cuff and hindlimb ischaemia models.

RT-PCR was used to determine gene expression of (A, B) HIF-1α, (C, D) VEGF, (E, F) CXCL12, and (G, H) MIF in the peri-arterial cuff and hindlimb ischaemia models. Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS. ^p<0.05 compared to AdGFP.
Figure 5.18. Protein expression of angiogenic markers in the hindlimb ischaemia model. Ischaemic hindlimb tissue was homogenised and assessed by Western blot to determine (A) HIF-1α, and ELISAs used to determine (B) VEGF, and (C) MIF protein. Data is expressed as mean ± SEM.
5.9. Discussion

The present study sought to determine whether the in vitro effects of 35K observed in Chapters 3 and 4 could be translated to in vivo murine models of angiogenesis. In contrast to the in vitro studies, we used an adenoviral gene transfer approach for these studies to overexpress soluble 35K systemically. We found that broad-spectrum inhibition of CC-chemokines by 35K inhibited inflammatory-driven neovascularisation in the peri-arterial cuff model as noted by reduced capillaries and arterioles. Conversely, 35K did not affect recovery of blood perfusion following induction of ischaemia in the hindlimb ischaemia model. Furthermore in ischaemia, capillary and arteriole density were not attenuated by broad-spectrum CC-chemokine inhibition, indicating that ischaemia-induced angiogenesis was preserved in vivo.

The peri-arterial cuff model was used to stimulate inflammatory-driven angiogenesis. This model involved the placement of a cuff around the vessel. The cuff is larger than the vessel allowing unrestricted blood flow through the vessel. Placement of the cuff around the femoral artery initiates a localised inflammatory response which triggers the recruitment of macrophages and the release of pro-inflammatory cytokines and growth factors that drive increased neovessel formation at the site of injury (Bhardwaj et al. 2011). New vessel formation was assessed in the adventitia of the femoral artery by histological staining for CD31+ endothelial cells (neovessels) and α-actin+ for smooth muscle cells (arterioles), which represent early and late neovessels. A significant inhibition in the number of neovessels and arterioles were seen in the mice injected with Ad35K when compared to the controls. We hypothesise that the initial suppression in the formation of neovessels by 35K then results in the subsequent reduction in the number of arterioles that were able to mature. It is also possible that broad-spectrum CC-chemokine inhibition has direct effects on smooth muscle cells and the maturation of neovessels into arterioles, as the inhibitory effect of 35K on arterioles was more striking than on neovessel formation.

Macrophages also play an important role in modulating inflammatory-driven angiogenesis by secreting pro-angiogenic cytokines and growth factors. Our in vivo model of inflammation-induced adventitial neovascularisation found that, whilst there was a trend for a reduction in adventitial macrophages and CD68 mRNA levels, 35K did not have a significant inhibitory effect on macrophage recruitment. This suggests that the inhibitory effect of 35K on neovascularisation in inflammation is via 35K suppressing the direct
effect of CC-chemokine activity on endothelial cells and angiogenesis rather than the indirect effect via the initial recruitment of macrophages. Additionally, 35K has high specificity binding to CC-chemokines only, and does not bind to other chemokine classes. Therefore the lack of significant reduction of macrophages in the tissue may be due to reduced chemokine activation of monocytes which are Ly6C^{high} or Ly6C^{low}. Ly6C^{high} monocytes have surface markers which are CCR2^{high} and CX_{3}CR1^{low}, thus 35K is more likely to prevent activation of these monocytes by binding to CCL2. However, Ly6C^{low} monocytes have surface markers which are CCR2^{low} and CX_{3}CR1^{high}, these are patrolling monocytes, which are not affected by CCL2/35K binding as they primarily bind to CX_{3}CL1 ligands (Burns et al. 2002).

The intima:media ratio is a determination of the extent of neointimal hyperplasia in the artery. Angiogenesis in the adventitia can drive this response (Khurana et al. 2004). However, no differences were seen in the intima:media ratio across all three groups. This suggests that inhibition of adventitial neovascularisation with broad-spectrum CC-chemokine inhibition occurs independently of changes in the neointimal area.

In contrast to the inhibitory effects that CC-chemokine inhibition had on inflammatory-driven angiogenesis in vivo, 35K had no effect on angiogenesis in ischaemia. In fact, there was an increase in arterioles. The hindlimb ischaemia model employed in this chapter is a well-established model of ischaemia-driven neovascularisation in vivo (Bonauer et al. 2009), where both angiogenesis and arteriogenesis can be investigated depending upon where the placement of the ligature, and whether the femoral vessels are completely excised. The current study ligated the femoral artery above both the epigastrica and profunda femoris before severing them distal to the ligation. The femoral artery and vein were also completely excised as distal as the popliteal. This method ensures that angiogenesis occurs rather than arteriogenesis and has been utilised by multiple research groups worldwide for this purpose; and was recently characterised in detail (Limbourg et al. 2009). Furthermore, the gastrocnemius muscle which is distal to the ligation and rich in neovessels was analysed, rather than the adductor muscle where arteriogenesis may occur. Therefore, the arterioles detected in the gastrocnemius muscle, of which there are few, have developed directly from neovessels.

Blood flow perfusion, as a marker of neovascularisation was not different between the Ad35K group and control cohorts. This suggests that broad-spectrum CC-chemokine
inhibition does not impact ischaemia-driven blood flow recovery. Furthermore, there were no differences in the number of neovessels in the hindlimb of the Ad35K injected mice compared to the controls. Interestingly, we observed a significant increase in the number of arterioles with the Ad35K group. Taken together, our two in vivo angiogenesis models showed that 35K specifically inhibits inflammatory-driven angiogenesis, but yet, has no effect on and is preserved in hypoxia.

The expression of two CC-chemokines, CCL2 and CCL5, were measured in the plasma as well as the tissues of these mice. We found that Ad35K injected mice had significantly higher circulating CC-chemokine levels when compared to both the PBS vehicle control and the AdGFP adenoviral control in both in vivo models. While intuitively, this might seem unexpected, previous studies have also reported similar observations (Bursill et al. 2003; Bursill et al. 2004). We then sought to determine if the chemokine activity in the plasma samples of these mice was inhibited using a monocyte migration assay. Interestingly we found that, despite the increased levels of circulating CC-chemokines in the plasma of the Ad35K mice, plasma CC-chemokine activity was reduced, as indicated by the reduced number of monocytes that migrate towards the Ad35K mouse plasma when compared to plasma from either PBS or AdGFP mice. Taken together, this suggests that 35K is bound to the CC-chemokines, thereby blocking the binding area of receptors on the surface of monocytes. Furthermore, when CC-chemokine levels were measured in the tissues, the reduction of CC-chemokines becomes evident. Given these observations, we propose that 35K protein is able to bind to CC-chemokines in the tissue and sequester them into the circulation resulting in an increase of circulating CC-chemokine levels. Alternatively, circulating CC-chemokine levels increase to compensate for the reduced activity, however 35K continues to bind to new CC-chemokines preventing increase in activity leading to elevated levels of circulating (but inactive) CC-chemokines which is still detectable by ELISAs. With the reduction of CC-chemokine activity, the monocytes are unable to migrate and differentiate into macrophages. This reduces the inflammatory response in the tissue, consequently reducing inflammatory-driven angiogenesis. We see this with a trend towards reduced expression of two inflammatory angiogenic markers, the active NF-κB subunit, p65, and macrophage marker CD68 in the Ad35K injected mice of the inflammatory in vivo model. However, given that inflammation occurs at the early stages of this model, the lack of change in mRNA levels
Post-translational modulation of HIF-1α is important in hypoxic cellular responses where under normoxic conditions, the prolyl hydroxylases (PHD1-3) function to hydroxylate HIF-1α, targeting it for ubiquitination and degradation. However, upon hypoxic stimulation, the E3 ubiquitin ligases Siah1 and Siah2 are upregulated, causing the inhibition of PHD1, PHD2, and PHD3. This prevents the degradation of HIF-1α allowing it to accumulate and translocate to the nucleus to drive hypoxia-responsive genes such as CXCL12 and VEGF to help mediate the cellular angiogenic response. There were no differences in Siah1, Siah2, PHD2 and PHD3 mRNA levels across all three treatment groups. Interestingly, while there was a significant increase in PHD1 mRNA levels in the Ad35K group, there were no differences in the protein levels of the PHD family suggesting that broad-spectrum CC-chemokine inhibition does not have any significant impact on the hypoxia-mediated angiogenic pathway.

The expression of key pro-angiogenic mediators HIF-1α and VEGF were also determined in the tissue of both inflammation and ischaemia models. VEGF is essential in angiogenesis under both conditions as it contains both hypoxia (HIF) and inflammatory (NF-κB) response elements in its promoter region (Ema et al. 1997; Forsythe et al. 1996; Levy et al. 1995; Shi et al. 2001). Additionally, HIF-1α has the NF-κB response element in its promoter region. Similar to the in vitro studies, VEGF mRNA levels was found to be inhibited in inflammation but remain unchanged under hypoxia. In contrast to our in vitro observations where 35K suppressed HIF-1α levels, overall there was no significant differences in HIF-1α in vivo. Given that mRNA levels were detected at the conclusion of the study and that HIF-1α is usually triggered at the early stage of inflammatory/ischaemic response, perhaps any effect of 35K on HIF-1α may be lost and an earlier time point may provide more information. Similar to our in vitro mechanism findings, 35K had no effect on the post-translational HIF-1α mediators, Siahs and PHDs in vivo. Although there is only a slight non-significant reduction of NF-κB this may be enough to affect HIF-1α and VEGF. VEGF is particularly affected by any changes to NF-κB as it has two response elements for NF-κB in its promoter region but only one hypoxic response element (Ramanathan et al. 2007). Furthermore, 35K may be conditionally regulating VEGF.

may not necessarily reflect the true action of 35K on inflammation and perhaps an earlier timepoint would be better suited to observe these effects in vivo.
through NF-κB in inflammation, whilst having no effects on the hypoxic response element in hypoxia.

Further analysis of macrophage migration inhibitory factor (MIF), a CXC chemokine that plays a role in angiogenesis in hypoxia, found it was unchanged in the hypoxic tissue. This would be expected as 35K specifically inhibits the CC-chemokine class. Interestingly, MIF was increased in our inflammatory-driven peri-arterial cuff model. MIF is also known to regulate the expression of macrophages in inflammatory conditions and this involves CCL2/CCR2 and CCL5/CCR5 CC-chemokine/receptor interactions (Gregory et al. 2006). Through inhibition of CC-chemokine activity by 35K, a compensatory elevation in MIF expression may therefore be occurring in our inflammatory model.

In conclusion, the inhibition of CC-chemokine class by 35K suppresses inflammatory-driven angiogenesis, but yet preserves hypoxia-mediated angiogenesis in vivo. Broad-spectrum inhibition of the CC-chemokine class may present as a therapeutic strategy to specifically target diseases associated with inflammatory-driven angiogenesis such as atherosclerosis and cancer, without causing the adverse side effects that occur with complete inhibition of angiogenesis across all pathophysiological contexts. However, the effects of broad-spectrum CC-chemokine inhibition in a system where angiogenesis is driven by both inflammation and hypoxia, such as that seen in cancer progression remains to be elucidated.
CHAPTER 6.
THE EFFECT OF BROAD-SPECTRUM CC-CHEMOKINE INHIBITION IN A MODEL OF TUMOUR NEOVASCULARISATION
6.1. Introduction

In cancer, tumour development is caused by a defect in the homeostatic environment of the cell, leading to abnormal proliferation (Cooper 2000). As the tumour cells become distant from nearby vessels, neovascularisation is critical for the development of new blood vessels to supply growth factors and oxygen allowing for tumour growth and expansion (Carmeliet & Jain 2000a). For this reason, angiogenesis is critical to the tumour environment. An imbalance between pro- and anti-angiogenic stimulators is compromised leading to excessive angiogenesis and exacerbation of tumour growth. Changes which are triggered by the imbalance include increased inflammation, increased cell proliferation, increased cell invasion, and decreased apoptosis, which ultimately leads to tumour growth and progression/development. Various signals which trigger this imbalance include low oxygen, increased inflammatory response and genetic mutations (Carmeliet & Jain 2000a). Hypoxia is a common characteristic of locally advanced solid tumours and has been linked to diminished therapeutic response and progression of malignant tumours (Vaupel & Harrison 2004).

As tumours are reliant on new blood vessel formation, angiogenesis is thought to be a good target for anti-tumour therapy. Current anti-angiogenic therapies available such as bevacizumab or TNP-470 inhibit VEGF or the endothelial cell cycle respectively (Ferrara, Hillan & Novotny 2005; Masiero, Figg & Kohn 1997; Teicher et al. 1994). However, the overall and complete inhibition of angiogenesis by these therapies causes a number of side effects including hypertension, haemorrhaging, vomiting, nausea, gastrointestinal perforations and reduced wound healing (Cabebe & Wakelee 2007; Mourad et al. 2008; Satchi-Fainaro et al. 2004). Thus an agent which inhibits pathological angiogenesis whilst preserving physiological angiogenesis is needed as an alternate therapeutic strategy. In cancer CC-chemokines regulate the migration of inflammatory cells to the tumour microenvironment. They are especially involved in the migration of tumour associated macrophages (TAM) to promote tumour neovascularisation (Mantovani et al. 2004; Okada et al. 2009; Rivas-Fuentes et al. 2015; Zhang, Lu & Pienta 2010a). Our findings from Chapter 5 show that broad-spectrum CC-chemokine inhibition by 35K reduced angiogenesis in the context of inflammation, whilst having no effects on angiogenesis in the context of hypoxia. In this study we seek to determine the effect of 35K in a disease pathology that involves neovascularisation in response to both inflammation and hypoxia, as seen in the context of cancer.
6.2. Method

6.2.1. Preparation of Lewis lung carcinoma cells

Lewis lung carcinoma (LLC) cells were grown to 70% confluency in Lewis lung carcinoma cell media (0.35% [w/v] D-glucose, 10% [v/v] foetal calf serum, in DMEM). The cells were trypsinised and resuspended in Hank’s Buffered Salt Solution (HBSS, H1387, Sigma Aldrich) and kept in a 37°C water bath until needed.

6.2.2. Tumour neovascularisation model procedure

As with the previous models, 6 – 8 week old male C57Bl/6J mice were injected via tail vein, with either PBS, 1 x 10^{11} vp AdGFP, or 1 x 10^{11} vp Ad35K, 3 days prior to procedure (n = 10 per treatment group). Mice were anaesthetised by inhalation of methoxyflurane. A 1 mL insulin syringe was used to inject 200 µL of a 1x10^{6} LLC suspension into the subcutaneous layer of the right flank of the mice. After injection, mice were monitored for signs of discomfort, and fed on standard diet and water.

6.2.3. LLC model sacrifice and tissue collection

Mice were sacrificed 12 days after injection of the LLC. Mice were sacrificed by overdose of methoxyflurane followed by cardiac puncture. Samples collected include the blood plasma, liver and tumour. Blood plasma and liver processing were completed as in previous chapters (Chapter 5, 5.5). A visible tumour was seen in the subcutaneous layer of the right flank of the mice. As it is highly vascularised, care was taken to excise the whole tumour by first removing tissue around the tumour. The tumour was then weighed and cut to be processed for histology and gene analyses.

6.2.4. Histology

For this study the tissue was placed in OCT and snap frozen at -80°C. Using the cryostat, 7 µm sections were taken from the tissue. Each slide had 3 sections per sample, cut slides were stored at -20°C until use.
Initial fixing of slides was performed in 4% (v/v) paraformaldehyde in PBS for 10 minutes, however we found that the sections did not remain adhered to the slide. To resolve this, StarFrost® hydrophilic slides (G311SF, ProSciTech) were used, however the sections continued to wash off the slide during fixing. Samples were instead fixed with methanol for 10 minutes. This method allowed the sample to remain attached for longer, however excessive washing during staining resulted in folding of the edges of samples. To obtain the best results, washing was kept to a minimum, any liquids were gently placed on to the sample, and liquid was removed by drawing up with a pipette rather than tipping off the slide.

To stain the slides after fixing, the samples were washed twice in PBS for 5 minutes each. Sections were incubated with the primary antibodies smooth muscle α-actin (1:1000, F3777, Sigma-Aldrich) and laminin (1:300, AB79057, Abcam), in antibody diluent with background reducing agent (S080983-2, Dako) in the dark at room temperature. Following incubation with primary antibody, the slides were washed twice in PBS for 5 minutes each, before 1 hour incubation with Alexa Fluor 350 secondary antibody (1:500, A21093, Invitrogen, California, USA) in antibody diluent. Coverslips were mounted onto stained slides in fluorescent mounting media. Slides were allowed to dry for 24 hours and kept in the dark before imaging. Samples were imaged at 10X magnification with 3 fields of view per section.

6.2.5. Gene expression

RNA from tumour samples were extracted using the Qiagen RNeasy Mini kit (74104, QIAGEN, Venlo, Netherlands). 400 ng of total RNA was reverse transcribed and real-time PCR was conducted as described in Methods (Chapter 2, Section 2.3.7).
6.3. Results

6.3.1. Confirmation of Ad35K gene transfer

As with the previous studies, Western blotting was used to confirm the expression and delivery of 35K by adenovirus in the plasma of mice from each group. Immunoprecipitation with anti-HA agarose was used to pull circulating 35K protein from the plasma. Figure 6.1A shows the detection of 35K protein in the plasma of the mice injected with Ad35K only, whilst no 35K bands were seen in the plasma from the PBS and AdGFP mice. Viral gene transfer was further confirmed by the presence of green fluorescence seen only in the livers of AdGFP mice (Figure 6.1B).

Figure 6.1. Confirmation of Adenoviral gene transfer
(A) Western immunoblotting was used to detect the presence of circulating 35K protein in plasma samples from mice injected with PBS, AdGFP or Ad35K in the tumour neovascularisation model. (B) Green fluorescence protein was detected in livers of mice injected with PBS, AdGFP or Ad35K to confirm the viral gene transfer of AdGFP. Scale bars represent 100 μm.
6.3.2. Tumour weights

Analysis of tumour weights (Figure 6.2) found no significant differences between the three groups. However, tumours from the Ad35K infused mice showed a trend for reduction in tumour weights when compared to PBS (11.5%) and AdGFP (13.0%).

Figure 6.2. Comparison of tumour weights.
Tumour weights from PBS, AdGFP and Ad35K injected mice were determined and compared. Data is expressed as mean±SEM.
6.3.3. Neovascularisation

Smooth muscle α-actin was detected by immunohistochemistry in the tumour samples to assess mature blood vessels in the tumour. Staining and analysis of smooth muscle α-actin (Figure 6.3) found no significant differences between PBS, AdGFP and Ad35K. However there was a 14.5% reduction in the Ad35K group when compared to AdGFP. Additionally, mice injected with AdGFP had increased smooth muscle α-actin staining (17.9%) compared to PBS injected mice.

Figure 6.3. Smooth muscle α-actin in Lewis lung carcinoma cell tumours
Immunofluorescence staining of smooth muscle α-actin (green) and laminin (blue) to indicate arterioles in the tumour tissue. The percentage of α-actin positive cells/tumour area was determined for PBS, AdGFP and Ad35K mouse tumours. Scale bars represent 200 μm. Data is expressed as mean±SEM.
6.3.4. Gene expression in tumour tissue

As tumour development and progression involves several key cellular processes including inflammation, proliferation, invasion and apoptosis, real-time PCR was used to measure markers of inflammation (p65, CCL2, VEGF, Bcl-2), cell proliferation (Ki-67, c-Jun), cell invasion (MMP2, MMP9), and cell apoptosis (p53, caspase 9, caspase 8, caspase 3). These will be described in further detail below.

6.3.4.1 Markers of inflammation

When inflammation is stimulated, the PI3K/Akt pathway is activated (Figure 6.4). This causes the increase of IKK to phosphorylate IκBα, allowing the translocation of the transcription factor NF-κB to the nucleus. NF-κB then activates the expression of pro-inflammatory factors such CC-chemokines and VEGF to enhance the inflammatory response. NF-κB is also able to activate Bcl-2, an anti-apoptotic (cell survival) gene. Excess inflammation in a tumour is a contributing factor that promotes tumour growth. In this model, to assess the effect of Ad35K on inflammation we determined the gene expression of p65, CCL2, VEGF and Bcl-2.

The expression levels of p65, CCL2, VEGF and Bcl-2 were not significantly different between the three treatment groups. Although not significant a number of trends were observed and are highlighted in Table 6.1. Unexpectedly 35K had no effect on the expression of CCL2. There was also no difference in the expression levels of p65, the active subunit of NF-κB (Table 6.1). While there was no inhibition of inflammation in the Ad35K mice, a non-significant increase in VEGF expression was observed, with a 34.3% and 130.0% increase seen when compared to PBS and AdGFP respectively. Interestingly, Bcl-2 expression increased by 95.7% and 71.2% in the Ad35K mice compared to PBS and AdGFP respectively. Furthermore, the adenoviral AdGFP control tumours exhibited a non-significant decrease (41.6%) in VEGF expression when compared to PBS.
Figure 6.4. Inflammation pathway

Inflammation activates the PI3K/Akt pathway to increase production of NF-κB, leading to the release of pro-inflammatory factors to exacerbate inflammation. NF-κB also increases the production of Bcl-2, responsible for cell survival.

Table 6.1. Gene expression of inflammatory markers in tumour tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBS value (mean ± SEM)</th>
<th>AdGFP value (mean ± SEM)</th>
<th>trend (vs. PBS)</th>
<th>Ad35K value (mean ± SEM)</th>
<th>trend (vs. AdGFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p65</td>
<td>100.0 ± 8.1</td>
<td>89.1 ± 40.8</td>
<td>-</td>
<td>96.8 ± 25.1</td>
<td>-</td>
</tr>
<tr>
<td>CCL2</td>
<td>100.0 ± 10.6</td>
<td>89.8 ± 35.7</td>
<td>-</td>
<td>93.7 ± 27.9</td>
<td>-</td>
</tr>
<tr>
<td>VEGF</td>
<td>100.0 ± 17.0</td>
<td>58.4 ± 32.1</td>
<td>↓</td>
<td>134.3 ± 43.5</td>
<td>↑</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>100.0 ± 10.3</td>
<td>114.3 ± 25.7</td>
<td>-</td>
<td>195.7 ± 64.9</td>
<td>↑</td>
</tr>
</tbody>
</table>

Data is expressed as mean±SEM.
6.3.4.2 **Markers of proliferation and invasion**

Cell proliferation and cell invasion are essential in tumour growth and metastasis. Figure 6.5 illustrates one pathway involved in regulation of proliferation and invasion, which involves both the ERK and JNK signalling pathways. To assess the effect of CC-chemokine inhibition on tumour cell proliferation and invasion, the expression levels of Ki-67 (a marker of cell proliferation), c-Jun, matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) were determined.

In this study, there was a non-significant increase in Ki-67 expression levels in the tumours of the Ad35K mice, with a 25.8% and 64.0% increase seen when compared to PBS and AdGFP respectively (Table 6.2). Similarly, the expression levels of c-Jun, which dimerises with c-Fos to activate transcription factor AP-1 and in turn increase proliferation, was increased in the Ad35K tumours (100.1% when compared to PBS and 84.8% compared to AdGFP). Interestingly, in the AdGFP tumours, the expression of Ki-67 decreased by 23.3%, whilst no changes were detected in c-Jun expression levels.

Other than proliferation, AP-1 is also responsible for the regulation of extracellular matrix degradation by MMP2 and MMP9. In the Ad35K tumours, MMP2 decreased by 39.4% when compared to the adenoviral AdGFP control group. However, in the Ad35K tumours, MMP9 levels increased by 104.8% and 89.4% when compared to PBS and AdGFP respectively. In addition, the expression levels of MMP2 was higher (64.1%) in the AdGFP tumours compared to PBS.
Figure 6.5. Proliferation and invasion pathway in tumours

MAPK pathways, ERK and JNK activate c-Jun and c-Fos respectively. Together they activate the AP-1 transcription factor to promote cell proliferation, and the expression of matrix metalloproteinases MMP2 and MMP9 which are responsible for extracellular matrix degradation.

Table 6.2. Gene expression of cell proliferation and cell invasion markers in tumour tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBS</th>
<th>AdGFP</th>
<th>Ad35K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>value</td>
<td>value</td>
<td>trend (vs. PBS)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>100.0 ± 8.5</td>
<td>76.7 ± 29.8</td>
<td>↓ 125.8 ± 25.4</td>
</tr>
<tr>
<td>c-Jun</td>
<td>100.0 ± 7.9</td>
<td>108.3 ± 41.6</td>
<td>- 200.1 ± 63.0</td>
</tr>
<tr>
<td>MMP2</td>
<td>100.0 ± 22.1</td>
<td>164.1 ± 82.1</td>
<td>↑ 99.4 ± 73.3</td>
</tr>
<tr>
<td>MMP9</td>
<td>100.0 ± 9.6</td>
<td>108.1 ± 34.7</td>
<td>- 204.8 ± 50.9</td>
</tr>
</tbody>
</table>

Data is expressed as mean ±SEM.
6.3.4.3 Markers of apoptosis

To determine the effect of Ad35K on apoptosis, gene expression of p53, caspase 9, caspase 8, and caspase 3 were determined. Apoptosis is initiated by the tumour suppressor gene p53 (Figure 6.6 Error! Reference source not found.) that then leads to caspase activation. Ad35K had no effect on the apoptosis-related genes that were tested in the tumours, compared to AdGFP infused mice. However, in the Ad35K mice, there was a non-significant trend for an increase in p53 expression (61.0%) compared to PBS and by 175.7% compared to AdGFP (Table 6.3). Downstream from p53, expression of caspase 9 was not significantly different across all three groups. However, caspase 8 levels significantly increased by 148.3% (p<0.05) in Ad35K tumours compared to PBS, whilst a non-significant increase of 113.9% was seen when compared to AdGFP. Furthermore, caspase 3 expression in the Ad35K tumours was non-simply elevated by 26.8% when compared to PBS and by 38.7% relative to AdGFP controls.
Figure 6.6. Apoptosis Pathway

Tumour suppressor gene, p53 is activated in apoptosis. This leads to the increase of pro-apoptotic gene, Bax, to suppress Bcl-2 production. Bax also causes the release of cytochrome C from the mitochondria to promote the release of caspase 9 leading to caspase 3 accumulation for apoptosis. Similarly, the increase of caspase 8 leads to elevated caspase 3 levels, ultimately driving apoptosis.

Table 6.3. Gene expression of apoptosis markers in tumour tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBS value</th>
<th>PBS value</th>
<th>trend (vs. PBS)</th>
<th>AdGFP value</th>
<th>AdGFP value</th>
<th>trend (vs. AdGFP)</th>
<th>Ad35K value</th>
<th>Ad35K value</th>
<th>trend (vs. AdGFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>100.0 ± 11.1</td>
<td>58.4 ± 19.9</td>
<td>↓</td>
<td>161.0 ± 38.7</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase 9</td>
<td>100.0 ± 8.1</td>
<td>117.1 ± 58.0</td>
<td>-</td>
<td>105.0 ± 41.0</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase 8</td>
<td>100.0 ± 5.6</td>
<td>116.1 ± 29.7</td>
<td>-</td>
<td>248.3 ± 59.3</td>
<td>↑*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase 3</td>
<td>100.0 ± 7.4</td>
<td>91.4 ± 31.7</td>
<td>-</td>
<td>126.8 ± 29.8</td>
<td>↑</td>
<td></td>
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</tbody>
</table>

Data is expressed as mean±SEM. Statistical analysis was performed using a One-way ANOVA with Tukey’s post hoc analysis. *p<0.05 compared to PBS.
6.4. Discussion

The present study sought to determine whether the in vivo inhibition of inflammatory-driven angiogenesis by 35K, as seen in Chapter 5, was also effective in a tumour context where neovascularisation is driven by both inflammation and hypoxia. 35K was delivered systemically by adenoviral overexpression of 35K. Overall, the effects of 35K were not striking and we only observed a trend towards a reduction in tumour size, which could be attributed to increases in the expression of apoptosis genes. The Lewis lung cancer model is an aggressive model of tumour formation, which may explain the lack of change in tumour formation; therefore our observations provide the impetus to perform future studies in alternate models of cancer.

The Lewis lung carcinoma model was used to assess tumour neovascularisation as the tumour that develops is highly vascularised and therefore relevant to the focus of this thesis on angiogenesis. Following the infusion of Lewis lung carcinoma cells subcutaneously into mice, the tumour is not visible for the first 8 – 9 days. The tumour then very suddenly grows visibly large over the next 1 – 2 days. This rapid tumour growth over the short time frame of just 1 – 2 days makes the optimisation of experiments to determine treatment effects very challenging. Despite this, trends for a reduction in tumour weight by broad-spectrum CC-chemokine inhibition using 35K were observed. This indicates that suppression of CC-chemokine activity has some tumour suppressive effects. Histological assessment of smooth muscle cell α-actin positive arterioles also supported this trend for a reduction in tumour weights. There was a trend for a reduction in arterioles in the Ad35K mice when compared to adenoviral AdGFP controls. Overall, however, 35K had no real effects on tumour development. This was unexpected, given the striking effects seen with 35K in reducing inflammatory-driven angiogenesis both in vivo (Chapter 5) and in vitro (Chapter 3). However, tumour development and progression also involves additional cellular processes including cellular invasion and apoptosis as well as hypoxia-driven angiogenesis, therefore 35K may not be able to modulate multiple cellular processes in such a way as to suppress tumour growth significantly.

Previous studies have reported the presence of CC-chemokines in various malignancies, including breast cancer (CCL2, CCL5), prostate cancer (CCL2) and leukaemia (CCL1, CCL3) (Lu & Kang 2009; Soria & Ben-Baruch 2008; Tanaka et al. 2005; Zhang, Lu & Pienta 2010a). These chemokines promote angiogenesis in the tumour.
In contrast to the suppression of tissue CC-chemokines by 35K in the models used in Chapter 5, we found that CCL2 was unchanged in the Ad35K treated mouse tumours, compared to controls. This is supported by a lack of change in tumour p65, indicating no reduction of inflammation. We propose that this inconsistency may be due to dysregulation of the tumour environment. Additionally 35K may not be having a strong inhibitory effect on CC-chemokines in the tumour environment due to the speed at which the model grows. Furthermore several CC-chemokine receptors are expressed on tumour cells (Balkwill 2004; Muller et al. 2001; Murphy 2001) with comparatively more chemokines in the tumour compared to other pathologies, thus making it more difficult for 35K to prevent CC-chemokine ligand and receptor binding. Without a decrease in tumour CCL2 levels by 35K, inflammatory cell recruitment would also not have been suppressed, as we would have hypothesised with 35K. Macrophages express VEGF and contribute to tumour neovascularisation, thereby a lack of change in inflammatory recruitment can also account for a lack of significant change in tumour weight by 35K. Interestingly, we also found that levels of the anti-apoptotic gene Bcl-2 were trending towards an increase in the Ad35K tumours, which would have been an additional factor in preventing the suppression in tumour weight.

Tumour progression involves cell proliferation and cell invasion. In Ad35K mice there was a trend for an increase in tumour Ki-67 expression, a known marker of cellular proliferation. Similarly c-Jun, associated with regulation of proliferation through AP-1 (Shaulian & Karin 2002), was higher, although not significantly, in the Ad35K mice compared to controls. An increase in c-Jun, will lead to an increase in the expression of the transcription factor AP-1 which in turn promotes extracellular matrix degradation by MMP2 and MMP9. Ad35K lead to a doubling of MMP9 expression in the tumours, which function to degrade the cellular “barriers” allowing for tumour cell invasion and metastasis. Aided by the increase in c-Jun and MMP9, proliferation and cell invasion may have been promoted in the Ad35K mice. As tumour weights were slightly lower in Ad35K mice it raises doubts as to whether this was occurring.

An imbalance between cell proliferation and cell death is characteristic of the tumour environment. Cell death by apoptosis is triggered in order to counteract the over growth of cells to rectify this imbalance. Upon further investigation of the tumours in this model, 35K was able to increase the expression of p53, the tumour suppressor gene. This leads to increase in Bax (pro-apoptosis) and the release of cytochrome C from the
mitochondria. This causes the increase of caspase 9, leading to increase of caspase 3 to increased apoptosis. Caspase 8 was also seen to increase. While no differences were seen in caspase 9 levels, Ad35K tumours had significantly higher levels of caspase 8 and a slight increase in caspase 3. The strong trends seen with the apoptosis markers could account for the slight reduction in tumour size. These results also suggest that 35K may be able to promote apoptosis in the tumours despite showing no signs of reducing inflammation or CCL2 expression.

As the Lewis lung cell model is a rapidly growing vascularised tumour model, perhaps this model was not the most appropriate to explore the effects of 35K and while the changes we see are minimal, and although not significant, the trends observed are promising as there is a possibility for these small changes to be enhanced through other modes of expression or delivery. Future studies could include the use with a membrane bound 35K as used in previous studies (Bursill et al. 2006) to induce localised expression of 35K on the Lewis lung carcinoma cells. This would allow for greater specificity and targeted expression of 35K within the tumour tissues. Alternatively, a less aggressive model of tumour neovascularisation may allow for better elucidation of the effects of 35K in the context of cancer.
CHAPTER 7.
THE EFFECT OF BROAD-SPECTRUM CC-CHEMOKINE INHIBITION IN A MODEL OF WOUND HEALING
7.1. Introduction

Wound healing is an intricate process requiring strict coordination between inflammation and cell growth in the wound. A balance must be maintained to prevent impaired wound healing. The in vivo studies in Chapter 5 have shown that broad-spectrum CC-chemokine inhibition can suppress pathological inflammatory-driven angiogenesis while having no effect on physiological hypoxia-mediated angiogenesis. However, it appeared that 35K had no effect in the tumour neovascularisation model (Chapter 6), although this could be attributed, at least in part, to the aggressiveness of the Lewis Lung cancer model. We now seek to determine the effects of broad-spectrum CC-chemokine inhibition in the murine wound healing model, which involves an initial inflammatory response coupled with physiological-mediated angiogenesis.

Wound healing is a multistep process composed of 3 distinct but overlapping stages: inflammation, proliferation, and remodelling. The first stage is the inflammatory phase, where neutrophils and macrophages are recruited to remove debris and begin the wound healing process. Clot formation, through platelet activation, occurs to prevent further blood loss. This is followed by the proliferation stage, where re-epithelialisation and the formation of granulation tissue begin to close the wound. During this stage angiogenesis is essential to bring inflammatory cells to the wound for debris removal and to provide support for proliferating and migrating cells, and the formation of granulation tissue (Gillitzer & Goebeler 2001). Finally, during the remodelling phase, fibroblasts reorganise the collagen matrix, forming a closed wound (Gurtner et al. 2008).

Inflammation is critical in the initial stages of wound healing, as it promotes fast healing without infection. However, when the inflammatory phase is prolonged, a distinct disadvantage is the increased scar formation that can occur. Excessive scar formation is due to an increased accumulation of macrophages due to the inflammatory response. Prolonged inflammation also delays wound healing times, which in turn increases the risk of infection. Wounds that exhibit impaired healing frequently enter a state of pathological inflammation, with most chronic wounds developing into ulcers linked to ischaemia, diabetes mellitus, venous stasis disease or pressure (Guo & DiPietro 2010). Chronic, non-healing wounds greatly impact a person’s quality of life and when left untreated sometimes lead to amputation resulting in enormous health care burden (Mathieu, Linke & Wattel 2006; Menke et al. 2007).
CC-chemokines regulate key processes involved in angiogenesis. They have been found to have direct effects on angiogenesis by stimulating tubulogenesis, and promoting endothelial cell migration (Galvez et al. 2005; Salcedo et al. 2001; Stamatovic et al. 2006; Strasly et al. 2004). Given the established role of CC-chemokines in inflammation and our recent observations of CC-chemokines in angiogenesis, manipulation of the CC-chemokine family could modulate either of these processes, which may in turn confer beneficial effects on wound healing. In human wounds, CC-chemokines CCL1, CCL2, CCL3, CCL4, CCL5 and CCL7 are expressed in the wound during the first week after injury and high levels of CCL2 have been found in human burns (Engelhardt et al. 1998; Gibran et al. 1997). Additionally studies in excisional wounds show localised expression of CCL2 and CCL3 in the epidermis, while CCL3 was also expressed in follicular epithelium, and sebaceous glands (Jackman et al. 2000). To date, current studies have focussed on the effect of a single CC-chemokine intervention, often with varying results. For example, CCL2 knockout mice have delayed re-epithelialisation and reduced angiogenesis in early stages, whilst CCL3 knockout mice followed relatively normal wound healing (Low et al. 2001). Similarly CCR1 knockout models showed no impairment despite CCR1 chemokine ligands being strongly upregulated in wounds (Kaesler et al. 2004).

These minimal changes are likely due to the redundancy in CC-chemokine signalling, where when a single CC-chemokine is inhibited another CC-chemokine can replace its function. Currently there has been no investigation on the effect of broad-spectrum CC-chemokine inhibition on wound healing. Therefore this study sought to determine the effect of broad-spectrum CC-chemokine inhibition by 35K on cutaneous wounds in a murine model that mimics human wound healing. For this study, the effect of topical application of isolated 35K protein was explored at two time points: (1) Day 4, which encompasses the early stages of wound healing including inflammation and angiogenesis, and (2) Day 10, the late stage of wound healing where inflammation/angiogenesis ceases and remodelling ensues.
7.2. Method

7.2.1. Isolation of 35K protein

35K protein was isolated from produced Ad35K viral media as outlined in Chapter 2, Section 2.2.5. Isolated 35K was dialysed into sterile PBS and filtered through 0.45 µm low protein binding syringe filter (Pall Corporation, NY, USA), then aliquoted to 200 nM and stored at -80°C until use.

7.2.2. Mouse model of wound healing

To explore the effect of broad-spectrum CC-chemokine inhibition, a murine model that closely mimics the human wound healing process was used (Dunn et al. 2013). This model was chosen to overcome the differences between the murine and human wound healing process. In the mouse, dermal wound healing occurs via contraction, while human wound healing occurs through re-epithelialisation. The introduction of splints in this murine model forces wound healing to occur via re-epithelialisation.

Before surgery, silicone splints and occlusive dressing were prepared. To prepare the silicone splint 10 mm diameter circles were cut from the 0.5 mm thick silicone sheet (P18178, Life Technologies, CA, USA). A biopsy punch (BIOPUNCH04, Livingstone international PTY, LTD, NSW, Australia) was used to create a hole in the centre of the circles to create an “O” shape. To prepare the occlusive dressings 10 mm diameter circles were created from OPSITE™ FLEXIFIX™ Transparent Film (66000041, Smith & Nephew, London, UK).

To perform the wound healing model, mice were anaesthetised by inhalation of methoxyflurane, pedal reflex were tested to confirm adequate analgesia before placing mice in the prone position. Using a biopsy punch to create a circle on the skin as a guide, two full thickness wounds including the panniculus carnosus, were excised on the dorsum of the mice, below the forelimbs, one on each side of the midline using surgical scissors. Backing from the silicone splint was removed, before the splint was secured around the wound with adhesive (super glue) and interrupted sutures, using 6-0 PROLENE™ sutures (8805H, ETHICON LLC, San Lorenzo, Puerto Rico). Wound diameter measurements were taken on the x-axis, y-axis and z-axis of the wounds using micro-callipers, before topical
application of either sterile PBS (vehicle control) or 200 nM 35K protein (n = 12 mice per time point). The clear dressing was applied on top of each wound. Following surgery, mice were injected subcutaneously with carprofen for pain relief, and mice were monitored for signs of discomfort. Wound measurement and application of treatment was conducted daily for the duration of the study. Blood perfusion was also measured by laser Doppler imaging (moorLDI2-IR, Moor Instruments, Devon, UK) throughout the study. Two cohorts were completed to determine changes at both the early-mid (Day 4) and late (Day 10) stages of wound healing.

7.2.3. Sacrifice

Mice were sacrificed 4 or 10 days following wounding by overdose of anaesthesia of methoxyflurane followed by right ventricular puncture. The wounds were excised and prepared for histological, protein or gene analyses.

For histology, wounds were cut in half directly through the middle of the wound. The sample was placed between two biopsy pads and into an embedding cassette, then fixed in 4% (v/v) paraformaldehyde overnight at 4°C before transferring to 70% ethanol prior to processing in graded ethanol concentrations before xylene and paraffin embedding. 5 μm sections were taken and stained for neovessels (CD31), arterioles (α-actin), macrophages (CD68) and collagen using Milligan’s trichrome according to techniques outlined in Chapter 2, Section 2.3.6.3.

For protein and gene analysis the remaining wound tissue was halved and stored in Eppendorf tubes at -80°C. Protein and gene levels of CC-chemokines and key angiogenic (HIF-1α, VEGF and CXCL12) and inflammatory (active p65 subunit of NF-κB and CD68) markers were detected using Western blot, ELISAs or quantitative real-time PCR as outlined in Chapter 2, Sections 2.3.7 – 2.3.8.
7.3. Results

7.3.1. Laser Doppler imaging and macroscopic measurement of wounds

Laser Doppler imaging (LDI) was used to determine blood flow perfusion in the wounds over the study period. For early-mid (Day 4) stage wounds, LDI was performed daily, whilst LDI for the late (Day 10) stage wounds was performed on Days 2, 4, 7 and 10. LDI was assessed by determining the ratio of 35K:PBS blood perfusions, where values over 100% indicate improved perfusion by the treatment (35K) compared to PBS controls. In Figure 7.1A, there was an increase in blood flow perfusion to the 35K wounds during the early-mid stage (Days 2 – 4), where angiogenesis is most important in the wound healing process. By Day 7 a decline in LDI perfusion was seen in the 35K wounds, however blood perfusion then increased by 19.8% (p<0.05) at Day 10 in the 35K treated wounds.

Wound closure measurements were taken daily to determine the difference in wound area over time for PBS and 35K treated wounds. Topical application of 35K protein promoted wound closure, particularly at Days 4 – 6 (p<0.05) compared to PBS as seen in Figure 7.1B.
Figure 7.1. Improved blood flow perfusion early post-wounding correlates with faster wound closure in 35K treated wounds.

(A) Laser Doppler imaging (LDI) was used to determine blood flow perfusion of the wounds. Measurements above 100% represent increased blood perfusion with the 35K treatment. (B) Wound closure measurements were taken daily to compare PBS and 35K treated wound areas. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05 compared to relevant PBS treatment.
7.3.2. **Histology**

7.3.2.1 **Neovascularisation**

To demonstrate angiogenesis in the wounds, neovessels and arterioles were assessed by detecting the presence of endothelial cells (CD31$^+$) and smooth muscle cells ($\alpha$-actin$^+$). Typically, angiogenesis increases in the early-mid stage producing a rich and complex neovasculature allowing for increased infiltration of inflammatory cells for debris removal. However, at the late stage of wound healing, angiogenesis has ceased and the newly formed vessels disintegrate, leaving a collagen rich scar. Consistent with this, 35K increased neovessels by 81.6% (p<0.05) compared to PBS in the Day 4 wounds, where angiogenesis is essential to wound healing (Figure 7.2). However, at Day 10 there was a 39.1% (p<0.05) decrease of neovessels in the 35K treated wounds compared to PBS, where angiogenesis has ceased. Furthermore, the arterioles detected in the wounds indicated a similar pattern as seen in the neovessels (Figure 7.3), where in the Day 4 wounds, there was a non-significant 34.2% increase in the number of arterioles in the 35K treated wounds, whilst by Day 10 35K treated wounds had 47.6% (p<0.05) less arterioles.
Figure 7.2. 35K treatment increases neovessels in the early stage wounds, but decreases neovessel content in late stage wounds.

Photomicrographs represent neovessels detected by staining for CD31\(^+\) (brown) in PBS and 35K treated wounds at Day 4 and Day 10. Scale bars represent 50 \(\mu\)m. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *\(p<0.05\) compared to relevant PBS treatment.
Figure 7.3. 35K treatment decreases arterioles in late stage wounds.

Photomicrographs represent arterioles detected by staining for smooth muscle $\alpha$-actin$^+$ (pink) in PBS and 35K treated wounds at Day 4 and Day 10. Scale bars represent 50 $\mu$m. Data is expressed as mean$\pm$SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05 compared to relevant PBS treatment.
7.3.2.2 Macrophage Infiltration

Macrophages play an important role in mediating inflammation in the wound healing process and have indirect effects on angiogenesis via their expression of VEGF. Histological analysis of macrophages revealed that at the early stage of wound healing, there were no differences in macrophage infiltration in wounds treated with 35K compared to PBS (Figure 7.4). There was, however, a non-significant trend for a decrease (24.8%) in wound macrophage content in the late stage (Day 10 post-wounding) cohort of 35K-treated wounds.

Figure 7.4. Macrophage infiltration is unchanged in wounds treated with 35K. Photomicrographs represent macrophages detected by staining for CD68⁺ (brown) in PBS and 35K treated wounds at Day 4 and Day 10. Scale bars represent 50 μm. Data is expressed as mean±SEM.
### 7.3.2.3 Collagen

In wound healing, collagen formation is the pre-requisite to scar formation. Collagen begins to form during the proliferation stage of wound healing to help fill the wound area. Typically, during this stage the wound is dominated by inflammatory cells indicated by the purple nuclear staining, which is depicted in the Day 4 wounds (Figure 7.5). We found no differences in the amount of collagen deposition at Day 4 in the PBS and 35K wounds. However in the Day 10 wounds, 35K significantly reduced the amount of collagen by 25.3% (p<0.05) compared to PBS.

**Figure 7.5.** 35K decreases collagen formation in late stage wounds. Collagen (green) was detected by Milligan’s trichrome staining in PBS and 35K treated wounds at Day 4 and Day 10. Scale bars represent 50 μm. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05 compared to relevant PBS treatment.
7.3.3. **CC-chemokine expression**

CC-chemokine expression during wound healing was determined through gene and protein analysis. Overall there was a decrease of CCL2 and CCL5 gene and protein expression with topical application of 35K protein to the wounds.

Gene expression of CCL2 at the early (Day 4) time point, decreased by 36.0% (p<0.05, Figure 7.6A), which subsequently translated to a 40.8% (p<0.05) decrease in protein expression in the 35K treated wounds (Figure 7.6C). Additionally, at the Day 10 time point, despite a non-significant 21.5% increase in CCL2 gene expression, protein expression decreased by 22.2% (ns) with topical 35K treatment compared to PBS treated wounds.

Similarly, at the Day 4 time point, while a non-significant decrease of 31.7% in CCL5 mRNA levels was seen in the 35K treated wounds (Figure 7.6B), CCL5 protein levels were significantly lower (36.5%, p<0.05, Figure 7.6D) compared to PBS treated wounds. However, 35K decreased both CCL5 gene (62.1%, p<0.05, Figure 7.6B) and protein (65.8%, p<0.01, Figure 7.6D) expression at Day 10.
Figure 7.6. CC-chemokine expression in the wounds.
Real-time PCR was used to measure gene expression of (A) CCL2 and (B) CCL5 in PBS and 35K treated wounds at Day 4 and Day 10. ELISAs were used to detect protein expression of (C) CCL2 and (D) CCL5 in PBS and 35K treated wounds at Day 4 and Day 10. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05, **p<0.01 compared to relevant PBS treatment.
7.3.4. Markers of angiogenesis and inflammation

Wound angiogenesis is primarily driven by the inflammatory response, but hypoxia also plays a role in promoting neovascularisation of the wound. To further elucidate the mechanistic effects of 35K on the wound healing process, the expression of key angiogenic markers (HIF-1α, VEGF and CXCL12) and inflammatory markers (p65 and CD68) were determined.

The mRNA levels of HIF-1α were not changed in the wounds of 35K treated mice although it was noted that there was a non-significant reduction (27.4%) in the Day 4 cohort (Figure 7.7A). Similarly, protein expression of HIF-1α was not changed at any time point (Figure 7.7B). Despite trends for a reduction, there were no changes in VEGF mRNA (Figure 7.7C) or protein (Figure 7.7D) levels. There was, however, a significant reduction in CXCL12 mRNA levels (55.6%, p<0.05) in Day 10 wounds treated with 35K, compared to PBS control wounds (Figure 7.7E).

Gene expression of the active subunit of NF-κB, p65, was shown to significantly decrease by 33.8% (p<0.05) at Day 4 and by 62.3% (p<0.05) at Day 10 compared to PBS treated wounds (Figure 7.8A). This correlated with the reduction in CC-chemokine expression that we see in these wounds (Section 7.3.3). No changes were seen in the levels of CD68 (Figure 7.8B), which correlated with the lack of differences seen in the macrophage histological observations in Section 7.3.2.2.
Figure 7.7. Expression of angiogenic markers in wounds.
Real-time PCR was used to measure gene expression of (A) HIF-1α, (C) VEGF, and (E) CXCL12, and protein expression was determined by (B) Western blot for HIF-1α and (D) ELISA for VEGF in PBS and 35K treated wounds at Day 4 and Day 10. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05 compared to relevant PBS treatment.
Figure 7.8. Gene expression of inflammatory markers in wounds.

Real-time PCR was used to measure gene expression of (A) active p65 subunit of NF-κB and (B) CD68 in PBS and 35K treated wounds at Day 4 and Day 10. Data is expressed as mean±SEM. Statistical analysis was performed using an unpaired two tailed t-test. *p<0.05 compared to relevant PBS treatment.
7.4. Discussion

The stages of the wound healing process are highly regulated and encompass the most complex biological processes that occur in human life. An imbalance or change to the process can greatly affect the outcome of wound healing, resulting in impaired wound healing which can often lead to severe unfavourable outcomes such as amputation; therefore highlighting the need to develop novel agents that promote wound repair. This study used a murine wound healing model that mimics the human wound healing process to explore the effect of topical application of 35K protein to treat cutaneous wounds. We found that broad-spectrum CC-chemokine inhibition by 35K promoted the rate of wound closure during the early stage (Day 4) and improved the state of the healed wound, producing less collagen and less neovessels compared to PBS control treatments, by the late stage (Day 10). Additionally, there was an overall reduction in the inflammatory state of the 35K treated wounds, which is accompanied by reduced CC-chemokines at the wound site.

Topical application of the broad-spectrum CC-chemokine inhibitor 35K promoted blood flow perfusion at the early stages of wound healing (Days 2 – 4). This was complemented by an increase in wound closure during the early-mid stage of healing (Days 4 – 6). At this stage blood flow perfusion is critical to wound healing to bring inflammatory cells to the wound site, changing the leukocyte subset from dominantly neutrophils to macrophages (Gurtner et al. 2008; Sunderkotter et al. 1994). This change is essential to accelerate debris removal and to promote wound healing. Macrophages release a host of growth factors and cytokines including in particular VEGF as well as TGF-β, TGF-α, bFGF, PDGF. Some of these stimulate collagen (Eming, Krieg & Davidson 2007). Consistent with previous studies in CCL2 and CCL3 knockout mice (Low et al. 2001), macrophage recruitment was maintained and collagen deposition reduced despite a reduction in neovessels in the Day 10 cohort of the present study. Additionally, increased perfusion increases oxygenation to help maintain the formation of the healed tissue (Jonsson et al. 1991). Typical at this early stage of wound healing, there was an increase of neovessels and arterioles in the 35K wounds, which in turn facilitated the increased perfusion detected by Laser Doppler imaging.

In contrast to the early stage of wound healing, the late stage (Day 10) wounds had reduced neovessels and arterioles with 35K treatment. At this stage, wounds have healed,
re-epithelialisation has occurred, inflammation has ceased, a scar has formed, and collagen remodelling from type III to type I collagen can begin (Lovvorn et al. 1999). Importantly as the wound healing process is near completion, angiogenesis ceases and neovessels disintegrate to allow for continued remodelling (Gillitzer & Goebeler 2001; Ilan, Mahooti & Madri 1998; Singer & Clark 1999). The pronounced reduction of neovessels and arterioles in our 35K treated wounds suggest that these wounds are at a more advanced stage where wounds have healed faster by Day 10 compared to PBS treatment. Consistent with this, the 35K wounds exhibited less collagen formation, filling the wound quickly and helping to close the wound to allow for reduced scar formation in the wounds. These findings are similar to previous studies in which reduced fibrosis was detected in kidneys and lungs of mice when CCR1 or CCR5 were knocked down or their signalling inhibited (Anders et al. 2002; Schuh, Blease & Hogaboam 2002).

Unexpectedly in the Day 10 wounds, 35K treatment caused an improvement in blood flow perfusion compared to PBS. This increase could be due to several factors. At this stage, remodelling is in its early phase, where the granulation tissue is disorganised and neovessels are still present but not as prominent as in the earlier stages. The increased perfusion in 35K treated wounds may be a factor of the less densely packed collagen, allowing more space for neovessels to increase the perfusion detected at the surface; whereas in the PBS wounds, a more tightly packed collagen was present preventing the detection of perfusion at the surface of the wound area. Additionally, the wound area measurement by Laser Doppler assesses the entire area with the circular splint, rather than only the healing wound area, which could result in the detection of residual perfusion around the wound.

Previous studies have shown an increase in the expression of CC-chemokines including CCL1, CCL2, CCL3, CCL4, and CCL5 in normal wounds, and particularly high expression of CCL2 one day after wounding (DiPietro et al. 1995; Engelhardt et al. 1998; Gillitzer & Goebeler 2001; Jackman et al. 2000), with more than 20% of total cells in the wound expressing CCL2 and by Day 2 hyperproliferating keratinocytes also express CCL2 (Gillitzer & Goebeler 2001). Increased expression of CCL2 was reported 6 hours after wounding compared to control, with persistent levels maintained during the early stages of healing (Jackman et al. 2000). In our study, the Day 4 and Day 10 wounds treated with 35K showed an expected overall decrease in the expression of CCL2 and CCL5. This is typically associated with decreased macrophages to the site of injury, however no
significant reductions in macrophages were observed. It has been previously reported that despite dominant expression of CCL2, these levels are considered relatively low and correlate with the observation of optimal monocyte accumulation with low levels of CCL2 (Gu et al. 1997), indicating that, a reduction but not complete ablation of CCL2 and consequently other CC-chemokine expression, may be beneficial in the recruitment of optimal levels of macrophages in the early stage. However, at the later stage (i.e. Day 10), the wound is preparing to enter remodelling so macrophages then undergo apoptosis and chemokine upregulation and angiogenesis are reduced. This may explain the consistent macrophage levels between PBS and 35K wounds despite the reduction in CC-chemokine levels. Even without significant reductions to macrophages in the wound, there was an overall reduction of inflammation in the 35K treated wounds as shown by reduction in p65 expression.

Consistent with the observations seen with macrophage infiltration, there was no change in VEGF expression in wounds treated with 35K compared to PBS. VEGF is involved in angiogenesis and the synthesis of collagen in the wound (Bao et al. 2009; Eming, Krieg & Davidson 2007). It is produced by wound fibroblasts, keratinocytes and macrophages (Eming, Krieg & Davidson 2007; Martin 1997; Singer & Clark 1999). As VEGF is not solely regulated through CC-chemokine activity, the reduced CC-chemokine expression in 35K wounds had minimal effects on VEGF, and therefore sufficient VEGF expression can be produced through other avenues. We also found no inhibition of HIF-1α gene and protein expression in the wounds, suggesting that there was sufficient oxygenation to the wounds to aid in the healing process. Despite no significant changes in HIF-1α, there was a surprising reduction of CXCL12 in the late stage of wound healing of our 35K treated wounds. At this point wounds have healed, thus the levels of CXCL12 may have declined as it is no longer needed at this point, particularly as the 35K treated wounds healed faster.

The present study found a significant decrease in mRNA levels of the active NF-κB subunit p65 at both time points. As NF-κB also drives other inflammation-responsive genes that may mediate macrophage recruitment, this suggests that the macrophages we have detected may be recruited by means other than solely through CC-chemokines and other NF-κB-driven genes. For example macrophages may be recruited by other chemokine classes as they can also express CXCR1 and CXCR2 receptors which bind CXCL8 (Patel et al. 2001). Additionally, macrophages can be divided into pro- and anti-
inflammatory phenotypes and both are involved in different stages of wound healing (Daley et al. 2010). With this in mind, there could be a possible shift in macrophage phenotype from pro-inflammatory to a more anti-inflammatory phenotype which is important in wound regulation (Daley et al. 2010).

In conclusion, this study shows that broad-spectrum CC-chemokine inhibition by topical application of 35K enhances wound closure through early promotion of neovascularisation. There were also reductions in the key inflammatory transcription factor NF-κB. Additionally, reduced collagen deposition in the Day 10 35K treated wound was indicative of reduced scar formation. Taken together, our findings showed that broad-spectrum inhibition of CC-chemokines may be beneficial in chronic wounds where inflammation is in excess, in order to promote wound healing and neovascularisation and reduce scar formation.
CHAPTER 8.
DISCUSSION
8.1. Introduction

Angiogenesis can have both beneficial and detrimental effects depending on the pathophysiological context. Current anti-angiogenic therapies are successful in suppressing the inflammatory-driven angiogenesis seen in pathological diseases such as atherosclerosis and cancer. However, physiological ischaemia-mediated angiogenesis that is essential for growth and regeneration, is also inhibited resulting in severe side effects including hypertension, haemorrhaging, vomiting, nausea, gastrointestinal perforations and reduced wound healing. This highlights the need for alternate anti-angiogenic therapies that can specifically target inflammatory-driven angiogenesis whilst having minimal effects on ischaemia-mediated angiogenesis. Increasing evidence has shown that CC-chemokines are implicated in inflammatory induced pathologies in which angiogenesis plays an important role. In contrast, the CC-chemokine class appear to have little to no role in ischaemia-mediated angiogenesis. Specific inhibition of the CC-chemokine class may therefore inhibit pathological inflammatory-driven angiogenesis, while preserving physiological hypoxia-mediated angiogenesis. The broad-spectrum CC-chemokine inhibitor ‘35K’ has already been shown to inhibit a host of inflammatory diseases including atherosclerosis, acute peritonitis and hepatitis, however a comparison of its role in both inflammatory and ischaemia-driven angiogenesis has not been tested.

This thesis investigated the effect of broad-spectrum CC-chemokine inhibition on angiogenesis using 35K. Treatment with purified 35K protein in vitro significantly reduced key functional angiogenic assays when subjected to an inflammatory stimulus, but had more modest or no effects in response to hypoxia. Mechanistically, in vitro studies revealed that 35K conditionally regulates VEGF, such that it is inhibited under inflammatory conditions, but yet preserved in hypoxia. These in vitro observations were also translated in vivo. Using an adenoviral delivery approach to overexpress 35K in mice, we observed that 35K effectively reduced the number of adventitial neovessels and arterioles in the peri-arterial femoral cuff model of inflammatory-driven adventitial angiogenesis. In contrast, 35K had no inhibitory effect on ischaemia-mediated angiogenesis in the hindlimb ischaemia model. The effect of CC-chemokine inhibition was also explored on tumourigenesis, where angiogenesis is driven by both inflammation and hypoxia. We found that, despite a trend for a reduction in tumour size, 35K had no effect on tumourigenesis in the Lewis lung carcinoma model. However, this lack of effect is likely due to the aggressiveness of the model used. Finally, given angiogenesis is critical in
the early stages of wound healing, topical application of 35K protein was tested in a murine wound healing model that mimics human wound closure. 35K improved the rate of wound neovascularisation and wound closure. The wounds also contained less collagen, indicating that there would ultimately be less scar formation.

8.2. Conditional regulation of angiogenesis by 35K *in vitro*

Endothelial cells (ECs) are one of the major cell types involved in angiogenesis and as they are mainly quiescent, angiogenesis needs to be triggered by a stimulating cytokine, such as the pro-angiogenic mediator VEGF (Carmeliet 2000; Karamysheva 2008). Endothelial cell proliferation, migration and tubule formation are key processes for the formation of neovessels (Carmeliet 2000). Upon VEGF stimulation, endothelial cells proliferate and migrate to sites of injury. They then assemble into tubular structures to form new blood vessels at the injury site. To examine this process, the effect of 35K in three *in vitro* functional angiogenic assays was assessed in inflammatory and hypoxic conditions. These studies, highlighted in Chapter 3, found that 35K strikingly inhibits proliferation, migration and tubule formation under inflammatory conditions, but these effects were more modest in response to hypoxic stimulation. The observations in response to inflammation support previous studies where incubation with single CC-chemokines (such as CCL2, CCL11 and CCL16) directly promotes endothelial cell tubule formation, migration and neovascularisation (Galvez et al. 2005; Salcedo et al. 2001; Stamatovic et al. 2006; Strasly et al. 2004). As expected, 35K has minimal or no effects under hypoxic stimulation as it inhibits CC-chemokines that have not been previously reported to have a role in ischaemia-mediated angiogenesis. Furthermore, the specificity of 35K to bind CC-chemokines means that it does not interact with the CXC-chemokine class (Burns et al. 2002). This is important as CXC-chemokines and, in particular, CXCL12 plays a key role in angiogenesis in response to hypoxia/ischaemia (Frangogiannis 2007).

In this study we chose to examine all three endothelial angiogenic processes to allow for a more complete understanding of the effect of broad-spectrum CC-chemokine inhibition on angiogenesis. Firstly, proliferation, the initial phase of angiogenesis after endothelial cell activation, was determined by directly measuring DNA synthesis using the Click-It EdU assay. This assay has advantages over other protocols as it does not require denaturing. This is due to the small size of the azide dye of the assay, which can easily
access the EdU through detergent permeabilisation. Additionally, this assay is more specific than other proliferation assays which count the number of “living cells” either by crystal violet staining, trypan blue exclusion or the MTT assay (Staton, Reed & Brown 2009), but rather assesses the transition from the G0-phase to S-phase of the cell cycle.

Secondly, endothelial cell migration is crucial to angiogenesis to recruit new cells to the site of injury. The scratch assay with monitoring by live cell imaging was chosen over the more conventional chemotaxis assay as it measures the cell to cell interaction to stimulate migration instead of the interaction with a stimulus. Thus the scratch assay is a better method to determine the effect of CC-chemokine inhibition by 35K, as there is no interference from an angiogenic stimulus. The assay was performed in 4 well chamber slides, thereby limiting the number of concentrations tested to one per treatment. The 50 nM concentration was chosen as higher concentrations showed complete inhibition of migration (data not shown). The benefit of choosing the chamber slide was the markings in the chamber slide allowed the scratches to be made at consistent intervals in each chamber and across all slides. Furthermore, as images were taken by the live cell imager over time, this allows for additional analysis such as the rate of migration or wound “closure” to be done.

Thirdly, tubulogenesis is the final step to forming new blood vessels. At this point endothelial cells have proliferated and migrated to the site of injury and must now form the early blood vessel structures, the capillaries. The basic tubule formation assay involves plating endothelial cells onto or into a layer of gel matrix (commonly collagen, fibrin or matrigel), which stimulates the attachment, migration and differentiation of endothelial cells into tubule like structures in a manner that simulates the in vivo situation (Kanzawa, Endo & Shioya 1993; Lawley & Kubota 1989). For this assay, cells were plated onto growth factor reduced (GFR) matrigel as opposed to the basement membrane matrigel. While not completely depleted of growth factors, GFR matrigel contains the same extracellular proteins with decreased amounts of EGF, IGF-1, PDGF and TGF-β, thus allowing for better testing of endothelial tubule formation without the strong interference of angiogenic growth factors that may be supplemented in the basement membrane matrigel (Taub et al. 1990). Endothelial tubule formation is observed for 4 hours, where cells will attach to the matrigel, migrate towards each other, align to the basement membrane (nucleus facing the basement membrane) and form tubules. This assay encompasses the key steps involved in angiogenesis, making the GFR matrigel
tubulogenesis assay a good in vitro test for angiogenesis in response to broad-spectrum inhibition of CC-chemokines by 35K.

Alternate assays that could be used to assess angiogenesis include the 3D gel assay, the chick choriallantoic membrane assay and the matrigel plug assay. However, these assays focus on the end product of new blood vessel formation and do not assess the individual processes involved in angiogenesis. Additionally, certain alternate assays such as the corneal angiogenesis assay, and the sponge plug assay do not allow the control of the angiogenic environment (inflammation/hypoxia) tested, thus not enabling the differential comparison of the effects of 35K. Despite the range of in vitro assays, a ‘gold-standard’ angiogenesis assay has yet to be developed; therefore, a combination of assays are required to identify the full scope of effects of a test protein or to identify the molecular and/or cellular events in angiogenesis (Staton, Reed & Brown 2009). The three assays used in this study, allowed for a more thorough elucidation of the effects of broad-spectrum CC-chemokine inhibition on angiogenesis in vitro in both inflammatory and hypoxic conditions.
8.3. 35K conditionally regulates key angiogenic marker VEGF *in vitro*

Given the differential effects of 35K on *in vitro* functional angiogenic assays, the studies outlined in Chapter 4 sought to elucidate the mechanism of 35K action by measuring key angiogenic markers *in vitro*. We found that 35K treatment reduced the expression of HIF-1α and VEGF in inflammatory conditions, whilst in hypoxia 35K had more modest inhibitory effects on HIF-1α, and no effect on VEGF. Further elucidation of the HIF-1α post-translational modulation pathway also revealed that 35K had no effect on the prolyl hydroxylases (PHD1-3). These results suggest that 35K conditionally regulates VEGF. This is possible due to the presence of response elements for both NF-κB and HIF in its promoter region. Similarly, HIF-1α also has NF-κB and HIF response elements in its promoter region. Accordingly a similar response pattern as VEGF may be expected with 35K. However, in response to hypoxia, HIF-1α is also post-translationally modulated by prolyl hydroxylases to promote HIF-1α degradation. It may be possible that 35K affects the post-translational modulation of HIF-1α under hypoxia. However, we found no effects on PHD1-3 with 35K under hypoxia. Therefore an alternate pathway in which VEGF is maintained in hypoxia despite reduced HIF-1α may be affected by 35K. Previous studies have found that while HIF-1α is the main transcription factor driving ischaemia-mediated angiogenesis, the NF-κB signalling pathway may also be activated in response to hypoxia. Under hypoxia, inhibition of PHDs increases NF-κB activation through phosphorylation of its upstream enzyme IKK (Cummins et al. 2006). As HIF-1α also has an NF-κB response element in its promoter region, it is proposed that 35K is most likely suppressing HIF-1α expression through its effects on NF-κB and not via direct cellular responses to hypoxia. There may also be additional activation of NF-κB in hypoxia that helps maintain VEGF levels following the suppression of HIF-1α by CC-chemokine inhibition.
8.4. 35K reduces inflammatory-driven adventitial neovascularisation whilst ischaemia-mediated neovascularisation is unaffected in vivo

To determine whether the differential angiogenic effects of 35K in vitro were also observed in vivo, two murine models were used to examine inflammatory-driven angiogenesis (peri-arterial cuff model) and ischaemia-mediated angiogenesis (hindlimb ischaemia model). For the in vivo studies, an adenoviral approach was used to overexpress 35K protein systemically. A single injection of Ad35K promoted persistent high-level expression of 35K avoiding the need for multiple dosing with recombinant protein. Previous studies report that the virus is expressed for at least 1 month in vivo post-injection (Ali et al. 2005). Our findings highlighted in Chapter 5, showed that broad-spectrum inhibition of CC-chemokines with 35K reduced inflammatory-driven adventitial neovascularisation but, in contrast, had no effect on ischaemia-mediated angiogenesis.

The murine peri-arterial cuff model was first adapted from a similar model in rabbits (Moroi et al. 1998). In this model the non-constricting cuff triggers intimal hyperplasia, and promotes neo-adventitial formation that contains neovessels formed three weeks after cuff placement. This model was selected for our studies as it produces a rapid angiogenic effect that is solely induced by the inflammatory stimulation caused by the placement of the cuff, which in turn promotes the recruitment of macrophages that secrete pro-angiogenic cytokines and growth factors, and promote neovascularisation. Furthermore, as opposed to a stent or graft model, there is a minimal chance of the incidence of thrombosis which could impact on the experimental outcome (Carmeliet, Moons & Collen 1998). The aetiology of this model is such that it exhibits many similar features as those seen in atherosclerosis.

The murine hindlimb ischaemia model is a well-established model of ischaemia-mediated neovascularisation (Bonauer et al. 2009; Limbourg et al. 2009; Niiyama et al. 2009). Both angiogenesis and arteriogenesis can be investigated using this murine model depending upon where the ligature is placed, and whether the femoral vessels are completely excised. Ligation of the femoral artery causes an ischaemic environment in the mouse hindlimb. To repair this arteriogenesis occurs in the femoral collaterals and angiogenesis occurs in the distal ischaemic muscles. The current study ligated the femoral artery above both the epigastrica and profunda femoris before severing them distal to the ligation. The femoral artery and vein were also completely excised as distal as the
This method ensures that in the gastrocnemius muscle there is significant hypoxia and that angiogenesis occurs rather than arteriogenesis. This model with the exact same surgical procedure has been utilised by multiple research groups worldwide for this purpose and was recently characterised (Limbourg et al. 2009). Furthermore, only the gastrocnemius muscle was analysed, which is distal to the ligation and rich in neovessels, rather than the adductor muscle where arteriogenesis may occur.

Increasing evidence suggests that CC-chemokines are important in inflammatory-driven angiogenesis. Previous studies have examined the effect of single CC-chemokine or CC-chemokine receptor knockout studies in various inflammatory-driven angiogenic pathologies, however these studies have reported conflicting results. For example, in 2008, a study of CCL3\(^{-/-}\) and CCR5\(^{-/-}\) mice show reduced corneal neovascularisation, and reduced macrophages compared to wild type mice suggesting an importance of CCL3 and its receptor CCR5 in this inflammatory model of angiogenesis (Lu et al. 2008). Contradictory to this, in a study done the following year in the sponge-induced model of inflammatory-driven angiogenesis, no changes in neovascularisation was seen in CCL3\(^{-/-}\) and CCL5\(^{-/-}\) mice (Barcelos et al. 2009). Additionally, treatment with Met-RANTES (CCR1/CCR5 antagonist) reduced neutrophil and macrophage influx in wild type mice, whilst improving vascularisation in a sponge-induced inflammatory angiogenesis model (Barcelos et al. 2009). These conflicting results may be due to the redundancies seen in the chemokine family. Furthermore, the sponge model is not exclusively a model of inflammatory-driven angiogenesis.

Chemokine ligands are able to bind to several chemokine receptors, and conversely, chemokine receptors are able to bind to several CC-chemokine ligands. However these interactions are restricted within each chemokine class, whereby chemokines and receptors will not bind to chemokines from different classes. Chemokine redundancy is essential for the chemokine network as it allows for the robust control of the inflammatory system, such that if one chemokine is inhibited another may replace its function. With this in mind, we used the broad-spectrum CC-chemokine inhibitor 35K to determine whether inhibition of the entire CC-chemokine class would be a better therapeutic approach for the inhibition of inflammatory-driven angiogenesis. Our findings suggest that by removing the influence of redundancies, inflammatory-driven angiogenesis can be better targeted and significantly suppressed. We propose that 35K mediates its actions via 3 ways: (1) binding CC-chemokines in the tissue and circulation, (2) causing
inactivation to prevent the recruitment of monocytes to the site of injury, and (3) removing bound CC-chemokines from the tissue to the circulation to reduce the expression of CC-chemokines at the injury site.

Broad-spectrum CC-chemokine inhibition has added advantages over current anti-angiogenic therapies used in cancer as it had no effect on ischaemia-mediated angiogenesis. This is significant as physiological angiogenesis is critical for repair and regeneration following a myocardial infarction or in wound healing. If angiogenesis is completely ablated across all pathophysiological contexts, normal repair and growth processes are inhibited, leading to a wide variety of side effects such as severe bleeding, proteinuria, arterial and venous thromboembolisms, hypertension, and gastrointestinal perforations, as seen with current anti-angiogenic therapies (Elice & Rodeghiero 2012; Keefe et al. 2011).

Taken together with our in vitro observations from Chapters 3 and 4, we find that broad-spectrum inhibition of the CC-chemokine class by 35K suppresses inflammatory-driven angiogenesis yet preserves ischaemia-mediated angiogenesis. Mechanistically, this may be via the conditional regulation of the key pro-angiogenic mediator VEGF in inflammation and hypoxia. Global inhibition of the CC-chemokine class may therefore present as an alternate therapeutic strategy to specifically inhibit diseases associated with inflammatory-driven angiogenesis such as atherosclerosis and cancer, without causing the adverse side effects that occur with complete inhibition of angiogenesis in all pathophysiological contexts.
8.5. **35K has no effect on tumour formation *in vivo***

Chapters 3 – 5 demonstrated that broad-spectrum CC-chemokine inhibition has differential effects on angiogenesis depending on the pathophysiological context. The studies conducted in Chapter 6 then sought to elucidate the effect of broad-spectrum CC-chemokine inhibition on tumour formation in which angiogenesis is driven by both inflammation and hypoxia. The Lewis lung carcinoma (LLC) model was chosen for these studies as it is a highly vascularised tumour model. LLC is a cell line established from the lung of a C57Bl/6 mouse bearing a tumour resulting from the implantation of primary Lewis lung carcinoma (Kellar, Egan & Morris 2015). The cell line is highly tumourigenic and is primarily used to model metastasis as well as to evaluate the efficacy of chemotherapeutic agents *in vivo* (Fisher et al. 2001; Perez-Soler 2004; Politi et al. 2006). The LLC cell line is typically injected subcutaneously into the back flanks of C57Bl/6 mice (1×10^7 cells per mouse) and within two weeks of incubation, tumours reach 2.2±0.4 mm (Fichtner et al. 2008; Politi et al. 2006). Overall, we found that the effects of broad-spectrum CC-chemokine inhibition by 35K on tumour size were not striking. This may be attributed to the aggressiveness of the tumour model chosen. Interestingly, however, the tumours in the Ad35K infused mice were slightly smaller in size and there was an overall trend towards an increase in apoptotic markers which could have attributed to this.

CC-chemokines regulate the infiltration of inflammatory cells in the tumour environment. Most notably, CC-chemokines are involved in the recruitment of tumour associated macrophages (TAM), especially CCL2 and CCL5 in breast cancer, prostate cancer lung cancer (Conti & Rollins 2004; Mantovani et al. 2004; Rivas-Fuentes et al. 2015; Sawanobori et al. 2008; Soria & Ben-Baruch 2008; Zhang, Lu & Pienta 2010a), and CCL7 in gliomas (Okada et al. 2009). As with macrophages, in response to inflammation TAMs are associated with angiogenesis, however they are also involved in the inhibition of anti-tumour responses (Mantovani et al. 2006; Pollard 2004). CC-chemokine levels are also found to be elevated in melanoma, breast cancer, colon cancer, oesophageal cancer, pancreas cancer, and prostate cancer (Harlin et al. 2009; Loberg et al. 2007; Luboshits et al. 1999; Monti et al. 2003; Ohta et al. 2002; Ueno et al. 2000). In the tumour environment, CC-chemokines are produced by tumour and stromal cells (Karnoub et al. 2007). CC-chemokines are increasingly linked with a range of tumours, with elevated CCL2, CCL5, CCL17, and CCL22 levels being associated with poor prognosis in human tumours (Lee et al. 2009; Luboshits et al. 1999; Niens et al. 2008; Ueno et al. 2000). Furthermore,
simultaneous expression of both CCL2 and CCL5 has been linked to advanced stages of breast cancer. With this in mind, it would have been anticipated that broad-spectrum CC-chemokine inhibition would suppress tumour progression.

In addition to increasing angiogenesis in the tumour environment, macrophages also release matrix metalloproteinases (MMPs). Previous studies show that CC-chemokines, mainly CCL2, CCL4, and CCL5 induce the production/release of MMP9 in macrophages (Robinson, Scott & Balkwill 2002). MMPs such as MMP9 are involved in extracellular matrix degradation and remodelling which aid in tumour cell migration and invasion. Additionally, previous studies found the increase of MMP9 in pancreatic cancer is critical for angiogenesis by stimulating VEGF production (Nozawa, Chiu & Hanahan 2006). Elevated levels of TAMs/macrophages in the tumour environment are greatly associated with poor prognosis in cancers/tumours (Bingle, Brown & Lewis 2002). Thus an agent that targets the regulators/recruitment of macrophages (such as CC-chemokines) in the tumour environment may result in a better prognosis and clinical outcome. Previous studies found there was a positive correlation between CCL2 and CCL5 levels, and macrophages in tumours This also correlated with a poor prognosis and increased cancer aggressiveness in human breast cancer (Azenshtein et al. 2002; Luboshits et al. 1999; Saji et al. 2001). In contrast, a previous study in the breast cancer cell line MCF-7, showed that activation of CCR5 receptor by CCL5 (Manes et al. 2003) resulted in the promotion of genes downstream of p53, a key apoptotic gene, suggesting that some CC-chemokines may also help to reduce tumour overexpression. Thus it may be possible that CC-chemokines are able to regulate pro- and anti-tumour processes in different tumours and in different conditions.

As mentioned, the lack of change in tumour development by 35K may be due to the aggressive and fast growing nature of the LLC model. Furthermore, there are multiple contributory factors that are involved in tumour neovascularisation that may not be influenced by the inhibition of CC-chemokines. Additionally, CC-chemokines are expressed and produced by several cell types, including tumour and stromal cells in the tumour environment, which means there are more recruiters/stimulators for CC-chemokines involved in the progression of tumour compared to other disease pathologies. In the Ad35K treated mice, there was little or no difference in inflammation, proliferation, and invasion markers, although slight increases were observed. This outcome could be attributed to the lack of CC-chemokine inhibition by 35K in the tumours. Surprisingly, we
observed a slight increase in key apoptotic markers including p53, caspase 8, and caspase 3 in mice overexpressing 35K in this aggressive tumour model, which aligns with the trend for a reduction in tumour size.

An alternate mode of delivery of 35K or a different tumour neovascularisation model may be needed to allow for a clearer elucidation of the effects of broad-spectrum CC-chemokine inhibition in tumour neovascularisation. For example, a local delivery approach of 35K could be used to direct 35K specifically to the tumour cells. This would ensure that there would be no competition of the circulating 35K with other circulating CC-chemokines. A membrane-bound 35K (mem35K) construct has been generated in which 35K is expressed on the cell whilst still being able to inhibit CC-chemokines *in vivo* and *in vitro* (Bursill et al. 2006). The use of mem35K in the LLC model would allow for the determination of the effects of localised 35K expression at the tumour site. We would hypothesise that localised 35K will bind to CC-chemokines produced by the tumour cells before they are able to express more CC-chemokines and subsequently recruit inflammatory cells to the growing tumour. Alternatively, pure 35K protein could be injected directly into the growing tumour.

Additionally, alternate tumour models that may be better suited to deciphering the effect of 35K on tumourigenesis could be used. For example, CT26 cells used in models of colorectal cancer and 4T1 mammary cells used in breast cancer models may be superior options. When injected subcutaneously, both cell lines also produce highly vascularised tumours. After 15 days, the 4T1 tumours produce smaller tumours with low CCL2 and high VEGF levels, whilst the CT26 cells produce tumours with high CCL2 and low VEGF that are comparatively larger (Viana et al. 2013). Using the CT26 cell line for tumour induction may be a better option as 35K would be able to specifically target CCL2 in these tumours. The larger tumour size compared to the 4T1 tumours after 15 days, also means that the experiment does not have to be prolonged reducing the length of discomfort that may be caused by the tumour growth.

Tumour growth stimulates the production of a wide variety of pro-angiogenic molecules, however, targeting a single angiogenic factor may cause a switch to increase the production of other factors. Current anti-angiogenic therapies inhibit the critical pro-angiogenic mediator VEGF that is known to stimulate angiogenesis in both physiological and pathological environments. Inhibition of VEGF causes a number of side effects which
can become quite severe. Thus, it may be beneficial to inhibit a group of pro-angiogenic molecules, such as CC-chemokines in order to reduce tumour angiogenesis. Currently, clinical trials have begun with single CC-chemokine receptor inhibitors. For example, mogamulizumab which targets CCR4 has been successful in clinical trials of adult T-cell leukaemia-lymphoma (Subramaniam et al. 2012). CCR4 binds to several important CC-chemokines including CCL2, CCL3, CCL5, CCL17, and CCL22, all of which are upregulated in cancer. Mogamulizumab treatment showed that inhibition of CCR4 and subsequently its interaction with several CC-chemokine targets had no adverse side effects, suggesting that inhibiting the whole CC-chemokine class may be a better approach in targeting tumour growth.
8.6. **35K improves wound healing and wound angiogenesis in vivo**

Wound healing is a multi-step process involving inflammation, proliferation, and remodelling. While current therapies provide some relief and facilitate wound healing, there is a need for the development of novel therapies that can further address the debilitating effects of impaired wound healing. CC-chemokines are particularly important during the inflammation and proliferation stages when there is an influx of inflammatory cells and increased angiogenesis. However, CC-chemokines are less important during the remodelling phase where inflammation has ceased and angiogenesis is no longer required. Angiogenesis plays a critical role in the early phases of wound healing, recruiting inflammatory cells to the wound and providing a microvascular network to maintain new tissue formation. Chapter 7 explored the effect of broad-spectrum CC-chemokine inhibition using a murine model that mimics the human wound healing process. For this study, daily topical application of 35K protein enhanced blood flow recovery and wound closure and reduced collagen content which is indicative of less scar formation. Therefore, broad-spectrum CC-chemokine inhibition may be an alternate therapeutic approach, particularly given the beneficial effects of 35K on inflammatory-mediated diseases (Ali et al. 2005; Bursill et al. 2003; Bursill et al. 2006; Bursill et al. 2004; Bursill et al. 2009; Seki et al. 2009) and that non-healing chronic wounds are largely attributed to pathological inflammation. Two time points were chosen for this study: an earlier time point (Day 4) to elucidate the effects of 35K on initial inflammatory response and angiogenesis, and a later time point (Day 10) to determine the effects of 35K on the remodelling phase.

CC-chemokine receptors are expressed by endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, epithelial cell and monocytes in the wound (DiPietro et al. 1995; Martins-Green, Petreaca & Wang 2013; Wetzler, Kampfer, Pfeilschifter, et al. 2000). During the inflammatory phase, neutrophils are initially recruited by CXCL8, however they undergo spontaneous apoptosis with the absence of inflammatory mediators (Conus et al. 2008). Thus neutrophils are followed by monocyte influx. This is mediated through CC-chemokine signalling and release from endothelial cells. Additionally, neutrophils are able to release CCL2 to aid in monocyte recruitment (Gillitzer & Goebeler 2001). The monocytes differentiate into macrophages that remove dead neutrophils, and release cytokines and growth factors to promote wound healing. Furthermore, macrophages release pro-angiogenic factors such as VEGF, to stimulate new blood vessel formation to the wound. Angiogenesis allows for the easy recruitment of cells and the
continued oxygenation of the wound preventing hypoxia. During the proliferation stage, granulation tissue begins to form, and re-epithelialisation occurs. CCL3, CCL4, CCL5 are produced by granulation tissue to recruit lymphocytes as a final defence against microbes and foreign bodies in the wound (Martins-Green, Petreaca & Wang 2013). The persistence of macrophages is important during the proliferation phase as it releases growth factors to stimulate keratinocyte production and migration at the wound edge. Finally during the remodelling phase, inflammation and angiogenesis has ceased, and fibroblasts re-organise collagen to create a scar.

Given the importance of CC-chemokines in wound healing, previous studies have looked at the inhibition of individual CC-chemokines, however, in contrast to the studies in Chapter 7, no significant beneficial effects to wound healing were seen. For example, CCL2−/− mice have delayed re-epithelialisation and reduced angiogenesis in early stages, whilst in CCL3−/− mice wounds healed normally (Low et al. 2001). Similarly CCR1−/− models showed no impairment in wound healing despite a strong upregulation of CCR1 in wounds (Kaesler et al. 2004). These studies suggest that the inhibition of a single CC-chemokine may not be as effective in wound healing due to the varied roles of CC-chemokines and the specificity in expression within the wound. Furthermore, there is also redundancy in CC-chemokine class signalling (Devalaraja & Richmond 1999; Mantovani 1999).

Broad-spectrum inhibition of CC-chemokines by 35K promoted the formation of neovessels and arterioles early in wound healing, whilst neovascularisation was suppressed at the later stage of wound healing. The augmentation of wound neovascularisation correlated with faster wound healing rates. Furthermore, suppression of inflammation at the early stage of wound healing and reduced collagen content is indicative of a better healed wound. A previous study has found impaired wound healing in a diabetic mouse model that was due to sustained prolonged expression of CCL2 in keratinocytes at the wound edge. This prolonged expression was associated with increased presence of macrophages that contribute to impaired wound healing, especially in the late stages (Day 13) (Wetzler, Kampfer, Stallmeyer, et al. 2000). Therefore a decrease/inhibition of CC-chemokines in the late stage of healing may be beneficial in the diabetic wound as seen in our study. Additionally, a study analysing wound fluid from venous ulcers was associated with decreased CCL2, CCL4 and CCL5 as the wound healed (Fivenson et al. 1997). Conversely, the inhibition of CC-chemokines in wound healing during the early phase as
seen in our study may not be beneficial to impaired wound healing in diabetes. A recent study found that delayed recruitment of macrophages to the diabetic wounds caused impaired healing, but could be corrected by treatment with CCL2 (Wood et al. 2014). However, the mice used in the current study were not made diabetic and therefore, whether broad-spectrum CC-chemokine inhibition also has similar effects in a diabetic setting remains to be explored.

Interestingly, no changes in macrophages were seen despite increased neovessels in the early stage and decreased neovessels in the late stage. This may be due to the need for an optimal level of macrophages for wound healing. Too many macrophages early in healing such as in diabetes result in prolonged inflammation and subsequently delayed healing. However, lower macrophage numbers will result in impairment of the healing process. This is because neutrophils have a finite life span in the circulation and tissue and are only involved in the initial immune response immediately following wounding. Following the initial immune response, macrophages replace neutrophils and are important for mediating the extended inflammatory response often seen with wound healing.
8.7. Future directions

The work generated in this thesis has provided some interesting and novel findings. There are, however, several questions that remain incompletely answered. Our observations have highlighted a role for broad-spectrum CC-chemokine inhibition in a clinical context, particularly in diseases associated with inflammatory-driven angiogenesis such as atherosclerosis and cancer. Specifically targeting inflammatory-driven angiogenesis in these diseases may allow for an alternate therapy that does not cause detrimental side effects seen with current anti-angiogenic therapies. However as cancer varies in both type and severity and tumour neovascularisation involves a complex plethora of critical cellular processes including both inflammatory-driven and ischaemia-mediated angiogenesis, proliferation and apoptosis, further studies are still required to explore the effects of broad-spectrum CC-chemokine inhibition in the tumour environment. The most obvious experiments are to try a less aggressive tumour model and a different mode of expression of 35K in the tumour environment. Localised expression of 35K in the tumours is the next step to determine the efficacy of 35K in tumours. An alternate mode of directly injecting tumours with 35K protein or a 35K-Fc fusion protein may be a better approach clinically.

Additionally, further investigation of broad-spectrum CC-chemokine inhibition in the context of wound healing could be explored. Wounds that exhibit impaired healing frequently enter a state of pathological inflammation, with most chronic wounds developing into ulcers and the incidence of these are associated with ischaemia, diabetes mellitus, venous stasis disease or pressure (Guo & DiPietro 2010). Chronic, non-healing wounds greatly impact a person’s quality of life and when left untreated sometimes lead to amputation resulting in enormous health care burden as can be seen with an increasing rise in the diabetic and/or aged population (Mathieu, Linke & Wattel 2006; Menke et al. 2007). We have shown that 35K is beneficial in wounds of wild type mice, so it would be interesting to see if 35K had the same or better beneficial effects in a model of impaired/delayed wound healing such as with diabetic or aged mice.
8.8. Summary and conclusions

In summary, the studies generated in this thesis demonstrate, for the first time, that broad-spectrum CC-chemokine inhibition by 35K could be an alternate therapeutic approach over current anti-angiogenic therapies. The novel findings generated both \textit{in vitro} and in two \textit{in vivo} models of angiogenesis showed that broad-spectrum CC-chemokine inhibition suppresses unwanted pathological inflammatory-driven angiogenesis whilst preserving desired physiological ischaemia-mediated angiogenesis. 35K may have significant advantages over current therapies as it inhibits only pathological angiogenesis leaving physiological angiogenesis unaffected and able to continue in healing and regeneration.

While 35K also showed promising effects in the LLC tumour neovascularisation model representing the combined and exaggerated inflammatory and ischaemic environments, further work needs to be performed to elucidate the effects of broad-spectrum CC-chemokine inhibition in the context of cancer. Finally, topical application of 35K promoted wound healing and wound angiogenesis suggesting that broad-spectrum CC-chemokine inhibition may be beneficial in chronic wounds where inflammation is in excess, in order to promote wound healing and neovascularisation and reduce scar formation.

Collectively, these findings highlight the potential therapeutic capacity for broad-spectrum CC-chemokine inhibition in the treatment of inflammatory-driven angiogenesis-associated diseases while preserving ischaemia-mediated angiogenesis that is critical for tissue neovascularisation such as in a myocardial infarction or promoting wound neovascularisation.
CHAPTER 9.
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