Investigating the role of angiotensin-(1-9) in neointimal formation

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Author's declaration

I declare that this thesis was written entirely by myself and is a record of the research performed solely by myself with the exceptions as detailed below.

- I trained and supervised Miss Gemma Greenshields as part of her BSc (Hons) project. Together we characterised the effect of Ang-(1-9) on vascular remodelling seven days after carotid ligation by haematoxylin and eosin staining and morphometric analysis of murine carotid arteries. VSMC proliferation was analysed by assessment of EdU incorporation *in vivo*. Characterisation of the effect on arterial fibrosis was performed by picrosirius red staining and subsequent image analysis.

- I also supervised Mr Andrew Irwin who completed his MSc project within our laboratory. Under my supervision, Mr Andrew Irwin performed the Elastic Van Geison staining of wire-injured murine carotid arteries presented in *Chapter 5*.

- Isolation of human saphenous vein smooth muscle cells and endothelial cells was performed by Mrs Elaine Friel at the University of Glasgow.

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Abstract: **El-Mansi S**, McKinney C, Bradshaw A.C, Nicklin S.A (2017) Assessing adenoviral-mediated delivery of Angiotensin-(1-9) to prevent human vascular smooth muscle cell migration. HUMAN GENE THERAPY 2017 28:A1–A36

Presentations

El-Mansi S, Robertson S, Irwin AJ, Bradshaw AC, Nicklin SA (2018) Gene therapy with Angiotensin-(1-9) inhibits neointima formation in a murine model of vascular injury. The International Vascular Biology Meeting, Helsinki, Finland. [Poster Presentation]

El-Mansi S, Irwin AJ, McKinney CA, Bradshaw AC, Nicklin SA (2017) Assessing adenoviral delivery of angiotensin-(1-9) to prevent human vascular smooth muscle cell proliferation and migration *in vitro* and neointima formation *in vivo*. The Scottish Cardiovascular Forum 2018 Trinity College Dublin, Dublin, Republic of Ireland [Oral Presentation]

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List of abbreviations

AAA	Abdominal aortic aneurysm
AAV	Adeno-associated virus
Ab	Antibody
ACE	Angiotensin converting enzyme
ACE2	ACE related carboxypeptidase
Ad	Adenovirus
Ad5	Ad serotype 5
AGTR2	Angiotensin two type two receptor [gene name]
Ala	Alanine
AMA	Aminopeptidase A
AMN	Aminopeptidase N
AMPKa	5' adenosine monophosphate-activated protein kinase
Ang	Angiotensin
ANOVA	Analysis of variance
Apo E	Apolipoprotein E
ARB	Angiotensin receptor blocker
Arg	Arginine
Arg	Arginine
aSMA	Alpha smooth muscle actin
Asp	Asparagine
AT_1R	Angiotensin two type one receptor
AT_2R	Angiotensin two type two receptor
AT_4R	Angiotensin two type four receptor
ATIP	AT_2R interacting protein
Bad	Bcl-2-associated death promoter
BCA	Bicinchoninic acid
BNP	Brain natriuretic peptide
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin

C02	Carbon dioxide
Ca2+	Calcium ions
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CAMK	Ca2+/calmodulin-dependent protein kinase
CAR	Coxsackievirus and adenovirus receptor (CAR) i
CAR	Chimeric antigen receptor
Cat	Catalogue number
CCL	CC chemokine ligands
CCR	C-C chemokine receptor
CDK	Cyclin dependant kinase
cDNA	Complimentary DNA
CHD	Coronary heart disease
CL	Carotid ligation
Col1a	Collagen 1 alpha [gene name]
Col3a	Collagen 3 alpha [gene name]
CPE	Cytopathic effect
Ct	Cycle threshold
CT	computed tomography
CVD	Cardiovascular disease
Cyc	Cyclin
DAMPS	Damage-associated molecular pattern molecules
DAPI	4',6-diamidino-2-phenylindole
dCt	Delta cycle threshold
DES	Drug eluting stents
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri phosphate
DOCA	11-Deoxycorticosterone
EC	Endothelial cell

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine
ELISA	Enzyme linked immuno-sorbent assay
Elk-1	ETS Like-1 protein
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinases 1/2
ETS	E26 transformation-specific
EVG	Elastic van geison
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
Fc	Fragment crystallizable region
FCS	Foetal calf serum
FRET	Förster resonance energy transfer
FX	Factor ten
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
Gly	Glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein couple receptor
GSK-3B	Glycogen synthase kinase 3 beta
HEK	Human embryonic kidney
His	Histidine
HMG-CoA	3-hydroxy-methylglutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
hr	Hour
HSP	Heat shock protein
HSPG	Heparin sulphate proteoglycans
HSV	Human saphenous vein
HSVEC	human saphenous vein endothelial cell

HSVSMC	Human saphenous vein smooth muscle cells
ICAM-1	Intercellular Adhesion Molecule 1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IHC	Immuno-histo-chemistry
IL	Interleukin
Ile	Isoleucine
IMA	Internal mammary artery
IP3	Inositol trisphosphate
IRAP	Insulin-regulated aminopeptidase
ISR	In-stent restenosis
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kb	Kilo base pairs
KC	Keratinocyte chemoattractant
kDa	Kilo Dalton
kg	Kilo gram
KO	Knock out
LAD	Left anterior descending
LCMS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
Leu	Leucine
LIMA	Left internal mammary artery
LoxP	Locus of X-over P1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mas R	Mas Receptor
MCP-1	Monocyte chemotactic protein 1
mg	milli gram
MHC	Myosin heavy chain
MI	Myocardial infarction

MIP	Macrophage inflammatory protein
MKP-1	MAP kinase phosphatase 1
mL	Milli litre
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mM	Milli molar
MMP	Matrix metalloproteinase
MrgD	Mas-related G-protein coupled receptor D
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MYH11	Myosin Heavy Chain 1 [gene name]
NaCl	Sodium chlorine
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NEP	Neprilysin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nano gram
nM	Nano molar
nm	Nano metre
NO	Nitric oxide
NOS	Nitic oxide synthase
NOX	NADPH oxidase
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PCI	Percutaneous intervention
PCNA	Proliferating cell nuclear antigen
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor

PDGFRB	Beta-type platelet-derived growth factor receptor
PFU	Plaque forming units
pg	Pico gram
Phe	Phenylalanine
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
POP	Prolyl oligopeptidase
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRAS40	Proline-rich Akt substrate
Pro	Proline
PTEN	Phosphatase and tensin homolog
PTP	Protein-tyrosine phosphatase
RAd	Recombinant adenovirus
RAS	Renin angiotensin system
Rb	Rabbit
Rb	Retinoblastoma protein
rh	Recombinant human
RhoA	Ras homolog gene family, member A
RIP	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROI	Region of Interest
ROS	Reactive oxygen species
SA-PE	Streptavidin-Phycoerythrin
Sca-1	Stem cell antigen one
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
Ser	Serine
SERCA	Sarcoendoplasmic reticulum (SR) calcium transport ATPase
SH2	Src Homology 2 (domain)

SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase-2
SHRSP	Stroke prone spontaneously hypertensive rats
SM22a	Smooth muscle protein 22 alpha
SMAD	Suppressor of Mothers against Decapentaplegic
STAT	Signal Transducer and Activator of Transcription proteins
SV	Saphenous vein
T	Tween
TBS	Tris-buffered saline
TGF-B	Transforming growth factor beta
Thr	Threonine
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF-a	Tumor necrosis factor alpha
TOP	Thimet oligopeptidase
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
UTC	Untransduced control
Val	Valine
VCAM-1	Vascular cell adhesion molecule 1
VGF	Vein graft failure
VP	Viral particles
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation
WI	Wire injury
µg	Micro gram
µM	Micro Molar
µm	Micro metre

Summary

During coronary artery bypass graft (CABG) procedures, the patient's autologous saphenous vein (SV) is grafted either side of an occlusion in the coronary artery. This allows oxygenated blood to bypass the narrowed section thus reperfusing the myocardium and relieving symptoms of coronary heart disease. Damage to the SV endothelium can promote the adherence of platelets and the formation of an occlusive thrombus that leads to early vein graft failure. Where thrombosis is limited, local inflammation and endothelial cell dysfunction can promote the inward migration of medial vascular smooth muscle cells (VSMC) . Migrated VSMCs, now residing in the intima, proliferate extensively forming an occlusive lesion known as the neointima. Neointimal VSMCs exhibit a synthetic phenotype and excrete components of extracellular matrix. Accelerated atherosclerosis can then lead to the total occlusion of the conduit resulting in myocardial infarction or requirement for further surgery. Approximately 40% of vein grafts fail over a period of ten years. The need to develop new therapies to prevent late vein graft failure (VGF) is therefore urgent.

The renin angiotensin system (RAS) provides vital modulation of blood pressure and vascular tone through the octapeptide angiotensin II (Ang II). Excessive Ang II signalling through the angiotensin II type I receptor (AT_1R) promotes pathophysiological processes important in the development and progression of vein graft disease, including but not limited to inflammation, VSMC migration and fibrosis. A non-canonical axis of the RAS exists to counterbalance the effects of Ang II. Angiotensin-converting enzyme-related carboxypeptidase 2 (ACE2) degrades Ang II to angiotensin-(1-7) [Ang-(1-7)] and angiotensin I (Ang I) to angiotensin-(1-9) [Ang-(1-9)]. Here, the effect of subcutaneous infusion of soluble Ang-(1-9) peptide was investigated in a mouse model of neointima formation induced by carotid artery ligation. In this model, subcutaneous delivery of Ang-(1-9) decreased circulating concentrations of the proinflammatory cytokine monocyte chemotactic protein-1 (MCP-1) (an effector of Ang II with a known role in vein graft remodelling) 14 days after ligation but not at the earlier 7-day time point. Infusion of Ang-(1-9) significantly attenuated VSMC proliferation as determined by immunofluorescence assessment of arterial proliferating cell nuclear antigen (PCNA) expression. However, Ang-(1-9) infusion

did not inhibit neointima formation as determined by histological staining and morphometric analysis. Next, VSMC were isolated from surplus sections of human SV donated by CABG patients. Platelet derived growth factor beta chain dimer (PDGF-BB) is a potent mitogen and plays a key role in the development of vein graft disease. Here, PDGF-BB induced both directional and chemotactic HSVSMC migration. In both migration models (scratch and Boyden chamber), PDGF-BB induced migration was attenuated by pre-incubation with Ang-(1-9). PDGF-BB also promoted potent pro-proliferative effects on HSVSMCs as determined by incorporation of a thymidine analogue (bromodeoxyuridine). At micromolar concentrations, Ang-(1-9) blocked the pro-proliferative effects of PDGF-BB. Downstream effects of PDGF-BB and Ang-(1-9) were assessed via an antibody-based protein array. At an acute time-point, PDGF-BB induced activation of the mitogen-activated protein kinase (MAPK) pathway as determined by phosphorylation of extracellular signal–regulated kinases (ERK1/2). Likewise, PDGF-BB elicited activation of the phosphoinositide 3-kinase (PI3K) pathway as detected by phosphorylation of protein kinase B (Akt) and proline rich Akt residue 40 (PRAS40). Preincubation with Ang-(1-9) selectively inhibited PDGF-BB induced ERK1/2 phosphorylation. This is in agreement with previously reported effects of the AT_2R. The MEKK inhibitor U0126 was then employed to demonstrate that selective inhibition of ERK1/2 prevented PDGF-BB induced HSVSMC proliferation. Therefore, it is suggested here that Ang-(1-9) confers therapeutic effects in VSMC *in vitro* and *in vivo* and could be a putative target for application in CABG. CABG surgeries are ideally suited to viral gene therapy owing to the opportunity to treat the vein *ex vivo* in the operating theatre before implantation. Therefore, local overexpression of Ang-(1-9) is achievable and may be beneficial in the setting of vein graft failure following CABG. Here, the effects of an adenoviral vector encoding an intracellular cleaved and secreted Ang-(1-9) peptide [RAdAng-(1-9)] was characterised. Direct transduction with RAdAng-(1-9) significantly inhibited HSVSMC migration induced by Ang II. Unexpectedly, RAdAng-(1-9) conferred pro-proliferative effects on HSVSMC and HSV endothelial cells (HSVEC). The hepatoma cell line HepG2 were then transduced with RAdAng-(1-9) and the effect of the conditioned culture media on recipient HSVSMC proliferation assessed. This aimed to mimic the subsequently performed *in vivo* model where the vector is delivered systemically resulting in liver transgene expression. HepG2 cells transduced with RAdAng-(1-

9) expressed and secreted the Ang-(1-9) fusion protein as detected by immunoblotting of the conditioned culture media. Conditioned media collected from RAdAng-(1-9) but not RAdControl transduced HepG2 cells inhibited serum induced HSVSMC proliferation. Subsequently, an *in vivo* proof of concept gene transfer study was conducted. Intravascular delivery of a control vector (Ad*Lac*Z) led to strong liver transgene expression (three days after infusion) and did not affect neointima formation following wire injury. Importantly, RAdAng-(1–9) administered intravenously 48 hours before wire injury surgery significantly inhibited neointima formation 28 days after endothelial denudation, whereas administration of saline or RAdControl had no effect.

These data elucidate novel therapeutic effects of Ang-(1-9) *in vitro* and *in vivo*. This is the first evidence that Ang-(1-9) can inhibit the effects of PDGF-BB in HSVSMC. Furthermore, this is the first evidence that gene therapy with Ang-(1-9) can be used to inhibit neointima formation *in vivo* . Taken together Ang-(1-9) may be a potential candidate for use in CABG. Further investigations should investigate how best to deliver this peptide and fully understand its mechanisms of action.

1 Chapter 1 General Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) imparts a truly astounding burden on public health and the global economy. Encompassing a wide variety of vascular and cardiac pathologies, CVD is a leading cause of premature death worldwide. In 2013 alone, CVD accounted for 17 million deaths with projected global mortality rates in excess of 23 million by 2030 (GBD, 2015).The most prominent contributor to CVD-related deaths is coronary heart disease (CHD) which was responsible for 1 in 4 deaths in 2010 (Lozano et al., 2012).

1.2 CHD and atherosclerosis

CHD is a result of the narrowing and loss of elasticity of the coronary arteries thus depriving the cardiac muscles of oxygenated blood. This is primarily caused by a multifaceted process known as atherosclerosis; a progressive inflammatory disease that involves the deposition and accumulation of fibrous and lipid rich plaques within the intima of arterial vessels (Ross, 1993). When this occurs in the coronary arteries, less oxygenated blood can reach the cardiac tissue leading to ischemia. Commonly this can result in angina. Exposure of the plaque through a thin layer of encapsulating fibrous tissue (known as the fibrous cap) may lead to thrombosis and clot formation (Virmani et al., 2006). Atherosclerotic plaque rupture is a leading cause (~60%) of stroke and myocardial infarction (MI) and can be fatal (Kubo et al., 2007). Erosion of the plaque can also occur and is a distinct process. Erosion is defined as acute thrombus formation within the intima of plaques with a proteoglycan and VSMC rich fibrous cap in the absence endothelium (exposure to the circulating blood) (Virmani et al., 2006). Like plaque rupture, erosion can lead to thrombi formation and sudden death (~30%) (Virmani et al., 2006). Furthermore, plaque erosion is also an important cause (~25%) of MI (Arbustini et al., 1999).

The initial stages of atherosclerosis involve the deposition and accumulation of low-density lipoproteins (LDL) within the blood vessel wall in the sub-endothelial space where they can be oxidised and aggregate (Skalen et al., 2002). It is thought that endothelial cells (ECs) can endocytose LDL before exocytosis of the LDL to the underlying sub endothelial space occurs in a process called transcytosis (Quest et al., 2004, Bian et al., 2014). ECs overexpress adhesion

molecules such as vascular cell adhesion protein 1 (VCAM-1) which can in turn lead to the infiltration of monocytes and macrophages which can proliferate locally to form a considerable fraction of the atherosclerotic lesion (Cybulsky and Gimbrone, 1991, Robbins et al., 2013). Resident macrophages also engulf lipoprotein-containing cholesterol which through endosomal trafficking to the endoplasmic reticulum (ER) is esterified to cholesteryl fatty acyl esters (Brown et al., 1980). Cholesteryl fatty acyl esters form intracellular lipid droplets giving cells a foam-like appearance (foam cells).ER stress can then promote programmed cell death (apoptosis) and poor clearance of dead cells (efferocytosis) can promote formation of a necrotic core within the lesion (Tabas, 2009).

The presence of a necrotic core is one of the classic hallmarks of plaque instability (Goldstein et al., 1979, Duewell et al., 2010). This in combination with a thin (<65 μm) overlying cap characterise a lesion that is at high risk of plaque rupture. Conversely, formation of a thick fibrous cap by smooth muscle cells is a marker of plaque stability, but the plaque is still susceptible to erosion (Virmani et al., 2000). When this cap is thin, the necrotic core can be exposed to the circulation. The necrotic core is highly thrombogenic and promote activation of coagulation cascades that can lead to the rapid occlusion of the coronary artery (Falk et al., 1995).

1.3 Medical treatment of CHD

Patients diagnosed with CHD are commonly prescribed medical therapy consisting of anti-platelet therapy to prevent blood clot formation and statins that lower lipids and cholesterols. Anti-platelet therapies include aspirin, prasugrel (Efient) and clopidogrel (Plavix) (Roe et al., 2012). The latter compounds inhibit platelet aggregation induced by adenosine diphosphate (ADP) and are more effective at lowering the risk of MI and stroke than aspirin treatment alone (CAPRIE, 1996).

The therapeutic benefit of statins is mediated by their inhibition of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which lowers levels of cholesterol and LDL in the circulation (Alberts et al., 1980). For example, when 6595 men with hyperlipidaemia (high levels of lipids) in the west of Scotland

were prescribed statins (pravastatin) the continuous treatment resulted in reduction of adverse cardiovascular events such as MI (31%) and CVD related mortality (32%) (Shepherd et al., 1995). A more recent and comprehensive meta-analysis of 22 trials (n=134 537) investigated the effect of statins on adverse cardiovascular events (MI, stroke, revascularisation) (Armitage et al., 2019). In this study, the response rate was defined as a ratio of the number of adverse events per 1 mmol/L reduction in LDL cholesterol. Overall, statins reduced the risk of adverse cardiovascular events by 21% (Armitage et al., 2019). Angiotensin converting enzyme (ACE) (see section 1.5) inhibitors are also prescribed to patients with acute coronary syndromes such as angina and a number of large scale trials have indicated their clinic efficacy (HOPE, 2000). In 1992, patients with unstable angina and reduced left ventricular ejection fraction were prescribed either placebo (n=3401) or the ACE inhibitor enalapril (Vasotec) (n=3396) (Yusuf et al., 1992). Enalapril reduced the risk of MI in patients with CHD by 23% (Yusuf et al., 1992). The HOPE (Heart Outcomes Prevention Evaluation) study recruited over 9000 patients with normal ejection fraction who were at risk of heart failure and prescribed them either placebo or the ACE inhibitor ramipril (Altace) (HOPE, 2000). Treatment of patients with ramipril reduced rates of MI (ramipril 9.9% vs 12.3% placebo), stroke (3.4% vs 4.9%) and risk of developing heart failure (9.0% vs 11.5%) (HOPE, 2000). A randomised, double blinded and placebo-controlled trial recruited 13,655 patients with stable CHD (EUROPA). In this multicentre trial, ACE inhibition with perindopril (Aceon) reduced the risk of CVD related death and MI related hospitalisation by 20%, with only 50 patients requiring interventional treatment to prevent an adverse cardiovascular event (Fox, 2003).

The renin angiotensin system (RAS) (see section 1.5) is also targeted therapeutically through the use of angiotensin receptor blockers (ARBs). Popular ARBs include candesartan (Atacand), valsartan (Diovan), losartan (Cozaar) and irbesartan (Avapro). Combinational therapy using a formulation that contains both the ARB valsartan and a neprilysin inhibitor sacubitril, has now superseded the ACE inhibitor enalapril. The PARADIGM-HF trial was a double blinded study that recruited 8442 patients with heart failure and compared the efficacy of valsartan/sacubitril (Entresto) combinational therapy to enalapril (McMurray et al., 2014). Valsartan/sacubitril combinational therapy was found to be more

clinically effective at reducing the rates of rehospitalisation and CVD associated mortality (McMurray et al., 2014).

Lastly, beta blockers are also commonly prescribed to treat presentations of CHD in order to reduce the heart rate. Beta blockers lower the myocardium's oxygen consumption requirements (Members et al., 2013). The beta blocker carvedilol (Coreg) reduced risk of death and non-fatal MI as compared to patients randomly prescribed a placebo treatment (Dargie, 2001).

1.4 Revascularisation strategies

Patients suffering from advanced presentations of CHD, such as coronary atherosclerosis, often require revascularisation strategies to restore blood flow to the cardiac muscle. Revascularisation strategies have been reported to improve long term outcomes (percentage survival and non-fatal MI) in patients with CHD as compared to medical therapy alone (Windecker et al., 2014). Revascularisation surgeries can be surgical or non-surgical as discussed below.

1.4.1 Percutaneous coronary intervention strategies

Percutaneous coronary intervention (PCI) also known as balloon angioplasty utilises a catheter attached device that inflates a balloon where luminal narrowing of the coronary artery has occurred. Briefly, a catheter is inserted in the groin and passed through the vasculature to the heart. Upon inflation the balloon forces the occlusive plaque outward, thus widening the lumen (transluminal dilatation). The balloon is then deflated and the catheter is removed. This is an effective and minimally invasive method capable of restoring blood flow to the myocardium (Gruntzig, 1978). However, subsequent re-occlusion of the coronary artery, known as 'restenosis' is a major limitation of this approach. Hence this method has now been superseded; PCI procedures now include the deployment of a stent which maintains the newly widened architecture (Sigwart et al., 1987). This addition to the procedure has been reported to improve the immediate success rate of the procedure (stenting: 96.1% vs 89.6% balloon angioplasty) and lower consequential restenosis rates by approximately ten percent (Fischman et al., 1994). However, bare-metal stents

(BMS) are naturally thrombogenic and require additional anti-coagulation therapy (Schatz et al., 1991, Schömig et al., 1996).

The next advancement in stent technology were balloon inflatable stents coated in a polymer to allow drug elution. Two compounds that have been utilised in drug eluting stents (DES) are sirolimus (also known as rapamycin [Rapamune]) and paclitaxel (Taxol) which both target the cell cycle but in distinct mechanisms. Paclitaxel has been found to inhibit mitosis via disrupting formation of normal spindle fibres and interrupting microtubules; cells unable to progress through mitosis then undergo apoptosis (Wang et al., 2000). Sirolimus has been reported to inhibit mammalian target of rapamycin (mTOR) a protein known to promote anabolism and inhibit autophagy (cell devouring) (Chung et al., 1992). Inhibition of mTOR leads to cells exiting the cell cycle at G_1 (cell cycle discussed in section 1.4.6.6). Sirolimus eluting stents have been demonstrated to reduce restenosis (21% vs 8.6%) and re-intervention rates (16.6% vs 4.1%) in comparison to BMS (Moses et al., 2003, Stettler et al., 2007). Moreover, six year follow up angiography studies also suggested that patients prescribed a DES to deliver a sirolimus derivative (zotarolimus) were less likely to require repeat revascularisation (DES 16.5% vs 19.8% BMS) (Bønaa et al., 2016).

Importantly however, some safety concerns have been raised regarding rates of stent thrombosis. In 2007, substantially higher rates of stent thrombosis following implantation with DES compared to BMS was reported (Stone et al., 2007). Between 30 days and 4 years following PCI 0.2% of patients implanted with a BMS experienced thrombosis. Contrastingly, 0.7% of patients implanted with sirolimus or paclitaxel DES experienced stent thrombosis (Stone et al., 2007). Further safety concerns have been raised regarding paclitaxel eluting stents; when implanted within the femoropopliteal artery, patients who received paclitaxel were at increased risk of death from any cause (Katsanos et al., 2018).

In an attempt to reduce thrombosis provoked by stents, bioresorbable stents have now been developed. However, when a clinical study randomly assigned 1845 patients either bioresorbable or BMS, it was found that the implantation of bioresorbable stents was significantly associated with an increased risk of thrombosis (3.9% vs 0.9%) (Wykrzykowska et al., 2017). Additionally, a meta-

analysis of over 10,000 patients implanted with bioresorbable or DES suggested patients with bioresorbable stents were at higher risk to MI (Odds ratio 2.06 vs 3.22) and stent thrombosis (Odds ratio 2.06 vs 3.98) (Lipinski et al., 2016). To summarise, PCI is an effective treatment for obstructive CHD, but major limitations remain.

1.4.2 Coronary artery bypass grafting

Another commonly prescribed revascularisation procedure is the coronary artery bypass graft (CABG) which utilises an autologous blood vessel to bypass the occluded coronary artery (Carrel, 1910). In order to perform CABG, the narrowed coronary artery is first identified by angiography. Next, the appropriate autologous blood vessel is chosen and in the case of vein grafting, the autologous saphenous vein is harvested from the leg. After which, the rib cage is opened, the heart is pharmacologically stopped using a potassium solution and a cardiopulmonary bypass circuit is set up which utilises venous and arterial cannulas, an oxygenator, a filter and a pump (Susak et al., 2016). This is called “on-pump” CABG. Surgeries that do not utilise this approach perform the graft while the heart is beating and this is referred to as “off-pump” CABG (Gundry et al., 1998). A meta-analysis of six clinical trials (BHACAS, Octopus, MASS III, CORONARY, GOPCABE, ROOBYSF) compared on- and off-pump CABG and suggested that on-pump CABG may be superior (Smart et al., 2018). However, guidelines for cardiology surgeons to this date have not changed and the choice of on-pump or off-pump is determined according to surgeon or institutional preference.

Finally, the harvested conduit is grafted within the vasculature of heart allowing perfusion of blood from the aorta to distal of the coronary occlusion thus bypassing the occlusion and reperfusing the cardiac muscle (Figure 1.1).

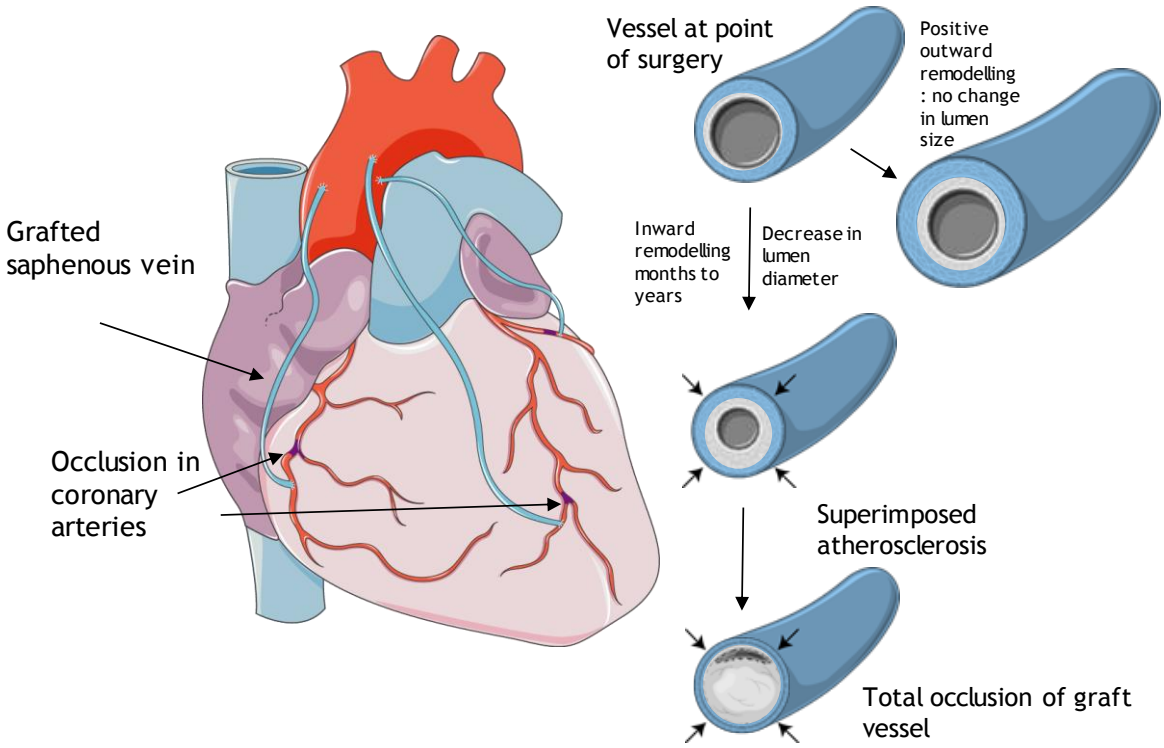

Figure 1.1 Schematic representation of the heart, CABG and vein graft failure.

Schematic depicting the anatomy of the heart after a multi-vessel coronary artery bypass graft procedure where venous segments (blue) are grafted between the aorta and the coronary artery (red) to bypass an occlusion (purple). The process of vein graft failure is illustrated [right] whereby the graft initially remodels outward to adapt to the arterial pressure with little change in lumen diameter. Migration inward of VSMCs and proliferation within the intima leads to inward remodelling and a decrease in lumen diameter. Accelerated atherosclerosis can lead to the total occlusion of the vein graft. Image created using Servier Medical Art available at https://smart.servier.com. No permission required**.**

1.4.3 Choice of conduit

In humans, veins and arteries have similar structural morphological features. Both have an outer adventitial layer, a middle medial layer and an internal intima which is lined at the luminal side with a single-cell thick layer called the endothelium. However, important distinctions do exist; arteries are adapted to a high-pressure system as they deliver blood from the heart to the tissues. Conversely, veins are adapted for a lower pressure circulation as they return oxygen depleted blood from tissue back to the heart. The most obvious structural adaption is the presence of venous valves that prevent back flow of blood given that blood pressure is lower (Portugal et al., 2014). Presence of valves can complicate CABG surgeries as they have the potential to cause perturbations in blood flow. Valves in venous grafts are pre-inclined to develop non-occlusive blood clots, therefore a section without valves can be chosen by the surgeon (Bulkley and Hutchins, 1977). SVs also have a less pronounced medial layer of VSMC and have larger ECs that form a more permeable membrane as compared to the left internal mammary artery (LIMA) (Cox et al., 1991). There are also functional differences between the endothelium of veins and arteries, for example; ECs from the LIMA are more effective at inducing vasodilation than those from the SV (Lüscher et al., 1988). These differences between veins and arteries may partially explain why different conduits used in CABG have different patency rates as discussed below (Goldman et al., 2004).

Arteries such as the LIMA are less prone to neointima formation as they are structurally similar to the left anterior descending (LAD) artery, but are more elastin rich and the average medial area is smaller (Prim et al., 2016). A previous study used diagnostic cardiac catheterization of patients 10 years after CABG with SV (1,074 patients) or LIMA (457 patients) and reported that LIMA grafts exhibit better long term patency than SV grafts (Goldman et al., 2004). However, the use of left or right IMA carries its own hazards and is associated with an increased risk of inflammation or infection of the chest (mediastinitis), thus the choice of conduit must be carefully considered (Culliford et al., 1976).

The radial artery (RA) is also used in CABG procedures. Until recently most trials comparing RA and SV grafts have been largely underpowered. Now, follow-up angiography of 1036 patients has associated the use of the RA with a decreased

risk of graft occlusion and MI but suggested no effect on overall mortality rates (Gaudino et al., 2018).

Despite a higher re-occlusion rate, the SV is still used in CABG surgeries primarily because there are only two short sections of IMA (the left and right) in each patient available to harvest. Moreover, in patients with multi-vessel CHD; a longer stretch of vessel is often required and surgeons still commonly utilise the great SV rather than performing multiple arterial grafts (Buxton et al., 2009, ElBardissi et al., 2012, Gaudino et al., 2015).

1.4.4 CABG vs PCI

Despite being introduced in the 1960s, CABG remains a gold standard intervention (Susak et al., 2016). Two large, multi-centre, randomised clinical trials comparing the efficacy of PCI and CABG have been performed in the last decade. The Future Revascularization Evaluation in Patients with Diabetes Mellitus: Optimal Management of Multivessel Disease (FREEDOM) trial investigated CABG and PCI outcomes in patients with diabetes (Farkouh et al., 2012). This five year follow up of 1900 patients prescribed either CABG surgery or PCI suggested that CABG was linked with a reduced frequency of MI and all-cause mortality (26.6% PCI vs 18.7% CABG) (Farkouh et al., 2012). However, CABG was associated with an increased frequency of stroke (2.4% PCI vs 5.2% CABG) (Farkouh et al., 2012).

The Synergy Between PCI With Taxus and Cardiac Surgery (SYNTAX) trial randomly prescribed patients with left main disease and multivessel disease either CABG surgery or PCI with paclitaxel eluting stents (Morice et al., 2014). In the SYNTAX trial, eighteen hundred patients were recruited and a retrospective analysis of cardiovascular events, mortality rates and requirement for repeat revascularisation determined that PCI was associated with an increased need for further surgery (PCI 26.7% vs CABG 15.5%) (Morice et al., 2014). No differences in MI were reported however CABG surgery was associated with increased risk of stroke (PCI: 1.5% vs CABG: 4.3%) (Morice et al., 2014).

Hanan et al., analysed 37,212 patients who were prescribed CABG and 22,102 patients who were prescribed PCI between the years of 1997 and 2000 in the

state of New York (Hanan et al., 2005). In this population, CABG was associated with a decreased rate of repeat revascularisation over a period of three years (PCI 27.3% vs CABG 4.6%) (Hannan et al., 2005).

The American College of Cardiology Foundation (ACCF) and the Society of Thoracic Surgeons (STS) Database Collaboration on the Comparative Effectiveness of Revascularization Strategies (ASCERT) study elucidated that in a cohort of patients above 65 years old; CABG (86,244 patients) was associated with a lower mortality rate than PCI (103,549 patients) four years after surgery (CABG 16.4% vs 20.8% PCI) (Weintraub et al., 2012). Additionally, a single-centre retrospective analysis of over 8000 patients reported that CABG was associated with increased rate of survival than BMS (PCI 76.3% vs 86.9% CABG) (Habib et al., 2015). Furthermore, when compared to PCI with DES, CABG was associated with increased survival at five and nine years post-surgery (Habib et al., 2015). Therefore, numerous clinical studies either indicate equivalence or advocate the use of CABG over PCI.

1.4.5 The unmet medical need to improve CABG outcomes

CABG surgeries as a treatment for CHD are hindered by re-occlusion rates and need for further surgery. The reported occlusion rates following CABG vary, however, they are consistently suboptimal. Fitzgibbon et al., recruited 221 patients and examined over 700 conduits by angiography between one and ten years post-CABG (Fitzgibbon et al., 1996). Over this period, the percentage of occluded grafts increased from 8% to 41% (at ten year follow up examination) (Fitzgibbon et al., 1996). Of the remaining ‘healthy’ grafts, 7% were narrowed at some locality of the vessel (Fitzgibbon et al., 1996). Goldman et al., reported similar occlusion rates; after 10 years 39% of SV grafts were occluded (Goldman et al., 2004). Interestingly, patency rates differed depending on the anatomical location with the vasculature of the heart. Graft occlusion rates were lowest within the LAD (31%) and highest in the right coronary artery (44%) (Goldman et al., 2004). Analysis of secondary outcomes of the PRoject of *Ex-vivo* Vein graft ENgineering via Transfection (PREVENT) IV trial revealed that 25.2% of CABG patients experienced VGF after just 18 months (1096/4343 patients) (Hess et al., 2014). Furthermore, after 10 years, approximately 50% of CABG patients required repeat revascularisation (Goldman et al., 2004). Taken together, these

clinical shortcomings provide much incentive to target VGF therapeutically. Over 200,000 CABG surgeries are performed each year in the United States (US) (Weiss and Elixhauser, 2014). Worldwide CABG is performed at a rate between 4 and 91 per 100,000 inhabitants (Head et al., 2017). Therefore, the unmet medical need to improve long-term outcomes is urgent.

1.4.6 The aetiology of vein graft failure

1.4.6.1 Early vein graft failure

The key events leading to VGF include: acute thrombosis, intimal hyperplasia and subsequent accelerated atherosclerosis (Harskamp et al., 2013). Early methods of harvesting the SV involved the removal of the perivascular tissue, thus causing intraoperative damage. Veins are then checked for leakages using hydrostatic dilatation which causes distention and damage to blood vessel. These processes have been reported to cause local inflammation (Khaleel et al., 2012) and activation of smooth muscle cells within the vein graft (Verma et al., 2014). The development of the “no touch technique” whereby surgeons remove the entire intact vessel (including perivascular tissue) has led to significant improvements in long term graft patency (Souza et al., 2002). Likewise, limiting pressure applied during hydrostatic dilatation reduces damage to the endothelium and limits intimal hyperplasia but this is not widely adopted (Li et al., 2014).

Further intraoperative damage to the SV graft occurs during the period between harvest and grafting. During this time, the vein graft experiences transient ischemia and as a consequence ECs produce less factors that normally act to quiesce the underlying smooth muscle e.g. prostacyclin and nitric oxide (Woodward et al., 2016, Mitchell et al., 2008). Even non-toxic dyes in surgical marker pens used to orientate the SV pre-implantation have been reported to exacerbate injury (Hocking et al., 2016).

Upon completion of CABG surgery, the endothelium will be damaged at the points of anastomosis and throughout the conduit caused by reperfusion upon restarting the heart (Weaver et al., 2012, Harskamp et al., 2013). This denudation of the endothelium exposes medial extracellular matrix (ECM)

components and thus enhances the adhesion of platelets (Figure 1.2). This in turn leads to the aggregation and accumulation of platelets that can form a thrombus and produce mitogenic, pro-thrombotic proteins such as platelet derived growth factor (PDGF) (Virmani et al., 2000). Consequently, thrombosis can lead to the occlusion of the vein graft. Fifteen percent of SV grafts experience thrombosis leading to early failure of the vein graft, typically within one month of surgery (Motwani and Topol, 1998). In some cases, acute thrombosis can lead to further complications such as pulmonary embolism and deep vein thrombosis (Viana et al., 2017).

Overall, damage to the SV before and immediately after implantation can lead to acute VGF. Furthermore, these events can set in motion pathophysiological processes that lead to a more gradual occlusion of the conduit as discussed in the following sections.

1.4.6.2 Late vein graft failure

It is widely agreed that late VGF is a result of intimal hyperplasia that leads to the development of an occlusive, hyperplastic and fibrotic neointimal lesion (also known as the neointima). Intimal hyperplasia describes the migration and proliferation of vascular smooth muscle cells (VSMC) that accumulate in the intima. This leads to thickening of the intima which impinges upon the lumen and can decrease the lumen area by up to 25% in just weeks (Motwani and Topol, 1998). This occurs as a consequence of conduit injury, which creates a pro-inflammatory environment. Upon denudation of the endothelial layer the underlying VSMCs are exposed to circulating pro-inflammatory cytokines and in response the VSMCs proliferate and upregulate production of mitogenic factors including but not limited to tumour necrosis factor alpha (TNF-a) and monocyte chemotactic protein 1 (MCP-1) (Schepers et al., 2006, Monraats et al., 2005). This process potentiates VSMC proliferation and promotes chemotaxis driven recruitment of inflammatory cells to the site of injury. This process allows infiltration of monocytes and macrophages into the intima further contributing to the creation of a mitogenic environment. Ultimately this leads to the narrowing of the blood vessel and predisposes the venous graft to superimposed atherosclerosis (Stary et al., 1992). In fact, just one year after surgery, foam cells can be identified in SV grafts (Kockx et al., 1994, Yahagi et al., 2016).

Subsequently, a necrotic core can develop in less than five years (Yazdani et al., 2012). This process as a whole is referred to as late VGF and accounts for 40% of surgeries that fail between one and ten years (Goldman et al., 2004). Neointima formation is a complex, multi-factorial process and the importance of VSMCs in this process is discussed in more detail below (Section 1.4.6.5).

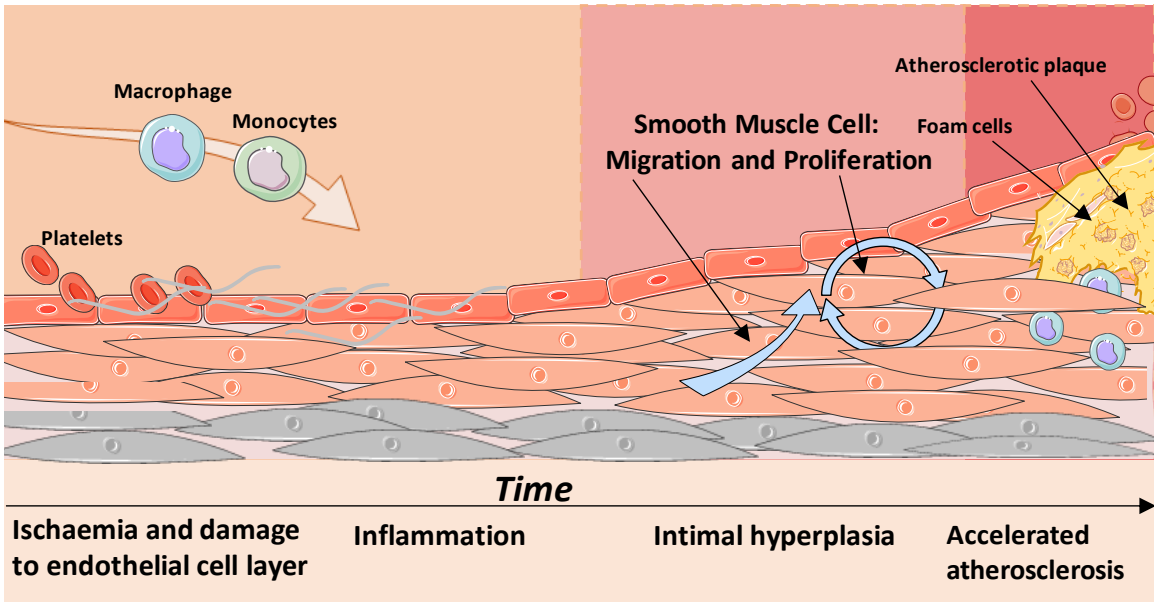

Figure 1.2 An overview of saphenous vein graft failure.
During CABG procedures the conduit experiences ischaemia in storage and damage to the endothelial cell layer upon grafting and reperfusion. Platelet activation at the site of injury and infiltration of immune cells creates a local proinflammatory niche. Production of PDGF-B by platelets and cytokines by immune cells elicits VSMC (grey) migration into the intima, toward the site of injury. Intimal resident VSMC (orange) upregulate production of mitogens and thus begin to proliferate. Proliferation of VSMC within the intima leads to intimal thickening and new layers of smooth muscle begin to impede upon the lumen. VSMC then synthesise ECM and the lesion becomes less hypercellular and more fibrotic leading to further expansion. Throughout this process infiltrating monocytes can differentiate into macrophage and uptake cholesterol containing lipoproteins to become foam cells thus promoting superimposed atherosclerosis. Together these processes can lead to the full occlusion of the grafted blood vessel. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.4.6.3 The role of ECs in VGF

The endothelium is the single layer of cells that acts as a barrier separating the circulating blood from the underlying smooth muscle cells in the vessel wall. It is also a sensory point of contact; ECs act to sense changes in blood flow and shear stress and convert these signals to biological responses (Davies, 1995, Gimbrone et al., 1997). The endothelium is vitally important in the control vascular tone. The endothelium under normal conditions promotes vasodilation through the catalytic action of endothelial nitric oxide synthase (eNOS) that metabolises L-arginine to nitric oxide (NO) which leads to production of cyclic guanosine monophosphate (cGMP) (Janssens et al., 1992). Increased levels of intracellular cGMP lead to decreased levels of Ca^{2+} thus promoting relaxation of VSMC and the blood vessel (Carvajal et al., 2000). Complications arise when control of vascular tone by ECs is impaired, such as when the endothelium is damaged during surgery or reperfusion injury.

ECs also contribute to the maintenance of a healthy vascular bed by quiescing neighbouring VSMC by virtue of their ability to produce NO (Garg and Hassid, 1989a). EC derived NO inhibits VSMC proliferation *in vitro* and in preclinical models of neointima formation where VSMC proliferation *in vivo* is exaggerated in eNOS KO mice (Yu et al., 2012). For these reasons, the damage or removal of the endothelium can rapidly induce migration and proliferation of VSMC, a phenomena that is reversed or partially reversed upon regeneration of the endothelium (Gimbrone, 1999).

Because the endothelium is the contact point between the blood vessel and the circulation, pro-inflammatory cytokines can bind to receptors on the cell surface promoting intracellular signal transduction leading to activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). NFκB activation leads to transcription of genes that encode vascular adhesion molecules that allow adhesion of platelets and infiltration of immune cells and therefore can evoke thrombosis and formation of an atherosclerotic plaque (Boyle et al., 1997).

It is also thought that ECs can transdifferentiate into mesenchymal cells that express SMC markers and contribute to a proportion of neointimal cells *in vivo*

after vein grafting (Cooley et al., 2014, Frid et al., 2002). Inhibition of this process and improving EC regeneration is being actively investigated as a therapeutic strategy for improving outcomes of interventional cardiac surgery (Cooley et al., 2014). In summary, ECs undoubtedly play a role in vascular remodelling and neointima formation mainly through mediating effects in the underlying smooth muscle. Endothelial denudation during revascularisation procedures therefore plays a prominent role during in-stent restenosis (ISR) and VGF (Kipshidze et al., 2004b).

1.4.6.4 Neointima formation and the role of VSMC in VGF

Neointimal formation is a key issue in interventional cardiology and leads to ISR, VGF and other pathologies (de Vries et al., 2016). Neointima formation is a mitogenic process that results in substantial accumulation of VSMC within the intima. Years of research have identified the importance of mature medial VSMCs in this process (Ross and Glomset, 1973).

Mature, contractile VSMCs are elongated in shape and organise themselves into concentric layers throughout the tunica media. VSMC synthesise collagen and elastin fibres that construct an ECM which is important for maintaining elasticity of the vessel. In larger blood vessels the tunica media is densely packed with layers of VSMC interlaced with ECM. But the primary function of VSMCs is to allow contraction (vasoconstriction) and relaxation (vasodilation) of blood vessels. For this reason, they express various myosin and actin proteins and possess extensive microtubule architecture meaning these cells can contract upon recognition of biological cues; this is a fundamental process in the regulation of vascular tone and blood pressure.

The biology of VSMC varies considerably through embryonic development and through disease process. In embryonic development, VSMC proliferate rapidly allowing the creation of the body's own expansive vascular tree (Cook et al., 1994). Upon maturation, VSMCs exist in a contractile state but their characteristics vary throughout different vascular beds (Shanahan and Weissberg, 1998). Moreover, VSMC heterogeneity has been reported to exist within the same vascular bed in mice (Dobnikar et al., 2018). Furthermore, VSMC do not differentiate terminally. Instead, VSMC remain plastic, capable of

undergoing trans-differentiation in response to biological cues such as experienced in disease settings (Owens et al., 2004). This is commonly referred to as plasticity and allows VSMC to repair blood vessels in response to injury or inflammation. In response to injury, VSMCs de-differentiate to lose their classical contractile phenotype and instead take on a pro-migratory, pro-proliferative and synthetic (producing ECM) phenotype (Owens et al., 2004). This de-differentiation is accompanied by a marked upregulation in expression of ECM related genes and by the loss or downregulation of genes that encode contractile SMC proteins such as alpha smooth muscle actin (αSMA), smooth muscle protein 22α (SM22a), SM-myosin heavy chain (MHC) and calponin (Gomez and Owens, 2012, Owens, 1995). Genetically modified mice that express fluorescent reporter genes downstream of SMC specific genes have been used to investigate the importance of VSMC phenotype switching during vascular injury. Lineage tracing studies performed using these mouse models showed that mature VSMCs rapidly proliferate after mechanical injury and this was responsible for the majority of the cells within the neointima (Nemenoff et al., 2011). Furthermore, the use of lineage tracing mice has revealed the presence of a rare population of stem cell antigen 1 (Sca-1) positive VSMCs that are predisposed to proliferate following activation (Dobnikar et al., 2018).

Although medial VSMCs appear to be key players in neointima formation, recent studies suggest that other cell types may *also* contribute. Some researchers hypothesise that arterial VSMCs migrate laterally and are the source of neointimal VSMCs within failing vein grafts (Hagensen et al., 2011, Liang et al., 2014, Hu et al., 2002). Additionally, adventitial fibroblasts have also been suggested as a possible source of intimal cells in a porcine arterial injury model (Shi et al., 1996). Lastly circulating progenitor cells have also been shown to contribute to neointima formation in mice. A parabiotic model that conjoined a wild type mouse and a transgenic mouse expressing green fluorescent protein (GFP) in every cell demonstrated that GFP positive progenitor cells were present in the circulation of the wild type mouse. When the wild type mouse was subject to femoral artery wire injury surgery, the resulting neointimal lesion contained GFP positive cells (Tanaka et al., 2008). However, it remains that the oldest and most popular theory highlight VSMCs as the prominent origin of cells within neointimal lesions. It follows therefore, that one strategy to target intimal

hyperplasia in revascularisation surgeries is to inhibit VSMC migration and proliferation whilst allowing normal EC function and re-endothelialisation. There are currently no therapeutics used in the clinic that directly target intimal hyperplasia and no conventional pharmacological agent has been proven to prevent late VGF (Okrainec et al., 2005). Standard secondary therapies following CABG target thrombosis by anti-platelet therapy and lowering LDL through statins (Kulik et al., 2015). In 2015, over 16,000 CABG procedures were performed in the UK alone representing an urgent need to improve the outcomes of CABG surgeries (BHF, 2018).

1.4.6.5 VSMC migration

VSMC migration and proliferation are key events in neointima formation leading to coronary artery occlusion, ISR and VGF (de Vries et al., 2016). VSMC migration from the media to the intima is thought to precede proliferation. VSMC migration following vascular injury is mediated by pro- or anti-migratory stimuli that elicits intracellular signalling events thus leading to degradation of ECM, cytoskeletal rearrangements, chemotaxis and directional migration. In order to migrate, VSMCs must first free themselves from the basement membrane and ECM. This is achieved by the upregulation of expression and activity of zinc-dependent endo-proteases called matrix metalloproteinases (MMPs) which act to degrade components of ECM, including collagen and non-collagen substrates. Degradation of collagen and ECM facilitates migration through the vessel by enabling detachment and the generation of new contact points (Carragher and Frame, 2004). MMP-2 and -9 (amongst others) have been associated with VSMC migration. Overexpression of MMP-9 promoted VSMC migration *in vitro* (Mason et al., 1999). While genetic deficiency of MMP-2 and -9 inhibited foetal bovine serum and PDGF-BB induced VSMC migration *in vitro* (Cho and Reidy, 2002). Furthermore, a lesser extent of intimal hyperplasia has been reported in transgenic MMP-2 and MMP-9 null mice as compared to the wild type (Cho and Reidy, 2002)

MMPs also have other key vascular functions such as angiogenesis (MMP-10) (Heo et al., 2010), VSMC proliferation (MMP-8) (Xiao et al., 2014) and apoptosis (MMP-7) (Williams et al., 2010). Inhibition of MMPs through overexpression of tissue

inhibitors of metalloproteinases (TIMPs) is a promising strategy to inhibit vein graft intimal hyperplasia as discussed later (section 1.7.3) (George et al., 2011).

Once freed from the ECM at the leading edge, VSMC must undergo cytoskeletal rearrangement. Actin polymerisation allows VSMC to form projections of the cytoskeleton at the leading edge called lamellipodia which allow VSMC to physically move (Louis and Zahradka, 2010, Pollard and Borisy, 2003). Actin-related protein (Arp) 2/3 is a complex that can initiate the formation (nucleation) of actin filaments *de novo* to enable movement (Pollard and Borisy, 2003). During migration, lamellipodia form temporary points of contact between the plasma membrane and ECM. Integrins are a family of receptors, that form heterodimers (one α and one ß subunit) that normally anchor VSMC to ECM. These transmembrane glycoproteins consist of an extracellular domain that binds to collagen and ECM components. A transmembrane region connects the extracellular domain to an intracellular domain that binds to the VSMC actin cytoskeleton, thereby facilitating signal transduction (Miyamoto et al., 1995). Nascent focal adhesions at the leading edge require integrins and can either quickly disassemble or mature into more secure contact points (Sun et al., 2014). Examples of cytoplasmic proteins tethered at these contact points are Focal Adhesion Kinase (FAK), paxillin and Src which play a role in signal transduction and mechanosensing (Gerthoffer William, 2007, Wang et al., 2001).

Just like VSMC contraction, myosin light chain (MLC) kinase plays a key role in migration; Ca^{2+} mediated phosphorylation of MLC kinase leads to contraction of MLC which allows for traction at the adhesion points. This is also a dependent on other kinases upstream, namely Rho-associated protein kinase (ROCK) (Amano et al., 1996, Wang et al., 2009). In addition to actin, microtubules also play a role in migration of VSMC. During migration, microtubules re-orientate and the nucleus is positioned to the opposite side of the direction of migration (Gomes et al., 2005). Finally, rear-end detachment of the VSMC from the ECM must then occur to allow for movement in the direction of the leading edge (Ridley et al., 2003). MMPs also play a role in mediating rear-end detachment (e.g. MMP-9) (Kirfel et al., 2004).

It is of note that VSMC migration only occurs when they have entered the G_1 phase (see below), therefore early cell cycle machinery is also important to migration as well as proliferation (Boehm and Nabel Elizabeth, 2001).

1.4.6.6 VSMC proliferation

Like all eukaryotic cells, in order to proliferate VSMC must enter and progress through the cell cycle. The cell cycle describes a precise series of events and check points whereby cells duplicate their genome and undergo cell division (mitosis). Briefly, when cells enter the cycle, they become more anabolic, expanding in size during the first phase of interphase called the gap 1 (G_1) phase. Next, cells enter the synthesis (S) phase where DNA replication occurs. After which, cells prepare for division of the cytoplasm through rapid protein synthesis in the gap 2 (G_2) phase. Mitosis (M) follows and describes the separation of identical chromosome pairs, division of the nucleus and finally division of the cytoplasm (cytokinesis) forming two daughter cells (Figure 1.3) (Malumbres and Barbacid, 2009).

VSMCs within the media are normally in the G_0 phase, displaying a contractile phenotype. However, in response to injury, mechanical stress and growth factors VSMC may enter the cell cycle and proliferate extensively. After injury VSMC upregulate the proteins essential for cell cycle progression including proliferating cell nuclear antigen (PCNA), cyclins (cyc) and cyclin-dependent protein kinases (CDKs) (Gordon et al., 1990a). Cyclins and CDKs form active complexes that control the “check points” throughout the cell cycle (Sherr, 1993, Koff et al., 1992). CDK4/cycD is required to enter the cell cycle and for transition from G_1 to S phase (Harbour et al., 1999). In the G_1 phase, VSMC prepare for DNA synthesis which is the key step of the following S phase. CDK4/cycD dimerization proteins under normal conditions exist in an inactive complex with E2F transcription factors and negatively regulates the cell cycle. When active, CDK4/cycD phosphorylates retinoblastoma protein (Rb) which dissociates from E2F. E2F then enters the nucleus and enhances transcription of the Myc gene and drives expression of cell cycle machinery thus controlling important S phase proteins, such as CDK2 and PCNA (a protein that binds around DNA and tethers DNA polymerase allowing replication) (Thalmeier et al., 1989, Dalton, 1992). Cells prepare for mitosis through protein synthesis and

reorganisation of microtubules into mitotic spindles in the G_2 phase. G_2 is regulated by CDK1/CycB and CDK2/CycA which continue to phosphorylate Rb and regulate histone synthesis (Ma et al., 2000). Finally, mitosis allows for chromosomes to separate in the M phase.

As previously discussed, inhibiting the cell cycle pharmacologically using sirolimus and paclitaxel eluting stents have been reported to reduce ISR with sirolimus providing more potent therapeutic effect than paclitaxel (Windecker et al., 2005). Post-operative MI was reduced in patients treated with sirolimus (6.2%) as compared to those treated with paclitaxel (10.8%) and likewise, the requirement for repeat revascularisation was also reduced (4.8% vs 8.3%) (Windecker et al., 2005). Sirolimus acts as a G_1 cell cycle inhibitor and paclitaxel promotes M-phase arrest (Figure 1.3) (Wessely et al., 2006). This highlights the importance of cell cycle control in revascularisation surgeries however conventional therapeutic strategies in CABG do not afford the use of continuous drug delivery systems such as DES.

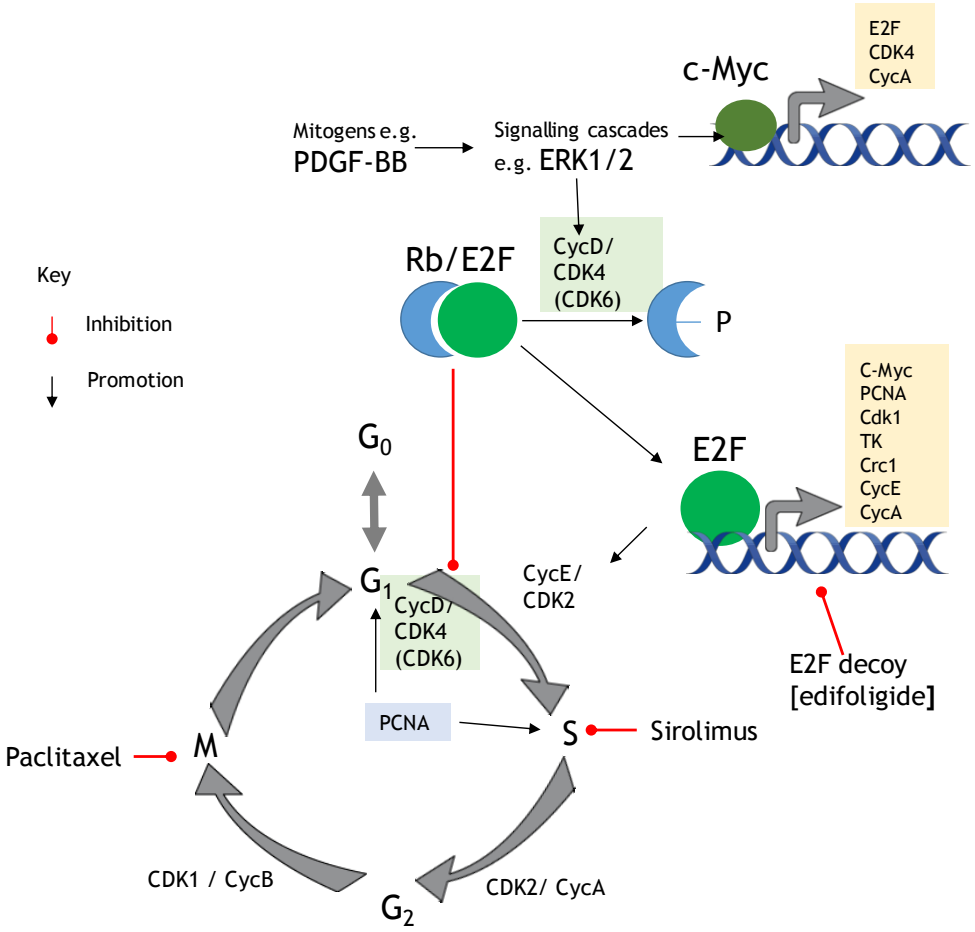

Figure 1.3 Cell cycle checkpoints in VSMC

The cell cycle consists of the following phases G_0, G_1, S, G_2, and M. Governing the progression of VSMC through each phase are CDKs and cyclins that promote the expression genes related to the cell cycle but also play other roles such as promoting the biosynthesis of histones. Mature VSMC in "healthy" vessels exist in a non-proliferative state known as G_0. Upon stimulation by injury or exposure to mitogens VSMC enter the cell cycle in G_1 and begin to growth larger. Entrance to the cell cycle (G_0-G_1) is controlled by E2F, CDK4 and CycD. When E2F dissociated from retinoblastoma protein (Rb) it promotes gene expression of c-myc and PCNA. PCNA is essential for replication of DNA and is therefore required for S phase where cells duplicate their genome. In the subsequent G_2 phase rapid protein synthesis occurs that will eventually allow the cell to split into two daughter cells in the M phase. Because VSMC proliferation is a hallmark of neointima formation and vein graft failure inhibition of the cell cycle is a therapeutic strategy. Paclitaxel inhibits mitosis (M phase), Sirolimus inhibits transition from G_1 to S and the E2F decoy prevents transcription of the cell cycle machinery. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.4.6.7 The role of PDGF in vascular remodelling after injury

In 1974, a factor in serum that was dependent on the presence of platelets was found to induce arterial VSMC proliferation (Ross et al., 1974). This was revealed to be platelet-derived growth factor subunit B (PDGF-B) which is a member of a family constructed of four isoforms: A, B, C and D. They are inactive as monomers and become active as dimers (e.g. PDGF-AA, PDGF-AB, PDGF-BB) (Raines, 2004). Early *in vitro* studies reported that PDGF-BB is particularly important in mediating proliferation of fibroblasts and VSMCs (Kohler and Lipton, 1974, Ross et al., 1974). Furthermore, gene expression of PDGF-B is upregulated in different vascular beds during disease processes exemplified in diseased human carotid arteries (Barrett and Benditt, 1987) and in human coronary arteries following PCI (Tanizawa et al., 1996). Inhibition of PDGF-BB signalling by the pharmacological receptor antagonist RPR101511A inhibited neointima formation in a porcine model of PCI (Bilder et al., 1999). PDGF-B is expressed to a high degree by intimal resident macrophage in humans (and other primates) and is likewise increased in monocytes taken from patients with high levels of cholesterol in their blood (Ross et al., 1990, Billett Michael et al., 1996). PDGF-B and it's receptor (PDGFRB) are also upregulated by VSMCs and ECs in response to changes in shear stress (Mondy et al., 1997). Thus, PDGF-BB is implicated in intimal hyperplasia that is driven by changes in haemodynamic conditions. This is relevant to CABG surgeries as the shear stress experienced by the SV when grafted within the vasculature of the heart is greater than its native environment. Furthermore, obvious changes in blood flow are present at the points of anastomoses. More turbulent blood flow at these points drive intimal hyperplasia; these locations of vein grafts and the coronary artery where the vein is grafted often display more pronounced neointimal lesions when examined by angiography or histology (Bassiouny et al., 1992).

Human saphenous vein VSMCs (HSVSMCs) express high levels of the beta-type platelet-derived growth factor receptor (PDGFRB) and therefore are more responsive to PDGF-BB than arterial VSMCs, this leads to increased levels of migration and proliferation *in vitro* (Turner et al., 2007, Yang et al., 1998). Therefore, PDGF-B signalling may be particularly important in the development and progression of vein graft disease as compared to arterial grafts or PCI. Additionally, inhibition of PDGF-B signalling in vein graft disease could be

particularly promising as it has been reported that blockade of this pathway in experimental atherosclerosis models does not affect VSMC commitment to the fibrous cap therefore maintaining plaque stability (Kozaki et al., 2002). Selectively targeting downstream components of the PDGF-BB/PDGFRB signalling cascade may be important in order to effectively inhibit intimal hyperplasia following CABG.

PDGF-BB mediates its effects through the PDGFRB which is a class III receptor tyrosine kinase (RTK) (Figure 1.4). PDGFRB receptor dimerization following ligand binding is important for activation by auto-phosphorylation (Kelly et al., 1991, Hammacher et al., 1989). PDGFRB can also be transactivated by other factors such as angiotensin II (Ang II) (Heeneman et al., 2000). Activation can lead to rapid phosphorylation of mitogen activated kinases (MAPKs), phosphoinositide 3-kinases (PI3Ks) and phospholipase C (PLC) pathways. Intracellular domains of the activated receptor act as points of interaction for these proteins via their SH2 (Src homology 2) domain (Raines, 2004).

The MAPK cascade is instigated by growth factor receptor bound protein 2 (Grb2) that complexes with another adapter protein, Sos (son of sevenless), that when recruited converts guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on the receptor bound G proteins (see section 1.5.3). This subsequently leads to activation of Ras and subsequently Raf which phosphorylates MEK1/2 (Kyriakis et al., 1992). PDGFRB activation leads to rapid phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) which upon nuclear translocation activates elk1 (E26 transformation-specific-like transcription factor) and SRF (serum response factor) subsequently regulating transition from G_1 to S-phase thus leading to proliferation of VSMC (Meloche and Pouyssegur, 2007). Migration of VSMC is partly dependent on ERK1/2 signalling however roles for p38 MAPK and JNK (c-Jun N-terminal kinase) have also been reported (Zhan et al., 2003b, Cospedal et al., 1999). *In vitro* studies utilising adenoviral vectors to overexpress dominant negative mutants of ERK1/2, p38 and JNK reported that downregulation of all three targets inhibited PDGF-BB induced migration (Zhan et al., 2003).

Intracellular PLC also plays a role in mediating the effects of PDGF-BB in VSMC. PLC-γ can be activated by PDGFRB to increase intracellular calcium ions release leading to MLC kinase activation and VSMC migration (Kundra et al., 1994a).

PDGF-BB mediated activation of PI3K leads to phosphorylation of Akt (Franke et al., 1995). Akt is a downstream effector protein of PI3K and has been reported to inhibit VSMC apoptosis (Allard et al., 2008). PDGF-BB induced Akt phosphorylation has also been linked to the dedifferentiation of VSMC through downregulation of classical SMC proteins aSMA and SM22a (Kaplan-Albuquerque et al., 2003). PDGF-BB/Akt pathway also mediates anti apoptotic effects in VSMC via the transcription factor NFκB (Romashkova and Makarov, 1999). NFκB is a transcription factor that is important for the induction of VSMC growth by mediating G_1/S transition (Zahradka et al., 2002). Inhibition of NFκB signalling has been achieved through use of gliotoxin and has been shown to inhibit neointima formation and VSMC proliferation and migration in response to PDGF-BB (Pozo et al., 2009).

Other, downstream regulators of these effects in VSMC are encoded by the immediate early genes ras, myc and c-jun (Irani et al., 1994, Barone and Courtneidge, 1995, Zhan et al., 2002b). Utilising an adenoviral vector encoding a dominant-negative form of c-Jun, it has been shown that c-Jun is key to allow transition from G_1 to S phase (Zhan et al., 2002a).

Taken together, there is strong evidence that PDGF-B plays a central role in vascular remodelling after interventional surgery.

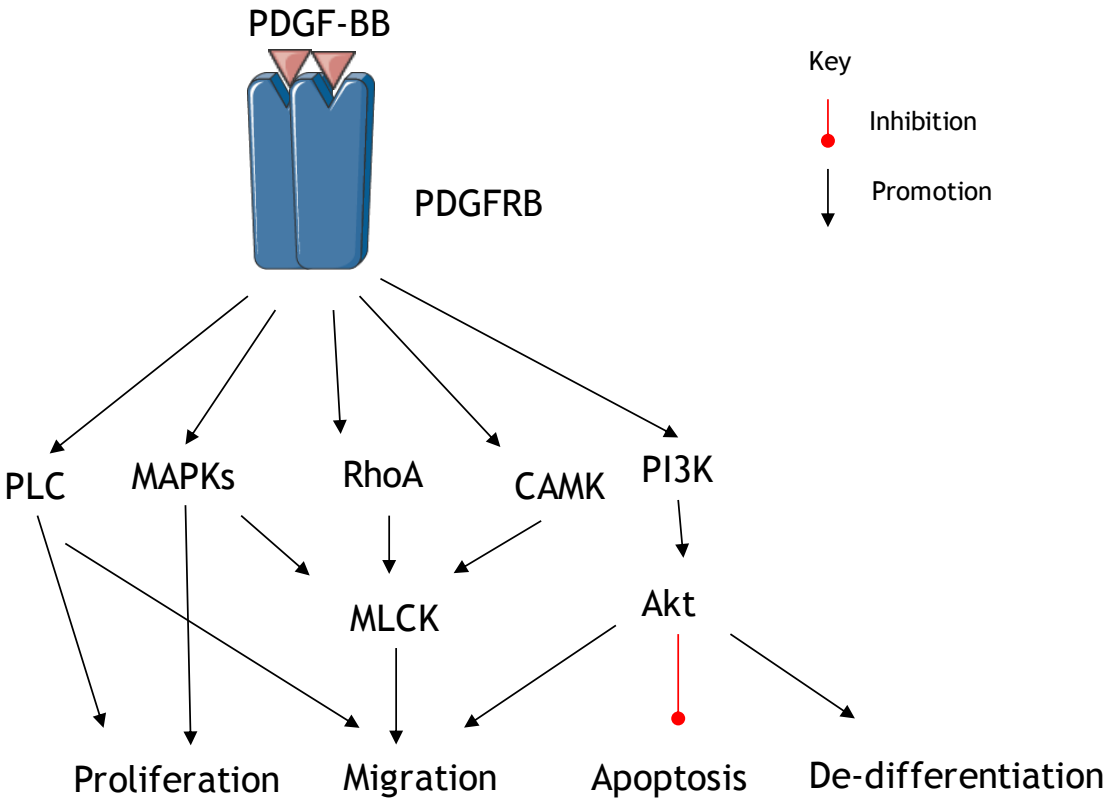

Figure 1.4 A simplified overview of PDGF-BB signalling VSMC

Activation of the PDGFRB results in activation of diverse signalling pathways that elicit a biological response. Activation of PLC and MAPK kinases elicit pro-proliferative responses. MAPKs, RhoA and CAMK also promotes VSMC migration. While the PI3K pathway promotes migration, de-differentiation and inhibits apoptosis. Image created using Servier Medical Art available at https://smart.servier.com. No permission required**.** Myosin light chain kinase (MLCK) Phosphoinositide 3-kinase (PI3K), Mitogen activated protein kinases (MAPKs), Phospholipase C (PLC), Ca^{2+}/calmodulin-dependent protein kinase (CAMK), Ras homolog gene family, member A (RhoA), Platelet Derived Growth Factor Receptor Beta (PDGFRB).

1.4.6.8 The role of local inflammation in vascular remodelling after injury

It is well known that inflammation plays a key role throughout the development and progression of VGF (Reviewed in de Vries et al., 2016). Firstly, damage incurred by the endothelium attracts circulating immune cells. Neutrophils bind to sections of the SV or LIMA where the ECs have been damaged or denuded (Schlitt et al., 2006). Studies using clinical samples have demonstrated that monocytes immediately adhere to SVs following grafting (Eslami et al., 2001). This is followed by invasion of monocytes within the vessel, a process that is modulated to a large extent by MCP-1 and its receptor, the CC-chemokine receptor-2 (CCR2) (Owens et al., 2015).

Denudation of the endothelium exposes the ECM components that are recognised as damage-associated molecular patterns (DAMPs) by B lymphocytes (Karper et al., 2011). This furthers the creation of a pro-inflammatory environment. Similarly, dendritic cells, macrophage and T lymphocytes have all been identified in autologous human vein grafts (Cherian et al., 2001). Infiltration of immune cells can lead to chronic local inflammation and expansion of the intimal lesion by releasing pro-proliferative cytokines such as MCP-1 and IL-1B as well as growth factors such as PDGF-BB; all of which are known to induce VSMC proliferation (Nelken et al., 1991, Viedt et al., 2002, Eun et al., 2015). In response, VSMCs themselves upregulate gene expression and production of chemokines and cytokines to further recruit immune cells as well as potentiate proliferation (Stark et al., 1997). As the neointima expands, resident intimal macrophage undergo apoptosis, this can lead to unstable lesions with the presence of a necrotic core (de Vries et al., 2016)

1.4.6.9 The role of systemic inflammation in vascular remodelling after injury

Preclinical studies demonstrate the importance of systemic inflammation in vascular injury processes. Raised systemic levels of inflammatory cytokines induced by lipopolysaccharide (LPS) exacerbates neointima formation in models of vascular injury (Danenberg Haim et al., 2002). In the clinic, CABG surgery itself is an invasive procedure and leads to a pronounced systemic inflammatory response (Karfis et al., 2008). Circulating inflammatory cytokines such as IL-1B inevitably come into contact with the endothelium, as well as the underlying

VSMC where the endothelium has been denuded. This can lead to EC dysfunction and inhibition of vasodilation (Bhagat and Vallance, 1997). The importance of inflammation is clearly highlighted by the fact that the ratio of pro- to anti-inflammatory cytokines and also of lymphocytes to monocytes have been reported to have prognostic value after CABG and PCI (Moreno et al., 2011, Oksuz et al., 2017, McBride and McBride, 1998). Increased lymphocyte to monocyte ratio is directly associated with VGF (Oksuz et al., 2017). In summary inflammation is ubiquitous and can be detrimental following acute vascular injury. Novel therapeutics should aim to also target inflammation in the setting of CABG.

1.4.6.10 Fibrosis during injury-induced remodelling

Months after surgery, the now established neointimal lesion changes to adopt a pro-fibrotic phenotype. Deposition of ECM at this late stage is known to contribute to the further narrowing and loss of elasticity of the vein graft (Jiang et al., 2009). In some cases the tunica media can be completely decellularized and instead become composed primarily of fibrotic tissue (Owens et al., 2015). In pre-clinical models this has been shown to be governed by transforming growth factor- ß (TGF-B) which promotes the production of collagens and fibronectins (Chen et al., 2000) as well as downregulating MMP expression and activity (Jiang et al., 2009b). Similarly, Ang II is well known to be pro-fibrotic through interplay with TGF-B and downstream SMAD signalling (Harvey et al., 2016, Wang et al., 2006, Rodriguez-Vita et al., 2005). Although inhibition of the process has been successful in preclinical studies, no anti-fibrotic therapeutics for use in revascularisation surgeries have reached the clinic (Reviewed in de Vries et al., 2016).

1.5 The renin angiotensin system

The canonical renin angiotensin system (RAS) is one of the most extensively studied hormonal systems within the human body. The RAS acts systemically and locally to govern blood pressure, vascular tone and cardiac function. The RAS also has secondary effects on inflammatory and fibrotic functions. It mediates all of these effects through a series of G protein coupled receptors (GPCRs), enzymes and peptide metabolites that have independent function.

When the kidney experiences reduced blood flow or perfusion pressure, it catalyses the cleavage of prorenin to renin (Figure 1.5) (Hsueh and Baxter, 1991). Increased systemic concentrations of active renin promotes conversion of angiotensinogen (produced by the liver) to the ten amino acid long angiotensin peptide known as angiotensin I (Ang I). ACE then cleaves Ang I to an eight amino acid long angiotensin peptide known as Ang II. Increased concentrations of Ang II leads to increased biosynthesis of aldosterone in the adrenal cortex. Aldosterone subsequently promotes reabsorption of sodium and water by the kidney thereby regulating blood pressure and volume (Figure 1.5). Ang II is also a potent vasoconstrictor and is of utmost importance in blood pressure control and also cardiac function, acting primarily through the angiotensin II type 1 receptor (AT_1R). However, when this system is dysregulated it can result in the development and progression of hypertension, CHD, abdominal aortic aneurysms (AAA) and restenosis.

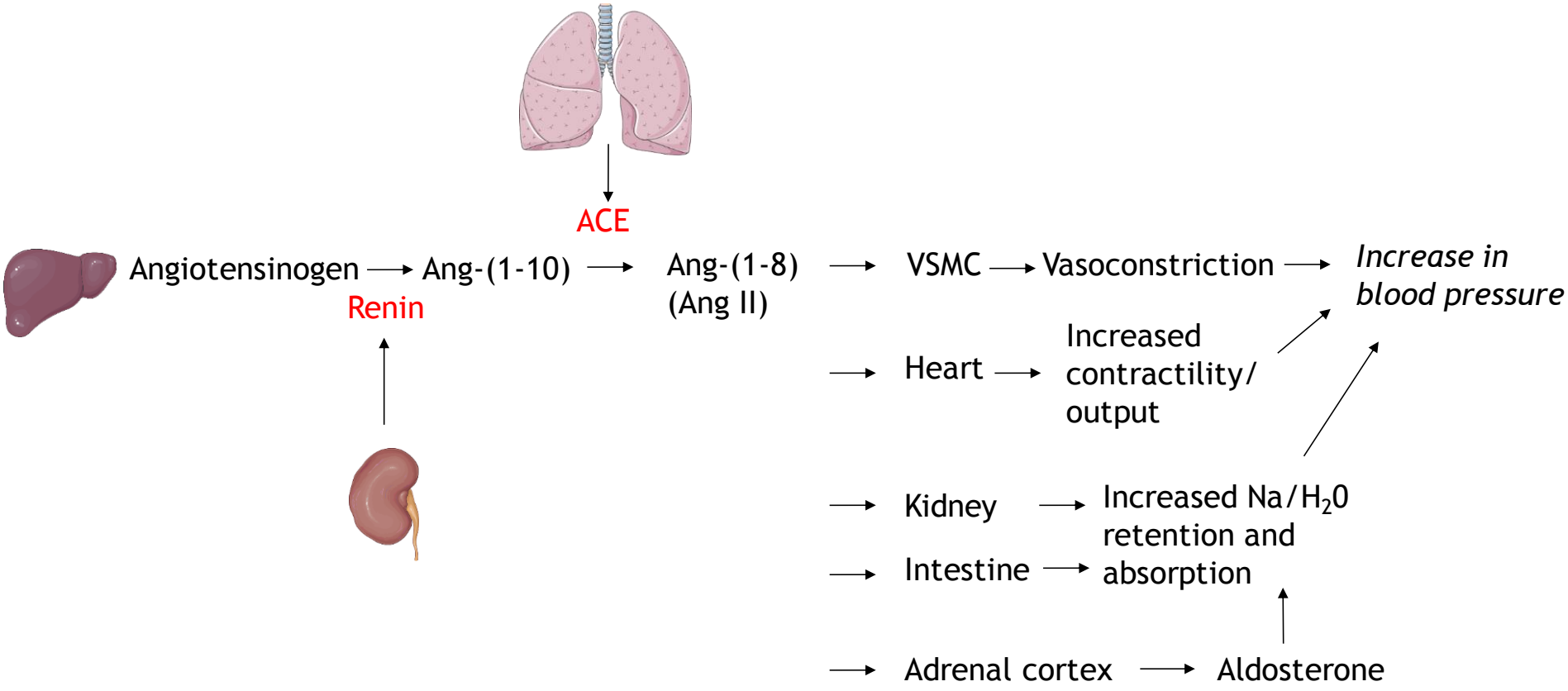

Figure 1.5 The classical RAS pathway (systemic).

Angiotensinogen is synthesised and secreted by the liver. Kidney derived renin is then responsible for the degradation of angiotensinogen to Ang-(1-10). ACE reduces Ang-(1-10) (Ang I) to the biologically active octapeptide Ang-II (Ang-1-8). Ang II acts on numerous cell types and systems in order to increase blood pressure. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.5.1 Renin

Renin was the first discovered member of the RAS, over 120 years ago. When renin was first purified from rabbit kidney tissue, intravenous injection of the extract led to an elevation in blood pressure in rabbits that had undergone surgical removal of the kidneys (nephrectomy) (Tigerstedt and Bergman, 1898). Renin is now known to be an aspartic protease that is produced in the kidney and converts angiotensinogen to Ang I, the precursor of Ang II.

1.5.2 Angiotensin Converting Enzyme

Fifty eight years after the discovery of renin, the biological role of ACE was elucidated (Skeggs et al., 1956). It was determined that the product of renin, Ang I (at the time known as hypertensin I) was converted by ACE to Ang II (hypertensin II) of which the vasoactive nature was already known (Skeggs et al., 1956). ACE is a membrane bound zinc metalloenzyme and is primarily produced in the capillaries of the lung (Bünning and Riordan, 1985). ACE is also expressed on ECs (Ryan et al., 1976, Lavrentyev Eduard et al., 2007). Importantly, disease conditions such as vascular injury and high glucose induce ACE expression in VSMC (Lavrentyev Eduard et al., 2007, Fishel et al., 1995). Notably, ACE expression is upregulated in intimal VSMC and resident macrophages in human coronary arteries that have undergone PCI. Modulation of Ang II signalling has proven an excellent strategy to treat cardiovascular conditions such as hypertension (Shah et al., 2008).

1.5.3 G protein coupled receptors (GPCRs)

GPCRs are the most abundant membrane bound receptors in human eukaryotic cells and are key to governing cellular responses to the RAS. These heptahelical receptors all contain an extracellular amino-terminal domain (N), three intracellular loops and an intracellular carboxyl-terminal tail. This structure allows extracellular cues to elicit intracellular signalling in order to promote biological effects. When a particular agonist binds to the extracellular domain of a GPCR, a conformational change occurs that allows for recruitment of intracellular effector proteins (Katritch et al., 2012). Signal transduction can be dependent of heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) of which four subclasses (Gs, Gi/o, Gq/11 and G12/13) have been

identified. Conversely, GPCRs can signal through G-protein-coupled receptor kinase (GRK) and arrestin (Gurevich and Gurevich, 2019).

G protein dependent GPCR signalling utilises the heterotrimeric G protein subunits Gα, Gß and Gγ. When inactive, Gα is bound to GDP and the G protein dimer ßγ (Gßγ). Activation of the GPCR promotes dissociation of GDP from Gα (Higashijima et al., 1987). Subsequently, the G protein subunits also dissociate from the receptor. GDP independent Gα can activate different signalling pathways through adenylyl cyclase (AC), PLC and phosphodiesterases (PDE) among others (Kristiansen, 2004). Gßγ can also activate members of integral signalling pathways that are linked to vascular disease such as MAPKs (Hilger et al., 2018). MAPKs are also key effectors of arrestin-mediated GPCR signalling. Arrestins are approximately 45-kDa in size. Arrestins, of which four families (1-4) exist (arrestin 1-4), serve a multitude of functions in GPCR signalling including termination of signalling, receptor endocytosis and activation of a plethora of intracellular signalling cascades (Cahill et al., 2017).

1.5.4 Angiotensin II and AT_1 Receptor signalling

Ang II is an octapeptide that is the main effector peptide of the classical RAS . Ang II governs many physiological processes in the vasculature, the most well-known of which is the control of blood pressure. As previously described, Ang II is produced by ACE from Ang I, a peptide that increases as a consequence of increased renin production (from pro-renin) by the kidney when it senses low blood pressure (Carey et al., 1997). Ang II is the first responder in this scenario. Ang II signalling via AT_1R promotes vasoconstriction of the arteries and arterioles that leads to an increase in peripheral resistance and increase in mean arterial blood pressure (Figure 1.5) (Ito et al., 1995).

Additionally, Ang II signalling in the renal proximal convoluted tubule promotes sodium and water reabsorption thereby increasing blood volume and subsequently cardiac output and arterial pressure (Schuster et al., 1984). To the same end, Ang II signalling in the adrenal cortex promotes aldosterone production that leads to potassium excretion and sodium absorption (Figure 1.5) (Oelkers et al., 1975). Furthermore, Ang II signalling in the brain (hypothalamus) stimulates thirst and therefore water intake (Davisson et al., 2000). Additionally,

Ang II stimulates excretion of antidiuretic hormone by the pituitary which increases water re-absorption by blood in the kidney (Scholkens et al., 1980). In this way, Ang II leads to higher concentration of water in the blood resulting in increased blood volume and pressure (Figure 1.1.5) (Scholkens et al., 1980).

1.5.4.1 The AT_1R

The AT_1R is a 359 amino acid long, seven membrane-spanning domain GPCR encoded on chromosome 3 (in humans) (Curnow et al., 1992). In humans, the AT_1R is expressed ubiquitously throughout the body including the brain, endocrine tissues, heart, lung, liver, kidney, vasculature, reproductive organs and skin. This is important in mediating the systemic control of the RAS. It is important to note that two copies of the AT_1R are expressed in mice and rats; the $AT_{1A}R$ and $AT_{1B}R$ have been reported as structurally identical however are expressed in different tissues, thereby modulating distinct functions (Elton et al., 1992). *In situ* hybridisation detected mRNA transcripts for the $AT_{1A}R$ in the liver, heart and lungs, while mRNA encoding the $AT_{1B}R$ were detected in the anterior pituitary (Gasc et al., 1994). Both $AT_{1A}R$ and $AT_{1B}R$ are expressed in the vasculature including the aorta where a role in the development of aorta aneurisms has been described for both receptors (Galatioto et al., 2018, Poduri et al., 2012)

Like many GPCRs, signalling downstream of the AT_1R is diverse and varies according to cell type, regulatory process and pathophysiology. Intracellular signalling cascades are key to regulating the effects of Ang II (Figure 1.6). The following sections describe Ang II/AT_1R signalling in the vasculature. AT_1R signalling through heterotrimeric G proteins is a key mechanism controlling vascular tone. Ang II signalling through the AT_1R recruits the G protein subclasses G_q and $G_{12/13}$ (see section 1.5.3). G_q activation by the AT_1R initiates protein kinase C (PKC) and phospholipase C (PLC) cascades that ultimately lead to VSMC contraction (Figure 1.6). PKC activation phosphorylates inositol trisphosphate (IP3) that in turn increases intracellular concentrations of calcium ions (Ca^{2+}) which activate myosin light chain kinase (MLCK). MLCK then phosphorylates MLC leading to VSMC contraction and vasoconstriction (Kanaide et al., 2003). While PLC activates RhoA/Rho kinase which has dual effect by inhibition myosin light chain phosphatase (MLCP) and activating Rho-associated protein kinase (ROCK)

that also phosphorylates MLC. It has also been reported the AT_1R can act as a mechanoreceptor and that G_q plays a role in mediating vasoconstriction that is independent of ligand binding (Mederos y Schnitzler et al., 2008). Lastly, PKC mediated phosphorylation of the carboxyl terminal tail leads to internalisation of the AT_1R and thus termination of Ang II/AT_1R signalling. Therefore, AT_1R signalling is transient, unlike the characteristics of its counter-regulatory twin, the AT_2R (as discussed in section 1.6.2 below).

1.5.4.2 Ang II in vascular remodelling

Vascular remodelling describes the structural modification of the blood vessel wall encompassing thickening of the vessel wall either through the cellular contents growing larger (hypertrophy), dividing (hyperplasia) or deposition of ECM. Ang II can promote all of these processes either directly or indirectly. For example, Ang II can promote vascular remodelling indirectly through sustained elevation of blood pressure (Safar et al., 1996). In fact, hypertension is fundamentally associated with blood vessel remodelling. In response to chronic high blood pressure, the architecture of the blood vessel wall is remodelled. Moreover, Ang II signalling also directly leads to vessel fibrosis by upregulating expression of collagens and fibronectins in VSMC *in vitro* (Kato et al., 1991) and *in vivo* (Himeno et al., 1994).

Ang II is well accepted to promote VSMC hypertrophy (Gibbons et al., 1992). Whether Ang II promotes a hypertrophic or hyperplastic response is thought to depend on the presence of other growth factors. For example, when VSMC were exposed to Ang II in the presence of a TGF-B_1 antibody: a pro-proliferative response was reported, whilst coincubation of Ang II and an immunoglobulin G (IgG) molecule resulted in no proliferative response (Gibbons et al., 1992). Moreover, the effect of Ang II on VSMC proliferation is also dependent on the origin (which vascular bed), species and experimental conditions. For example, Ang II does not induce proliferation of human aortic VSMCs. Contrastingly, Ang II promotes proliferation of aortic VSMC isolated from rats (Dugourd et al., 2003, Zhang et al., 2016) and proliferation can be induced indirectly via Ang II mediated upregulation of PDGF-B (Deguchi et al., 1999).

AT_1R signalling through beta arrestin dependent mechanism are key to evoking VSMC migration and proliferation (Min et al., 2005). This occurs via AT_1R mediated activation of intracellular kinases such as p38 MAPK, ERK1/2 and Akt (Figure 1.6) (Kendall et al., 2014, Wei et al., 2003). AT_1R mediated phosphorylation of ERK1/2 in VSMC results in activation of the transcription factor Elk1 (Godeny and Sayeski, 2006). Phosphorylation of the transcription factor Elk1, leads to upregulation of pro-proliferative genes such as serum response factor (SRF) and immediate early genes such as c-Fos (Marais et al., 1993). AT_1R mediated phosphorylation of p38 MAPK evokes migration of VSMC by activating the transcription factor c-Src (Mugabe et al., 2010). Therefore Ang II/AT_1R signalling converges substantially with PDGF-BB signalling as previously discussed (section 1.4.2.7).

Preclinical models of neointima formation have effectively highlighted the important role for Ang II in pathological vascular remodelling (Laporte and Escher, 1992, Lee et al., 2018). In rats, chronic Ang II infusion promoted carotid artery VSMC proliferation after both sham and balloon angioplasty procedures (Daemen et al., 1991). Furthermore , expression of the AT_1R is increased following aortic balloon angioplasty in rats (Viswanathan et al., 1992). Ang II mediated activation of NFκB can lead to production of MCP-1 and other inflammatory cytokines that have been implicated in VGF and which themselves can lead to enhanced human VSMC proliferation (Viedt et al., 2002, Fu et al., 2012b). Blocking Ang II signalling in human subjects can decrease concentrations of circulating inflammatory cytokines. For example, the ACE inhibitor enalapril significantly reduced plasma MCP-1 concentrations after just three days in patients who were hospitalised following MI (Soejima et al., 1999). Additionally, a 22 patient study in which CABG patients received high dose therapy with ACE inhibitors and statins demonstrated that systemic inflammation induced by on-pump CABG can be significantly decreased (Radaelli et al., 2007). Furthermore, twelve weeks of treatment with the ARB irbesartan reduced circulating concentrations of TNF-α by over 50% in patients with atherosclerosis (Navalkar et al., 2001).

It is clear that Ang II/AT_1R signalling has diverse effects in diverse target tissues and dysregulation of the RAS by chronic Ang II signalling can lead to the pathophysiology associated with VGF.

1.5.4.3 Ang II in vein graft disease

In a canine model of vein grafting, ACE activity doubled after surgery leading to VSMC proliferation (Yuda et al., 2000). Infusion of the AT_1R antagonist L-158,809 inhibited VSMC proliferation in the aforementioned study (Yuda et al., 2000). Similarly, reducing Ang II bioavailability through ACE inhibition [captopril (Capoten)] reduced neointima formation in a rabbit model of vein grafting (O'Donohoe et al., 1991). While ARBS have also been used to inhibit porcine coronary artery VSMC proliferation *ex vivo* (Wilson et al., 1999).

Yet clinical trials assessing the long-term outcomes of CABG patients have been disappointing (Rouleau et al., 2008, van Diepen et al., 2018). 2553 patients were randomly assigned the ACE inhibitor accupril or placebo as treatment following CABG. No therapeutic effect on stenosis were reported (Rouleau et al., 2008). Moreover in the first 3 months following surgery ACE inhibition was associated with increased risk of primary outcomes that were defined as death, MI, angina, rehospitalisation, stroke or requirement for revascularisation (Rouleau et al., 2008). Likewise, sub-analyses of the PREVENT IV (see section 1.7.3) trial showed ACE inhibitors and ARBS had no effect on CABG outcomes after 2 years (Goyal et al., 2007). Therefore, with no robust clinical evidence suggesting ARBs and ACE inhibitors have any effect in CABG; novel and more effective strategies to inhibit vein graft remodelling are urgently needed.

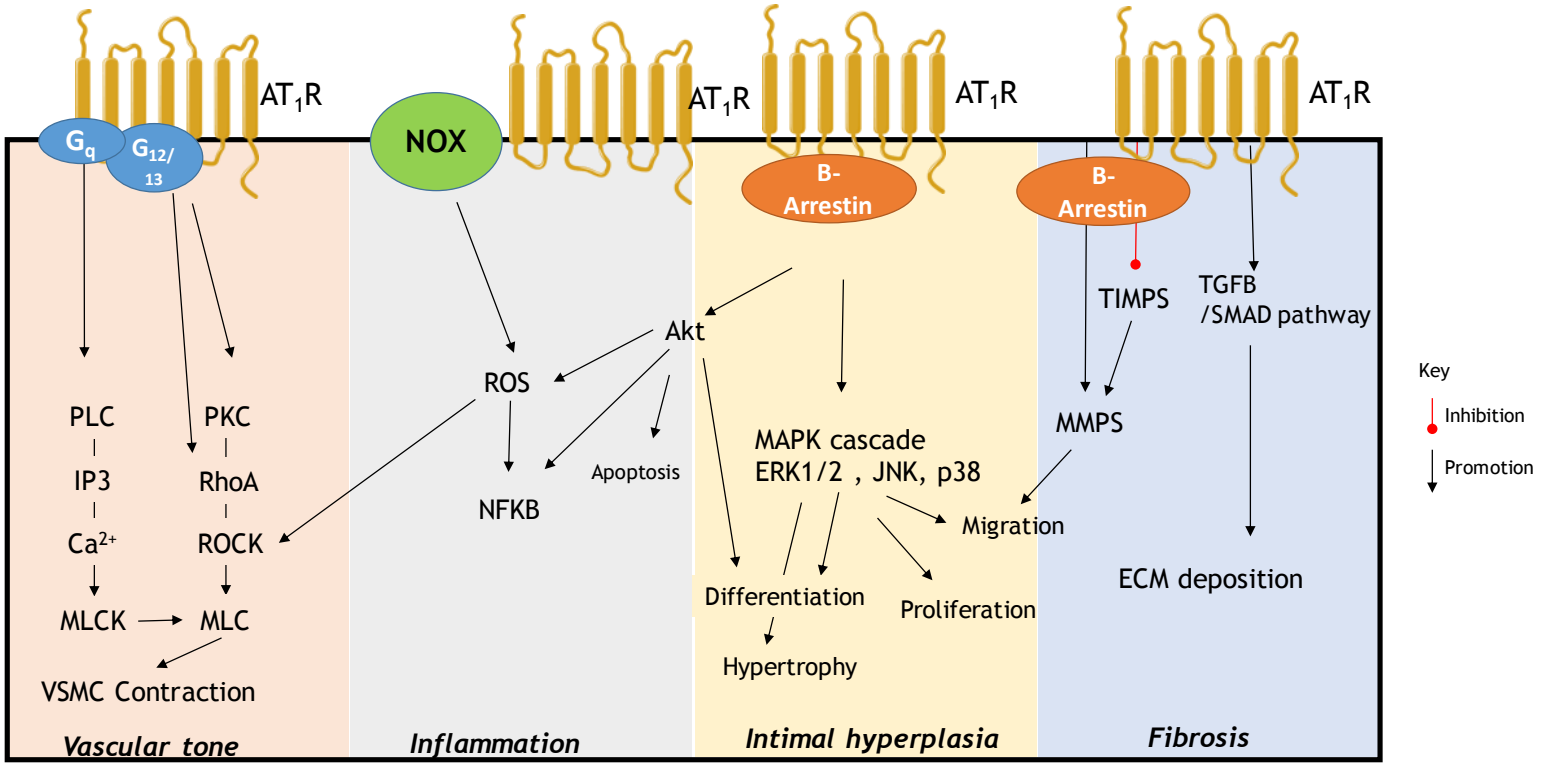

Figure 1.6 A schematic outline of the effects of AT_1R signalling in the vasculature.

Ang II signalling through the AT_1R activates diverse signalling cascades. G protein mediated AT_1R signalling is in part responsible for the control of vasculature through the PKC and PLC pathways that ultimately lead to VSMC contraction. NAD(P)H oxidase (NOX) pathways lead to generation of ROS and activation of the inflammatory transcription factor NFKB. VSMC proliferation and migration are by in large promoted by beta arrestin mediated activation of ERK1/2, p38, JNK and the PI3K/Akt pathway leads to inhibition of apoptosis as well promoting de differentiation of VSMC from contractile phenotype to a synthetic pro-proliferative phenotype. Lastly activation of MMPs through downregulation of TIMPs leads to increased migration. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.6 The counter-regulatory renin angiotensin system

Many years after the classical axis of the RAS was discovered, evidence of a non-canonical axis began to surface. This axis acts naturally to counter-regulate the effects of Ang II and is now known to be composed of peptide metabolites and a carboxypeptidase that shares similarities with ACE (Figure 1.7) (Donoghue et al., 2000).

1.6.1 Angiotensin-converting enzyme-related carboxypeptidase 2

Angiotensin-converting enzyme-related carboxypeptidase (ACE2) is an essential regulator of blood pressure and cardiac function (Crackower et al., 2002). ACE2 acts by hydrolysing the His-Leu bond in Ang I and in doing so releasing the nonapeptide angiotensin-(1-9) [Ang-(1-9)] (Donoghue et al., 2000). Despite sharing some homology with ACE, ACE2 does not catalyse the cleavage of Ang-(1-9) to Ang II (Vickers et al., 2002). However, human ACE2 isolated from cardiac tissue has been shown to hydrolyse Ang II to Ang-(1-7) (Lin et al., 2004). This dual effect of decreasing Ang II and increasing levels of the protective Ang-(1-7) peptide is thought to be the driving force behind ACE2's potent protective effects in the cardio-vasculature.

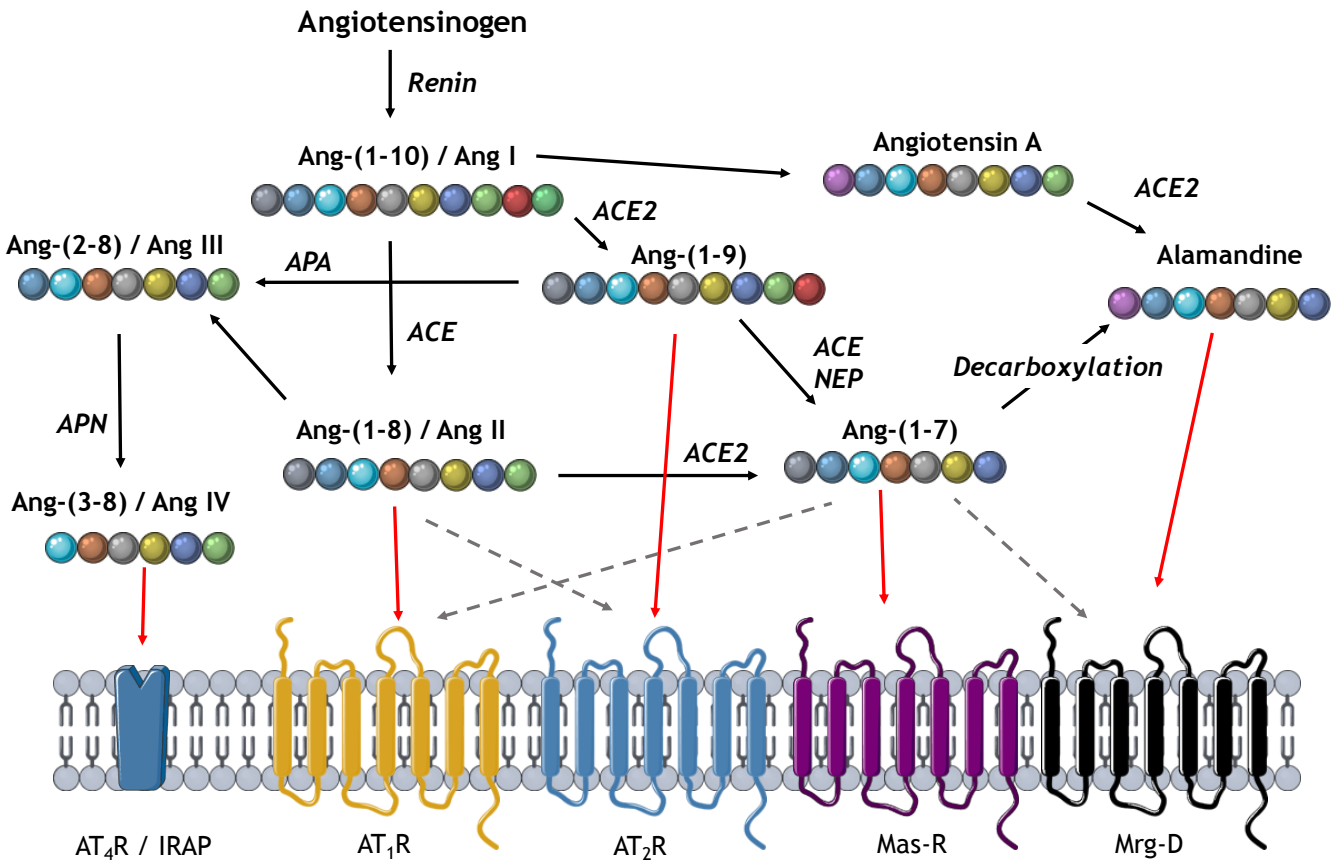

Figure 1.7 A schematic outline of angiotensin peptide interaction and metabolism. Ang II exerts its effects predominantly via the AT_1R to govern effects important to the pathophysiology of vein graft failure. Ang I and II are converted into counter-regulatory peptides by the action of ACE2. Ang-(1-7) primarily exerts effect through the Mas receptor, whilst Ang-(1-9) is thought to elicit AT_2R signalling. Arrows in red indicate the highest affinity interaction with a receptor. Grey dashed arrows indicate other known interactions. Black arrows indicate peptide metabolism. Image created using Servier Medical Art available at https://smart.servier.com. No permission required. Angiotensin II type I receptor (AT_1R) Angiotensin II type II receptor (AT_2R). Angiotensin I Am converting Enzyme 2 (ACE2), insulin-regulated aminopeptidase (IRAP), neprilysin (NEP), aminopeptidase A (APA), aminopeptidase N (APN), Mas-related G protein-coupled receptor member D (MrgD).

1.6.2 The angiotensin type 2 receptor (AT_2R)

The AT_2R is a seven membrane spanning domain GPCR which shares ~34% amino acid sequence homology with the AT_1R (Inagami et al., 1992). Despite this similarity, a large body of evidence shows that the AT_2R antagonises the effects of Ang II/AT_1R signalling in cardiovascular remodelling (Forrester et al., 2018). The first indication that this was the case was the finding that AT_2R stimulates protein tyrosine phosphatases (PTP); an enzyme that dephosphorylates tyrosine residues on important signalling components such as ERK1/2 (Figure 1.8) (Bottari et al., 1992, Brechler et al., 1994).

The AT_2R has several ligand binding sites and the crystal structure has been resolved (Asada et al., 2018). Unlike AT_1R signalling, which is transient, AT_2R signalling can be sustained. It is thought that this is because the AT_2R is poorly internalised (Widdop et al., 2002, Hein et al., 1997). Previous work elucidated the structural reasons for the atypical effects of AT_2R, suggesting that Helix VIII can change orientation to sterically block the recruitment of certain G proteins and ß-Arrestins whilst maintaining an active state (Zhang et al., 2017a). Furthermore, an important structural feature is the third intracellular loop which is particularly important in mediating the effects of the AT_2R. Amino acid deletions within this region (240-244) led to the attenuation of SHP-1 activation and ERK1/2 inhibition (Lehtonen et al., 1999).

1.6.2.1 AT_2R receptor interactions

It is known that the AT_2R mediates additional effects by interacting with other membrane-bound receptors. Usually present in monomeric form at the cell surface, it has been reported that AT_2R can form a homodimer through disulphide bonding (Cys^{35}-Cys^{290}) (Miura et al., 2005). This interaction allows the AT_2R homodimer to be active in the absence of a ligand (Miura et al., 2005). The AT_2R can also form a heterodimer with the AT_1R to dampen effects of Ang II (AbdAlla et al., 2001), an interaction that has been confirmed using Förster resonance energy transfer (FRET) studies (Inuzuka et al., 2016). The AT_2R also inhibited ERK1/2 phosphorylation after heterodimerisation with the bradykinin b2 receptor (Abadir et al., 2006). Lastly, compelling evidence suggested a functional relationship between the AT_2R and the Mas-R. Firstly, AT_2R receptor

antagonists have been demonstrated to reverse the effects of Ang-(1-7) (Villela et al., 2015). Co-transfection of fluorescently tagged AT_2R and Mas-R vectors were then used to provide evidence that the two receptors could form a heterodimer by FRET (Leonhardt et al., 2017). The AT_2R cysteine residue 35 was found to be responsible for this interaction. Functional assays revealed that upon stimulation with Mas-R and AT_2R agonists, astrocytes upregulated CX3C chemokine receptor-1 mRNA expression. Importantly, this effect was not observed in astrocytes isolated from AT_2R or Mas-R KO mice (Leonhardt et al., 2017).

1.6.2.2 AT_2R in cardiac pathologies

In cardiac pathologies the role of the AT_2R has been debated. Previous findings have described that deletion of the AT_2R in mice attenuated pressure overload-induced cardiac remodelling (Senbonmatsu et al., 2000). Accordingly, AT_2R overexpression led to pathological effects, promoting cardiomyopathy (Nakayama et al., 2005). Contradictory findings provided evidence that overexpression of the AT_2R improved heart function after MI (Yang et al., 2002). Furthermore, AT_2R KO mice exhibited decreased rates of survival following MI (Adachi et al., 2003). Elsewhere it has been suggested that the AT_2R agonist C21 prevented cardiac fibrosis in Stroke Prone Spontaneously Hypertensive Rats (SHRSP) (Widdop et al., 2016). Therefore, controversy exists as to the role of the AT_2R in the heart. Generation of transgenic mice that overexpressed the AT_2R to varying degrees (one, four or nine copies) in the heart have helped explain the previously mentioned conflicting findings (Xu et al., 2014). Mice overexpressing one and four copies of the AT_2R were protected after MI as determined by echocardiography. However, this was abolished in mice overexpressing AT_2R to higher degrees (nine copies) (Xu et al., 2014). Thus, it may be the case that expression levels of AT_2R are key to regulating therapeutic effects and this should be considered in future studies (Xu et al., 2014).

In diseases of the vasculature, the therapeutic effects of the AT_2R are less controversial. A landmark study reported that in experimental models of AAA, AT_2R signalling mediates therapeutic effects (Habashi et al., 2011). The authors reported that aortic ERK1/2 and SMAD signalling were exacerbated in AT_2R KO mice and it was suggested that the beneficial effect of AT_1R antagonists is in

part mediated by subsequent redirected signalling through the AT_2R (Habashi et al., 2011).

1.6.2.3 AT_2R in vascular remodelling

The discovery that AT_2R activates PTPs and that PTPs are potent regulators of ERK1/2 subsequently led to reports of an anti-proliferative effect of AT_2R i*n vitro* (Pulido et al., 1998, Stoll et al., 1995). This anti-proliferative effect was also reported *in vivo*; local overexpression of the AT_2R inhibited neointima formation following carotid artery balloon angioplasty in rats (Nakajima et al., 1995). AT_2R stimulation was later reported to be linked with activation of the tyrosine phosphatase, Src homology region 2 domain-containing phosphatase-1 (SHP-1) (Cui et al., 2001, Bedecs et al., 1997). Activation of SHP-1 led to apoptosis of rat VSMC (Cui et al., 2001). In agreement with these findings, vanadate, a pharmacological PTP inhibitor, reversed AT_2R-induced apoptosis in an ERK1/2 specific manner (Yamada et al., 1996). Furthermore, this study and others elucidated the importance of mitogen-activated protein kinase phosphatase-1 (MKP-1) in programming cell death (Horiuchi et al., 1997, Yamada et al., 1996).

The C-terminal of the AT_2R can also bind an AT_2R-interacting protein (ATIP) and this has been reported to lead to activation of SHP-1 (Figure 1.8) (Nouet et al., 2004). A more recent study reported that an AT_2R agonist (C21) inhibited intimal hyperplasia in a rat model of vascular injury. In this study, ATIP activated peroxisome proliferator-activated receptor gamma (PPAR-γ) (Figure 1.8) (Kukida et al., 2016). Overexpression of ATIP in mice also inhibited vascular remodelling suggesting it is important in mediating the anti-proliferative effects of AT_2R (Fujita et al., 2009). It is also known that PPARγ inhibits expression of MMPs in human VSMC to inhibit migration (Marx et al., 1998). Similarly, another AT_2R interacting protein includes an MMP inhibitor, TIMP3, that will be discussed later as a target for use in vein graft procedures (see section 1.7.3) (Kang et al., 2008). The AT_2R also plays a protective role in vascular cells by reducing reactive oxygen species through activation of NOS (Li et al., 2016, Peluso et al., 2018).

1.6.2.4 Pharmacological targeting of AT_2R

It can be said that AT_2R signalling shows great potential as a drug target, and this notion was embodied by the synthesis of pharmacological agents capable of selectively targeting the AT_2R (Wan et al., 2004b, Rice et al., 2014). Most notably, an orally available non-peptide AT_2R agonist, C21, has been extensively studied and has been very useful for studying AT_2R biology (Wan et al., 2004b). The application of C21 in pre-clinical models of cardiovascular disease has been prolific. C21 has been reported to have therapeutic effect in models of MI, vascular injury and more recently pathologies related to VSMC function such as AAA (Kaschina et al., 2008, Kukida et al., 2016, Lange et al., 2018).

It was thought that AT_2R stimulation had no effect on systemic blood pressure however in female rats, systemic infusion of C21 blunted deoxycorticosterone acetate (DOCA)/salt induced hypertension (Dai et al., 2016). Moreover, in the N(ω)-nitro-L-arginine-methyl ester (L-NAME)-induced hypertension model; C21 led to a decline in collagen deposition and aortic stiffening (Paulis et al., 2012). In atherosclerosis models C21 inhibited high fat diet induced vascular inflammation (Sampson et al., 2016). Furthermore, C21 acted therapeutically in the SHRSP by improving EC function and reducing oxidative stress and inflammatory cell infiltration (Rehman et al., 2012). In the femoral artery ligation model, inflammatory cytokines such as MCP-1 and IL-1B were increased in the injured arteries and these were even further elevated in the injured arteries of AT_2R KO mice (Wu et al., 2001). Kukida and colleagues utilised C21 in the same model to demonstrate that AT_2R/ATIP1 mediated activation of PPARγ led to the inhibition of vascular remodelling after cuff placement (Kukida et al., 2016).

Promisingly, oral doses of C21 (0.3 to 100 mg) (Wan et al., 2004b) had no adverse effects in a randomised, double blind phase I trial (Steckelings et al., 2017). While another AT_2R agonist, EMA401 has been shown to be well tolerated in humans and provided relief to patients with neuropathic pain (Rice et al., 2014).

To summarise, the AT_2R is a complex GPCR, that appears to function in a protective manner by interacting with other GPCRs and inhibiting ERK1/2

signalling through activation of PTPs. Given the previous success of ARBs in hypertension, the AT_2R represents a legitimate drug target and future studies surrounding AT_2R agonists may lead to exciting new therapeutics.

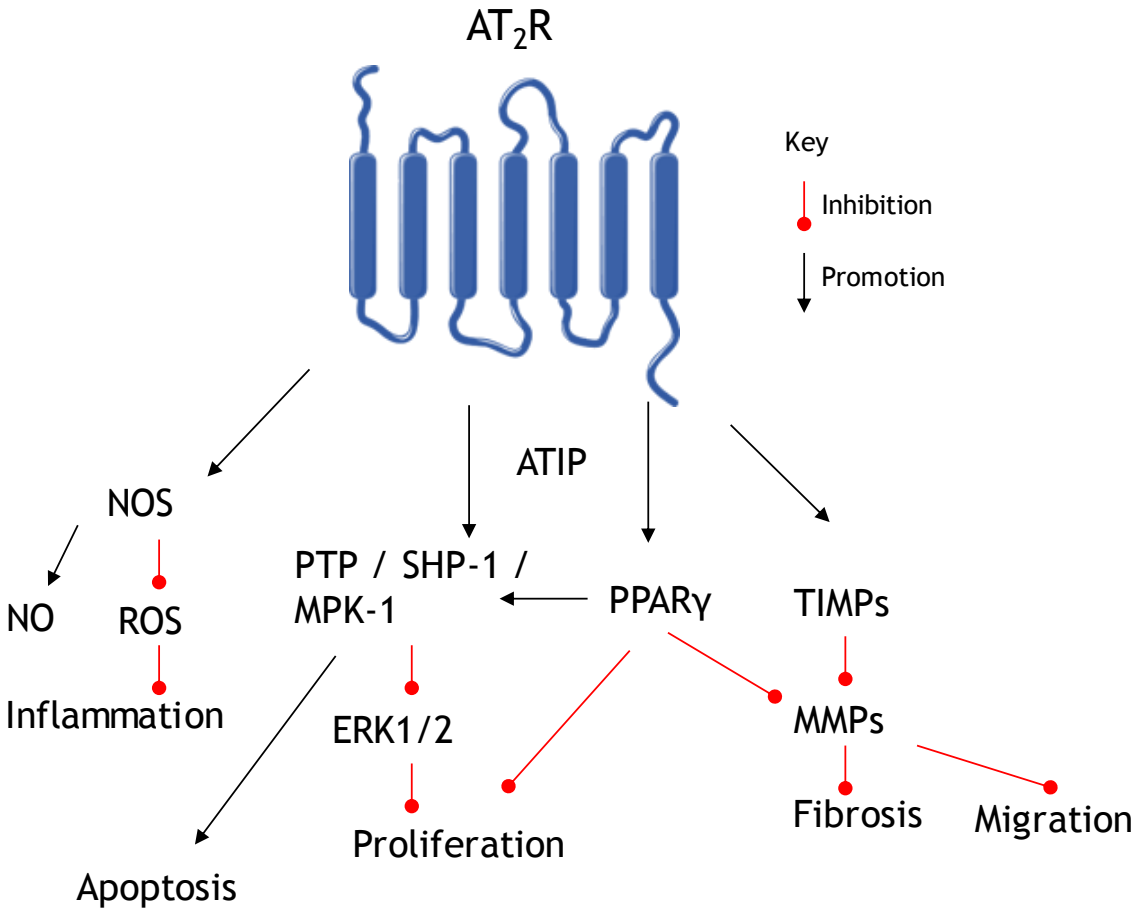

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Figure 1.8 A basic overview of AT_2R signalling.

The AT_2R has been reported to mediated pro apoptotic, anti-proliferative and anti-inflammatory effects in a number of cell types including VSMC. Primarily AT_2R mediated effects rely of phosphatases such as protein tyrosine phosphatase (PTP), Src homology region 2 domain-containing phosphatase-1 (SHP-1) and Mitogen-activated protein kinase (MPK-1). Activation of which can lead to subsequent de-phosphorylation of tyrosine kinases and MAPK kinases**.** AT_2R mediated activation of nitric oxide synthase (NOS) produces the vasodilator nitric oxide (NO). It is thought that the AT_2R can modulate fibrotic process via regulation of matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinases (TIMPs). Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.6.3 Mas Receptor

The gene encoding the Mas receptor (Mas-R) was originally identified as a proto-oncogene that encodes a hydrophobic GPCR with seven membrane spanning domains (Young et al., 1986). Shortly thereafter, Mas-R was misleadingly reported as a receptor for Ang II (Jackson et al., 1988). Santos et al., then utilised radioligand binding assays in combination with functional assays and Mas-R KO mice to demonstrate that the Mas-R is the dominant receptor for Ang-(1-7) (Santos et al., 2003).

The Mas-R can antagonise the AT_1R by forming a heterodimer with the AT_1R (Kostenis et al., 2005) The Mas-R, which is endocytosed after Ang-(1-7) stimulation (Gironacci et al., 2011) also antagonises the effect of Ang II via activation of intracellular phosphatases and NOS (Gava et al., 2009).

1.6.4 Angiotensin-(1-7) (Asp^1–Arg^2–Val^3–Tyr^4–Ile^5–His^6–Pro^7)

Angiotensin-(1-7) (Ang-(1-7) is a seven amino acid long angiotensin peptide that is produced by the enzymatic degradation of Ang II by ACE2. Another source is Ang-(1-9), which can be hydrolysed by neprilysin (NEP) and ACE to form Ang-(1-7) (Rice et al., 2004). In healthy humans, circulating concentrations of the heptapeptide Ang-(1-7) are approximately 70 pg/mL (Shen et al., 2019). The half-life of exogenous Ang-(1-7) in human tissue is approximately 30 minutes; whilst in rat plasma it is as short as nine seconds (Yamada et al., 1998, Petty et al., 2009).

In a landmark study, Santos et al., reported that Ang-(1-7) had an independent role, functioning through the Mas receptor (Mas-R) in order to control fluid balance (Santos et al., 2003). Ang-(1-7) is also well known for its vasodilator action (Faria-Silva et al., 2005). Ang-(1-7) mediated aortic vasodilation and fluid balance control after water loading and this was prevented in Mas-R KO mice (Santos et al., 2003). In human resistance vessels Ang-(1-7) inhibited Ang II induced vasoconstriction (Ueda et al., 2000). The Mas-R is expressed in the endothelium and acute stimulation with Ang-(1-7) leads to vasodilation and production of NO, whilst this effect is abolished when co-administered with the Mas-R antagonist A779 (Faria-Silva et al., 2005). Furthermore Ang-(1-7) displays

potent cardiovascular protection by activating NOS in EC and mediating the inhibition of EC adhesion molecules (ICAM-1/VCAM-1) (Zhang et al., 2013, Liang et al., 2015). Chronic Ang-(1-7) infusion inhibited hypertension in the SHRSP whilst this effect was attenuated upon coadministration of A779 (Sullivan et al., 2010).

It is of note that the Mas-R is not the only receptor that is ligated by Ang-(1-7). Ang-(1-7) has been reported to bind the AT_1R (Gironacci et al., 1999) despite lacking the Phe^8 residue that is important for Ang-II binding to the AT_1R (Noda et al., 1995). Teixeira et al., elucidated that Ang-(1-7) had similar AT_1R binding affinity as Ang II [Ang II 8.81 ± 0.25 vs Ang-(1-7) 6.66 ± 0.14 kP_i] (Teixeira et al., 2017). However, Ang-(1-7) mediated AT_1R signalling involved only beta arrestin engagement with little to no involvement of canonical G protein signalling. Ang-(1-7) was next shown to have protective effects in a mouse model of cardiac hypertrophy which could only be abolished with co-administration of both the AT_1R inhibitor losartan and the Mas-R antagonist A779 (Teixeira et al., 2017). This highlights the importance of the angiotensin peptide amino acid sequence in mediating distinct effect through different receptors.

In the setting of vascular injury, Ang-(1-7) has been shown to prevent HVSMC proliferation and migration *in vitro* (Freeman et al., 1996). *In vitro* studies have illustrated that Ang-(1-7) downregulated the AT_1R (Clark Michelle et al., 2001) and inhibited Ang II induced PI3K/Akt and ERK1/2 signalling in order to prevent VSMC proliferation, migration and inflammatory processes (Zhang et al., 2016). *In vivo,* Ang-(1-7) infusion by osmotic mini pump produced an anti-atherosclerotic effect in apolipoprotein E knock out ($ApoE^{-/-}$) mice as well as attenuating neointima formation after vascular injury and stenting (Langeveld et al., 2005, Jawien et al., 2012). Moreover, osmotic mini pump infusion of Ang-(1-7) [25 µg/kg/hr] inhibited VSMC ERK1/2 signalling and concomitant intimal hyperplasia in a rat jugular vein graft model (Wu et al., 2011).

Studies indicate that Ang-(1-7) may have broader clinical benefits beyond the vasculature. In previous clinical trials Ang-(1-7) has been shown to increase haematopoietic recovery in breast cancer patients who had received chemotherapy (Rodgers et al., 2006). However, a phase I sarcoma trial delivering Ang-(1-7) subcutaneously led to toxicities at high doses of Ang-(1-7)

[700 µg/kg] that in some cases led to stroke. Promisingly however, at a lower dose the trial reported that one patient experienced a 19% reduction in tumour burden that did not respond to conventional therapy (Petty et al., 2009). In a phase II clinical trial in sarcoma patients Ang-(1-7) was administered subcutaneously (20 mg daily) (Savage et al., 2016a). At this dose it was well tolerated and Ang-(1-7) was significantly increased in human plasma after 4 hours (Savage et al., 2016a). Overall, the study failed to replicate the therapeutic effect on biomarkers seen in the previously phase I trial (Petty et al., 2009).

These studies clearly demonstrate the need to refine the use of these angiotensin peptides by either chemical modification to enhance half-life or developing novel delivery strategies such as gene therapy for continuous delivery. The former has already been investigated by creation of Ang-(1-7) analogues with thioester bridges and cyclic non-natural amino acid substitutions (Wester et al., 2017, Kluskens et al., 2009). As of 2015, recruitment had begun for the Phase III clinical trials using the Ang-(1-7) formulation DSC127 (NCT01830348/NCT01849965) in topical treatments of diabetic foot ulcer (Rodgers et al., 2015). Moreover, a gene therapy expression cassette has been developed to deliver an intracellular cleaved and secreted mature Ang-(1-7) peptide, which will be discussed later (see section 1.7.4).

1.6.5 Angiotensin-(1-9) (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9)

Substantial evidence of the protective effects of Ang-(1-9) in the heart and vasculature has accumulated over the past decade. Ang-(1-9) is generated from Ang I by ACE2, cathepsin A and carboxypeptidase A (Donoghue et al., 2000, Jackman et al., 2002). In humans, plasma Ang-(1-9) concentrations are low (0.4 fmol/mL) as compared to other angiotensin peptides (e.g. Ang II ~13.9 fmol/mL) (Lawrence et al., 1990). While in rats, plasma concentration of Ang-(1-9) are again low at approximately 1 fmol/ml (Campbell et al., 1993). In rat tissues; concentration of Ang-(1-9) vary substantially with the highest concentrations being detected in adrenal tissue (62 fmol/mL) and aorta (19 fmol/mL) (Campbell et al., 1993).

In some disease states Ang-(1-9) levels are increased. For example, in a rat MI model, plasma concentrations of Ang-(1-9) doubled one week after MI but were unchanged 8 weeks post-coronary artery ligation (Ocaranza et al., 2006). In patients with acute respiratory distress syndrome (ARDs) increased plasma concentrations of Ang-(1-9) were reported in surviving patients (Reddy et al., 2019). Therefore, Ang-(1-9) concentrations vary throughout the body, by disease state and could be utilised as a biomarker in certain conditions.

It was originally understood that Ang-(1-9) mediated cardio-protection by competitive inhibition of ACE and elevated NO signalling through bradykinin cross-talk (Jackman et al., 2002). Later, an investigation into the effect of an ACE inhibitor (enalapril) on cardiac remodelling after MI, showed that both MI and sham operated rats treated with enalapril exhibited an approximately six-fold increase in plasma concentrations of Ang-(1-9) (Ocaranza et al., 2006. This increase was not seen with regard to Ang-(1-7) and was the earliest indication that Ang-(1-9) is a vasoactive peptide with independent cardio-protective effects (Ocaranza et al., 2006).

Ocaranza et al., later described that Ang-(1-9) inhibited cardiac hypertrophy *in vitro* and in rats which had undergone MI through coronary artery ligation (Ocaranza et al., 2010). Using a radioligand binding assay, Ang-(1-9) was shown to compete with radiolabelled Ang II for the AT_2R, thus describing for the first time, the affinity of Ang-(1-9) for the receptor (Flores-Muñoz et al., 2011). Subsequently, AT_2R antagonism (using a non-peptide AT_2R inhibitor, PD123 319) was used to distinguish the AT_2R-dependent inhibition of cardiomyocyte hypertrophy *in vitro* as well as cardiac fibrosis in SHRSP *in vivo* (Flores-Munoz et al., 2012). Very recently, Ang-(1-9) infusion has been demonstrated to decrease cell death and infarct size after MI and reperfusion injury, again, this effect was reported as dependent on the AT_2R (Mendoza-Torres et al., 2018).

Emerging evidence indicates that Ang-(1-9) may also play a protective role in the pulmonary vasculature. In human pulmonary arterial ECs Ang-(1-9) enhanced the release of NO through the bradykinin system (Jackman et al., 2002). In monocrotaline (MCT)-induced pulmonary hypertensive rats, Ang-(1-9) inhibited vascular remodelling and damage to the endothelium. These effects were blocked by the AT_2R antagonist PD123,319 thus bolstering the evidence to date

that Ang-(1-9) mediates its vasculoprotective effects via the AT_2R (Cha et al., 2018). Interestingly, Ang-(1-9) infusion decreased the concentrations of the inflammatory cytokines IL-6, MCP-1 and IL-1B in the plasma of MCT treated rats (Cha et al., 2018).

Recently, Ocaranza et al., have utilised Ang-(1-9) mini pump infusion and DOCA-salt hypertensive rats in order to elucidate an anti-inflammatory effect of Ang-(1-9) (Gonzalez et al., 2018). In this model, rats have one kidney surgically removed and subsequent treatment with DOCA in combination with salt loading induces hypertension and cardiovascular remodelling (Selye et al., 1943). Subsequent upregulation of adhesion molecules and cytokines leads to a proinflammatory state (Neves et al., 2005, Blasi et al., 2003). Chronic infusion with Ang-(1-9) significantly inhibited monocyte infiltration in the aorta, cardiac tissue and kidney of DOCA-salt hypertensive rats (Gonzalez et al., 2018).

Therefore, amounting evidence suggests Ang-(1-9) is protective in the heart, lungs, vasculature and inflammation.

1.6.6 Alamandine (Ala^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7)

The most recent member of the counter-regulatory RAS to be discovered is alamandine (Lautner et al., 2013). Alamandine confers vasoactive effects by signalling through the Mas-related G-protein-coupled receptor, member D (Mrg-D) (Lautner et al., 2013). Alamandine is formed by ACE2 mediated enzymatic cleavage of angiotensin-A (Ala^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8). Alamandine therefore differs from Ang-(1-7) by just one amino acid substitution at the N terminal. Additionally, alamandine can also be formed by decarboxylation of Ang-(1-7) (Hrenak et al., 2016). When infused systemically alamandine leads to hypotensive, anti-fibrotic effects (Lautner et al., 2013). After this discovery, alamandine has quickly been applied in other models of cardiovascular disease. Alamandine has now been shown to inhibit hypertensive cardiac remodelling as well as having a protective effect in reperfusion injury (Park et al., 2018a, Oliveira et al., 2019, Liu et al., 2018).

Although only two of these alanine containing angiotensin peptides (Ang-A and alamandine) have been discovered, it is thought that a whole network of these

peptides may exist (e.g. ala-Ang-(1-9), ala-Ang-I). Prominent researchers in this field have even begun to refer to them by a new name, the “alatensins”(Santos et al., 2019).

1.7 Gene therapy

The clinical use of angiotensin peptides is hindered by their rapid half-life. Overexpression of angiotensin peptides via gene transfer approaches can facilitate continuous expression *in vitro* and *in vivo*. Gene therapy utilises diverse vectors to overexpress a therapeutic gene or inactivate a faulty gene. These include viral and non-viral vectors. The main viral vectors utilised for gene therapeutics are adenoviral, adeno-associated virus (AAV), retroviral and lentiviral vectors. Each has its own associated advantages and disadvantages. Adenoviral vectors (section 1.7.1) contain a double stranded DNA genome (7.5 kb capacity) which does not integrate into the host DNA instead existing episomally (Wold and Toth, 2013). Adenoviral vectors induce a host immune response through leaky expression of viral proteins presenting on the host cell major histocompatibility complex-I (MHC-I) and therefore expression is transient (Yang et al., 1994). AAVs (capacity 4 kb) evoke less of an immune response, the viral genome integrates into host genome, but induction of transgene expression is slower and the duration of which is longer (McCarty et al., 2001). Lentiviral vectors contain a single stranded RNA genome (8 kb capacity) which integrates into the host genome causing long term transgene expression with low toxicity (Escors and Breckpot, 2010). Retroviral vectors also contain a single stranded RNA genome but can only infect dividing cells, furthermore the expression cassette integrates in a more random fashion (Escors and Breckpot, 2010).

Gene therapy can be used to treat innate immunodeficiency disorders, haemophilia , severe central nervous system (CNS) conditions such as X-linked adrenoleukodystrophy (ALD) and now also several forms of leukaemia (Cartier et al., 2009, George et al., 2017, Park et al., 2018b). Most success has been found in previously untreatable inherited monogenic diseases. The first Food and Drug Administration (FDA) approved, directly administered therapy was voretigene neparvovec (Luxturna); a treatment for the genetic ocular disease caused by a mutation in the retinoid isomerohydrolase gene (*RPE65*). Restoration of *RPE65* expression via an AAV significantly improved vision in patients with the *RPE65*

mutation (Russell et al., 2017). More recently, the FDA have approved a single dose gene therapy for spinal muscular atrophy (SMA) following a trial that utilised an AAV vector to replace the faulty gene (survival motor neuron 1) associated with the condition (Mendell et al., 2017). This therapy (Zolgensma) has gained some notoriety as it is now the most expensive drug on the market however the price of treatment is still less than that estimated for lifetime treatment of SMA.

The majority of gene therapy trials to date have focused on cancer (65%), while only 6.9% of trials have focused on cardiovascular disease most of which are cardiac related (Ginn et al., 2018). Two main gene therapy strategies exist in the cancer field; *in vivo* administration of oncolytic viral vectors or *ex vivo* gene transfer followed by adoptive transfer. Talimogene laherparepvec (T-VEC) is a genetically engineered herpes simplex virus with dual function and is the first FDA approved oncolytic virus. T-VEC selectively replicates and lyses tumour cells, secondly T-VEC produces granulocyte macrophage colony-stimulating factor (GM-CSF) in order to potentiate systemic tumour targeted immune responses (Liu et al., 2003). Patients with skin cancers (melanomas) respond well to T-VEC treatment and results in increased 'durable response rates'; a final outcome that describes therapeutic effect on tumour burden and increased survival (Andtbacka et al., 2015). T-VEC is administered by direct injection into the tumour, promisingly however anti-tumour effects have been seen in tumours elsewhere in the patients anatomy as a result of a systemic immune response (Andtbacka et al., 2015). In a single-centre study, T-VEC treatment led to complete remission of 43.5% of patients with advanced melanoma (Perez et al., 2018).

Secondly, *ex vivo* gene and cell therapy strategies have shown remarkable success rates in patients with drug refractory CD19+ B-cell leukaemia and lymphomas (Park et al., 2018b). Following leukapheresis, autologous (Grupp et al., 2013) or allogeneic (Kochenderfer et al., 2013) $CD4^+/CD8^+$ T cells can be transduced *ex vivo* with viral vectors (lenti- or retroviral) encoding a chimeric antigen receptor (CAR) that acts to target cytotoxic T cells to cancerous (CD19+) B cells. CARs combine the recognition specificity of an antibody (extracellular single chain variable fragment), fused to a CD3ζ (cluster of differentiation 3

zeta) chain to allow activation of the T cell (first generation CAR). Second (and third) generation CARs include the addition of one (or two) costimulatory domains such as 4-1BB and CD28 which act to inhibit apoptosis and T cell exhaustion thus improving longevity and engraftment *in vivo* (Imai et al., 2004, Finney et al., 2004)

Adoptive transfer of anti CD19 CAR T cells has since been proven to result in sustained remission of relapsed refractory chronic lymphocytic leukaemia (CLL) (Porter et al., 2015, Grupp et al., 2013) acute lymphoblastic leukaemia (ALL) (Lee et al., 2015) and follicular lymphoma (Kochenderfer et al., 2013). Remarkable remission rates of 81% have been reported in a global trial for anti CD19 CAR T cells for the treatment of children and adults with ALL (Maude et al., 2014). Taken together, CAR T cell therapy is thought by many to have revolutionised haematology. These recent success stories have renewed the interest in gene and cell therapy and thus the stage is set for advancements in the field of cardiovascular research as well but progress this area has been slower.

1.7.1 Adenoviral vectors

In order to effectively utilise gene therapy, extensive investigation into the choice of gene is required but the choice of vector can be equally important. Adenoviruses (Ad) are non-enveloped, DNA viruses with a linear double strand (36 kb) genome organised into 4 transcriptional units; early regions one to four (E1-4) and a late transcription unit (MLP). Structurally, adenoviral capsids are composed of 240 trimeric hexons. 12 pentameric penton bases and 12 trimeric fibers which extend from the penton base (Nicklin et al., 2005) (Figure 1.9). Species C serotype 5 (Ad5) is by far the most commonly used viral vector in research. Deletions in E1 in the Ad5 genome produces a replication-deficient Ad vector, which can incorporate a <5 kb expression cassette thus providing flexibility for the introduction of therapeutic transgene in diverse pathologies (Davison et al., 2003). Further deletions made to E2 and E3 regions allowed for a larger expression cassette (14 kb) (Amalfitano et al., 1998). Lastly the final generation of adenoviral vector has the entire viral genome excised with the exception of the 5′ and 3′ inverted terminal repeats (ITRs) and a packaging signal (Alba et al., 2005)

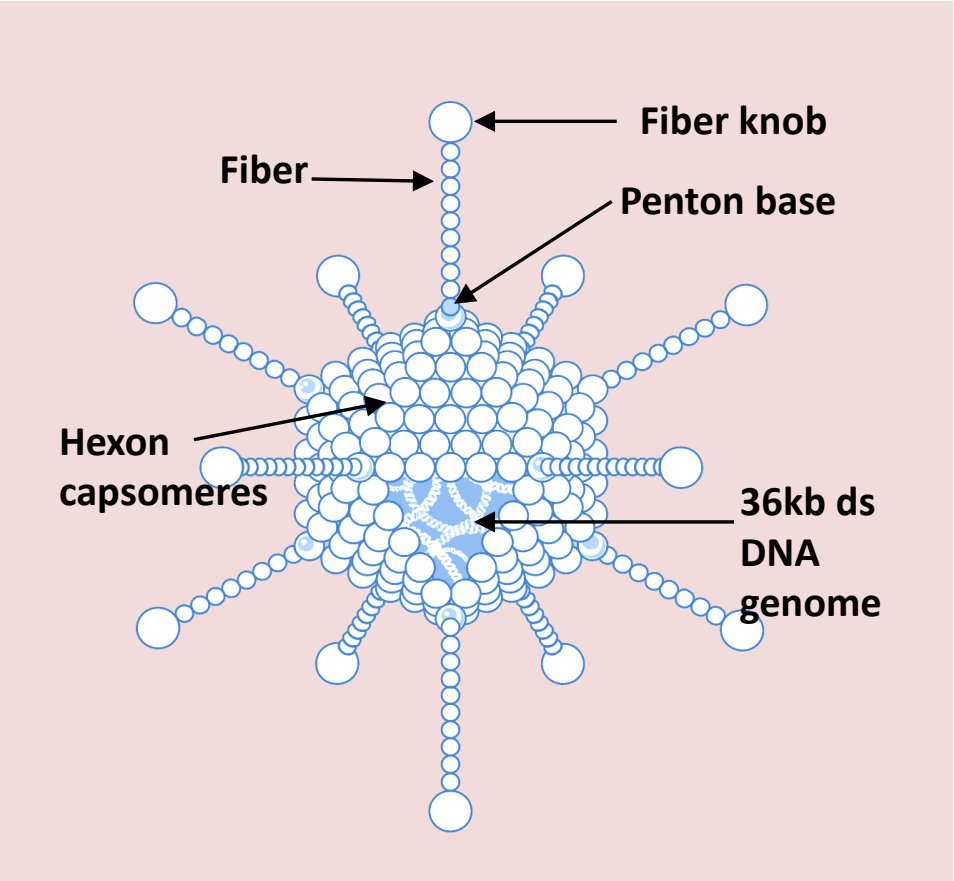

Figure 1.9 Adenovirus structure
Ad viruses contain a linear double stranded DNA genome (36kb). Their capsid is composed of 240 hexon capsomeres, 12 penton bases, and 12 protruding fibers. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

In vitro, cell attachment occurs initially through the coxsackievirus and adenovirus receptor (CAR) which interacts with the fiber knob (Roelvink et al., 1999) (Figure 1.10 (1)). When delivered locally most tissues and cell types, including VSMC and ECs can be transduced very efficiently with Ad5 vectors, however levels of transgene expression correlate to CAR expression (Bergelson et al., 1997). Subsequent binding of an Arg-Gly-Asp (RGD) motif in the viral penton base to cellular integrins (αvß3 or αvß5) promotes endocytosis via clathrin coated pits (Figure 10 (2)) (Wickham et al., 1993). Acidification of the endosome releases the virion (Figure 10 (3-4))and the viral DNA is released to the host cell nucleus facilitating transgene expression (Figure 10 (5-6)) (Seth, 1994, Trotman et al., 2001).

After systemic delivery *in vivo*, coagulation factor X (FX) binds to hexon proteins in the Ad5 capsid. Ad5/FX complexes target Ad5 vectors for liver transduction via heparan sulphate proteoglycans (HSPGs), thus compromising efficacy of tissue-specific gene transfer (Waddington et al., 2008). For example, intravascular deliver of Ad5 vectors to non-human primates resulted in 95% liver transduction (Sullivan et al., 1997). However, FX interaction with Ad5 prevents neutralisation by the classical complement pathway (Xu et al., 2013).

An innate immune response to adenoviruses is pre-existing in most human subjects. Furthermore, in E1 deleted recombinant adenoviral vectors, while transcription of viral proteins is mostly attenuated, leaky expression can occur and this is thought to evoke immunogenic processes leading to the clearance of adenoviral vector transduced cells (Yang et al., 1994). *In vivo*, viral antigens presented by MHC I on transduced cells recognised by cytotoxic T cells (Yang et al., 1995). Moreover, if the transgene is not of the same species, then the product will also be recognised by the immune system as foreign (Tripathy et al., 1996). In summary, adenoviral vectors are effective tools for gene transfer and this transgene expression is transient.

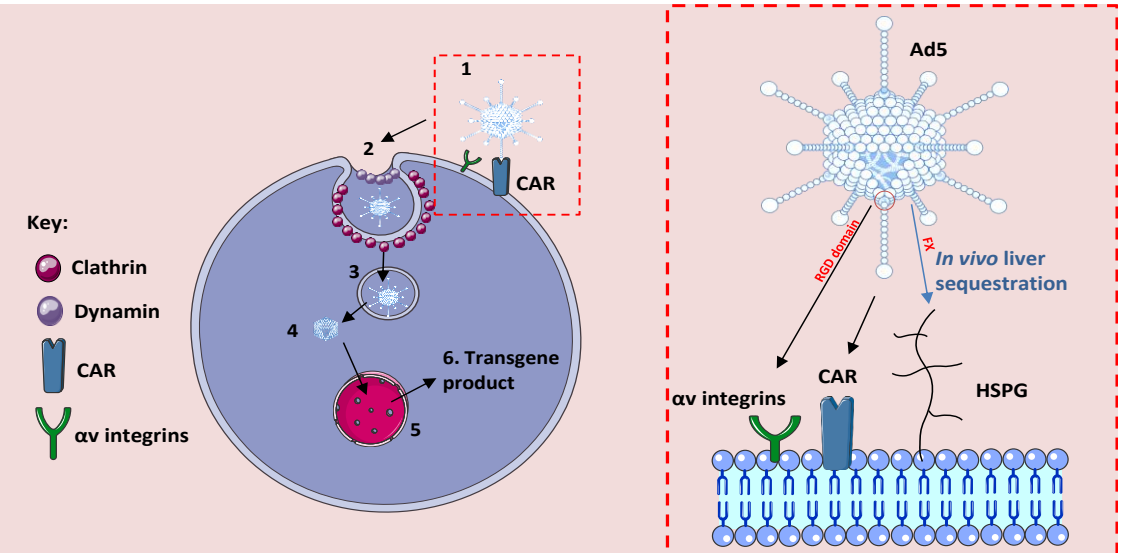

Figure 1.10 Ad vector internalisation by host cells
In vitro Ad vectors typically initially attach to host cells through the knob interacting with CAR. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.7.2 Cardiovascular gene therapy

The field of cardiovascular research has seen exciting developments in gene therapy with a plethora of promising pre-clinical trials. However, due to the complexities of pathophysiology and co-morbidities; gene therapy in CVD has made far slower progress on the clinical stage.

Patients with angina are often prescribed medical therapy or revascularisation surgeries. However, 5-10% of these patients will develop refractory angina, therefore gene therapy strategies are being investigated as a new treatment for these patients (Henry et al., 2014). One strategy is to promote the generation of new blood vessels (angiogenesis) within the myocardium to resupply the ischaemic area (Ferrara and Alitalo, 1999). Vectors encoding genes related to these processes have been investigated in the clinic. Different forms of vascular endothelial growth factor (VEGF) have been studied extensively. The REVASC trial recruited 67 patients with refractory angina to be administered Recombinant adenovirus (RAd)-VEGF-A or placebo in an attempt to improve myocardial perfusion (Stewart et al., 2006). Symptoms of angina as determined by an exercise test (time to ST depression) was significantly decreased in patients treated with RAd VEGF-A (Stewart et al., 2006). The NORTHERN (NOGA angiogenesis Revascularization Therapy: assessment by RadioNuclide imaging) trial tested plasmid mediated delivery of VEGF A in 93 patients with ischaemic heart disease but failed to show any beneficial outcomes as evaluated by myocardial perfusion or exercise stress tests (Stewart et al., 2009). In a related ischaemic pathology, intramuscular delivery of RAd VEGF-A in the RAVE (Regional Angiogenesis with Vascular Endothelial growth factor in peripheral arterial disease) trial for patients with peripheral artery disease also had no therapeutic effect (Rajagopalan et al., 2003). The RAVE trial recruited 105 patients to be randomly assigned a placebo dose or twenty intramuscular injections of RAd VEGF-A. Exercise tests and questionnaires identified RAd VEGF-A did not improve quality of life (Rajagopalan et al., 2003).

VEGF-D adenoviral gene therapy is currently being investigated in human subjects and has been shown to be well tolerated, increase ejection fraction after one year (a phase IIa trial) (Hartikainen et al., 2017). Twenty-four patients with refractory angina were treated with RAd-VEGF-D and six patients received a

placebo dose. One year after treatment, myocardial perfusion was assessed and indicated that patients treated with RAd-VEGF-D experienced higher rates of myocardial perfusion (Hartikainen et al., 2017). 180 patients with refractory angina who are not suitable candidates for revascularisation surgeries are soon to be recruited to expand this trial (*ClinicalTrials.gov*: NCT03039751).

The other major gene investigated for transfer into the myocardium is sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) which mediates intracellular calcium handling by cardiomyocytes. SERCA2a is downregulated during ischaemic heart failure and over expression via viral mediated gene transfer (AdSERCA2) restored cardiac function in a mouse model of heart failure (Kho et al., 2012, Miyamoto et al., 2000). The Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID) trial utilised local delivery of an AAV encoding SERCA reported the safety profile of the therapy in patients with advanced heart failure (Jessup et al., 2011). Some benefit was reported in this initial trial with patients receiving AAV SERCA2a spending less mean time (>3 days) in hospital in the following year (Jessup et al., 2011). The subsequent phase IIb trial (CUPID2) was the largest gene transfer study to date for patients with heart failure, with 123 patients treated with AVV SERCA2a and 127 patients treated with a placebo (Greenberg et al., 2016). The primary endpoint in this study was heart failure related readmission to hospital and secondary end points were terminal events. Exploratory endpoints included N-terminal pro-B-type natriuretic peptide (BNP) levels and exercise tests. Disappointingly, this larger scale trial provided no evidence of the treatment (AVV SERCA2a) being clinically beneficial (Greenberg et al., 2016).

The authors postulate that these negative results observed in CUPID2 may have been a result of differences in patient population as well as efficiency of transgene expression (Greenberg et al., 2016). For example, some patients who received AVV SERCA2a were deemed to have marginally more advanced or symptomatic HF than those who received the placebo dose (Greenberg et al., 2016). Differences in the AAV product delivered in the CUPID trials included the proportion of empty capsids. A lower number of empty capsids were present in the product used in CUPID1, therefore transgene expression in CUPID2 may have

been compromised if the product was less potent (Greenberg et al., 2016). Induction of transgene expression to therapeutic levels in heart failure patients is a key challenge in this field. Furthermore, elevated brain natriuretic peptide (BNP) levels were included in the selection criteria for CUPID2 but not CUPID1. BNP has been shown to inhibit SERCA2 expression (Toischer et al., 2010, Toischer et al., 2008, Kogler et al., 2006). This aspect of the CUPID2 clinical trial design has been criticised and thought by some to possibly explain the negative results (Zhai et al., 2018). Therefore, some of the setbacks experienced in the field of CVD gene therapy may not be as 'black and white' as they appear.

1.7.3 Gene therapy in CABG

CABG offers a unique opportunity to treat the venous graft *ex vivo*. In the operating theatre there is a surgical window of around 30 minutes, between removal of the patient's SV and its implantation into the coronary vasculature (Figure 1.11) (Wan et al., 2012).

The PREVENT investigators performed extensive characterisation of a decoy antisense oligonucleotide against E2F to inhibit VSMC proliferation in the setting of vascular injury. Early preclinical studies in rats were promising (Morishita et al., 1995). Sprague Dawley rats were subject to carotid artery wire injury surgery, following which E2F antisense oligonucleotides (edifoligide) were locally delivered into the vessel lumen. This single treatment attenuated neointima formation and this effect was sustained to 8 weeks after wire injury surgery (Morishita et al., 1995). In a related pathology, edifoligide was shown to prevent neointima formation in cardiac transplant tissue in non-human primates (Kawauchi et al., 2000). Edifoligide also inhibited neointima formation in a porcine model of balloon angioplasty (Nakamura et al., 2002).

Edifoligide was utilised in a series of clinical trials to test its effect on vein graft neointimal hyperplasia following CABG and related surgeries such as peripheral vein grafting which is performed on patients with critical limb ischaemia. The first PREVENT trial assessed the effect of *ex vivo* gene transfer of E2F decoy to human infrainguinal vein grafts (Mann et al., 1999). PREVENT I recruited 41 patients that randomly received either placebo (17) or edifoligide (16).

Edifoligide reduced vein graft stenosis and requirement for repeat revascularisation at 12 month follow up (Mann et al., 1999).

PREVENT II recruited 200 patients due to receive a CABG with SV (PREVENT-II, 2001). Vein graft stenosis was assessed by intravascular ultrasound post-operatively after 12 months. Vein graft stenosis was reduced by 30% in patients that received edifoligide as compared to placebo treated patients (PREVENT II, 2001).

PREVENT III recruited 1404 patients with critical limb ischaemia however a one year follow up study suggested that edifoligide had no effect on graft patency as compared to the placebo group (Conte et al., 2006).

The PREVENT IV trial recruited over 3000 patients with presentations of CHD (Lopes et al., 2012, Alexander et al., 2005, Mann and Conte, 2003). This was employed in the first and only large-scale clinical trial to assess the effect of gene therapy to prevent VGF. Edifoligide was deemed safe and effective in downregulating E2F. Edifoligide decreased DNA replication, c-myc and PCNA expression in HSVs by approximately 70% (Mann and Conte, 2003). Disappointingly this had no effect on long term outcomes of CABG procedures, with no reduction in repeat interventions 12 to 18 months after treatment (Alexander et al., 2005). Edifoligide also displayed no beneficial effect when the rate of mortality, MI, revascularisation and rehospitalisation were compared to placebo groups (Lopes et al., 2012). This emphasises the need for new strategies to inhibit VGF. Although unsuccessful, valuable information continues to be mined from these trials. For example, Hess et al., directly linked factors such as surgery duration with poorer outcomes (Hess et al., 2014). Furthermore, Harskamp et al., found that intraoperative preservation of the vein grafts in buffered saline solution was associated with a lower rate of VGF than venous grafts that were preserved in a blood-based solution (Harskamp et al., 2014).

It is noteworthy that antisense oligodeoxynucleotides have also failed in clinical trials for related pathologies such as ISR . The investigation Thoraxcenter of antisense DNA using local delivery and IVUS after coronary stenting trial tested targeted suppression of c-myc via antisense knockdown but did not reduce neointima formation following PCI (Kutryk et al., 2002). When restenosis rates in

77 patients were quantified by follow up angiography there was no difference between patients treated with a placebo and those treated with oligodeoxynucleotides against c-myc (Kutryk et al., 2002).

Importantly, new gene therapies are still being actively investigated. Early studies reported human SVs could be effectively transduced with a reporter adenoviral vector resulting in high-level transgene expression (*LacZ*) in both venous ECs and VSMCs after 14 days (George et al., 1998). Directly treating grafts in this way prevents systemic exposure of the patient to viral particles thus improving the safety profile of therapy. Additionally, transgene expression is transient due to immune responses directed against adenoviral proteins in infected cells, this prevents possible complications caused by overexpression of a gene over a long period of time (Newman et al., 1995). In the context of CABG this could be advantageous as gene therapy can satisfy an early therapeutic window to prevent VGF whilst having long lasting effects, an effect first seen in preclinical studies testing antisense oligonucleotides targeting PCNA (Morishita et al., 1993). Otherwise, if required the duration of transgene expression can be extended to approximately three months via the use of 'gutless' adenoviral vectors that provoke less potent humoral immunogenic processes in the vessel wall (Wen et al., 2004). Therefore, adenoviral vectors could be promising tools for use in coronary artery interventions.

A key issue in developing gene therapy for VGF is finding the right candidate gene for delivery. Experimental gene therapy studies have investigated the transfer of genes that prevent thrombosis, EC dysfunction, inflammation and VSMC proliferation/migration (see table 1.1). Probably the most promising target for use in CABG is TIMP3. For the past 20 years adenoviral mediated delivery of TIMPs 1-3 have been extensively investigated as a strategy to inhibit neointima formation. In early studies, RAdTIMP1 inhibited HSVSMC migration in an *ex vivo* organ culture model (George et al., 1998). To this end RAdTIMP1 inhibited neointima formation over 14 days while having no significant effect on VSMC proliferation or apoptosis (George et al., 1998). RAdTIMP3 was later demonstrated to inhibit formation of neointimal lesions by approximately 84% in the 14 day *ex vivo* organ culture (George et al., 2000). RAdTIMP3 also inhibited neointima formation 28 days after vein grafting in a porcine model, this effect

was associated with *both* inhibition of migration and promotion of vascular VSMC apoptosis (George et al., 2000, Baker et al., 1998). Baker et al., previously delineated the subtle differences between TIMP1-3: soluble and viral mediated delivery of TIMP-3 promoted VSMC apoptosis and inhibited migration while TIMP1 had no effect on proliferation and TIMP2 inhibited proliferation and migration but had no effect on apoptosis *in vitro* (Baker et al., 1998). *In vivo*, RAdTIMP2 did not affect VSMC apoptosis and had no effect on neointima formation thus demonstrating the need for a dual inhibitory effect on migration and proliferation for maximal therapeutic benefit (George et al., 2000). Importantly, RAdTIMP3 transduced vein grafts exhibited sustained attenuation of neointima formation three months after grafting following local adenoviral-mediated delivery of TIMP-3, despite the transient nature of adenoviral gene transfer (~30 days) (George et al., 2011). This research is now being pursued at the clinical stage with patients being recruited for phase I (12 patients) and phase II (120-160 patients) (Yla-Herttuala and Baker, 2017).

Target	Gene	Vector	Model	Effect	Citation
Thrombosis	Hirudin	Adenovirus	Porcine balloon angioplasty	Inhibited mural thrombus formation	(Meyer et al., 1994)
Thrombosis	Hirudin	Adenovirus	Porcine balloon angioplasty	Inhibited NI formation by 35%	(Rade et al., 1996)
EC function	eNOS	Adenovirus	Rabbit carotid balloon angioplasty	Inhibited NI formation following balloon angioplasty	(Kullo et al., 1997)
EC function	eNOS	Adenovirus	Swine coronary angioplasty	Inhibited NI formation follow coronary angioplasty	(Varenne et al., 1998)
Inflammation	7ND (MCP-1 inhibitor)	Adenovirus	Canine vein graft model	Inhibited NI formation at 4 weeks	(Tatewaki et al., 2007)
Inflammation	CC-Chemokine inhibition (35K) and TIMP1	Adenovirus	Rabbit vena cava into carotid artery interposition	Reduction in NI at 4 weeks	(Turunen et al., 2006)
Inflammation	35K	Adenovirus	Vena cava into carotid artery interposition [ApoE$^{(-/-)}$ mice]	Reduction in NI and SMC content at 2 weeks	(Ali Ziad et al., 2005)
VSMC	PTEN	Adenovirus	Canine aortocoronary bypass	Reduction in NI at 3 months	(Hata et al., 2005)
VSMC	p53	Adenovirus	Human saphenous vein organ culture system	Reduction in NI 68% at day 7	(Wan et al., 2004a)
VSMC	p53	Adenovirus	Porcine saphenous vein carotid interposition vein graft model	Reduction in NI at three months after vein graft	(George et al., 2001)
VSMC	TIMP3	Adenovirus	Porcine saphenous vein carotid interposition vein graft model	58% reduction in NI at 28 days	(George et al., 2000)
VSMC	TIMP3	Adenovirus	Porcine saphenous vein carotid interposition vein graft model	Inhibited NI formation at 3 months	(George et al., 2011)

Table 1.1 Adenoviral gene therapy strategies employed in pre-clinical studies to inhibit neointima formation (NI)

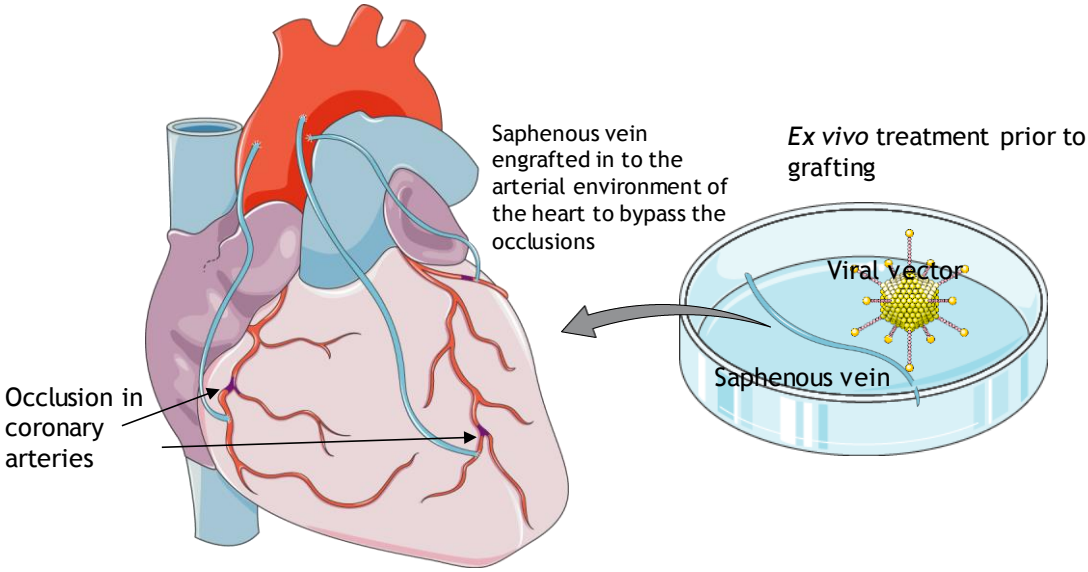

Figure 1.11 Gene therapy to prevent vein graft failure
Coronary Artery Bypass Graft (CABG) procedures are well suited to gene therapy as the venous graft can be treated in the operating theatre before transplantation. Ex vivo luminal delivery of adenoviral vectors transduced both the endothelium and the underlying smooth muscle. Effective transduction can be achieved in thirty minutes intraoperatively. Saphenous veins can be washed in saline solutions to remove any residual viral particles. Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.7.4 Gene therapy as a novel tool to counter-regulate the RAS

1.7.4.1 Antisense oligonucleotides

Early gene therapy studies aimed to manipulate the RAS using antisense oligonucleotides that bind to mRNA strands that encode members of the classical RAS such as AT_1R and ACE. Administration of the oligonucleotides *in vivo* have been reported to decrease experimental hypertension (Wielbo et al., 1996) and this is still an active field of research (Uijl et al., 2019).

In the late 1990s, viral vectors encoding antisense oligonucleotide were used in a number of studies to target the AT_1R to attenuate hypertension in the SHRSP (Iyer et al., 1996, Gelband Craig et al., 1999, Tang et al., 1999). AAV vectors have also been utilised to deliver AT_1R antisense therapy to reduce blood pressure (Phillips et al., 1997). Local gene delivery to the hypothalamus or lateral ventricles via intracranial injection decreased blood pressure in SHRSP rates over a period of nine weeks (Phillips et al., 1997).

The next advancement arrived upon the discovery of the alternative counter-regulatory axis of RAS. With the exception of alamandine and its receptor MrgD, gene therapy has been used successfully introduce counter-regulatory members of the RAS. This can be achieved in a number of ways utilising different viral vectors or plasmids that encode either non-secreted, secreted or cleaved products. Similarly, the mechanism and route of delivery can be adapted according to the pathology.

1.7.4.2 Gene transfer of ACE2

Viral vectors have been designed to overexpress a membrane bound and a secreted form of ACE2 (Huentelman et al., 2004). Viral vectors that encode for a membrane bound ACE2 have been tested the most thoroughly. In preclinical models of atherosclerosis local adenoviral-mediated delivery of ACE2 to the aorta inhibited formation of atherosclerotic lesions by suppressing infiltration of immune cells via MCP-1 downregulation as well as inhibiting VSMC proliferation and migration (Zhang et al., 2010). Moreover, Ad-ACE2 increased stability of atherosclerotic plaques in a rabbit model of atherosclerosis (Dong et al., 2008).

Adenoviral and AAV mediated delivery of ACE2 inhibited fibrotic processes in the liver and heart respectively (Mak et al., 2015, Ma et al., 2017).

Counter-regulating the RAS has also been pursued as a strategy to reduce inflammation. For example, lentiviral delivery of ACE2 inhibited MCT induced pulmonary hypertension and simultaneously inhibited the increases in pro-inflammatory cytokines normally induced in this model of PAH (Yamazato et al., 2009). Furthermore, intraocular delivery of AAV-ACE2 was therapeutic in a murine model of uveitis (Qiu et al., 2016). Intraocular delivery of AAV-ACE2 also improved condition of mice with diabetic retinopathy again through inhibiting inflammatory processes (Verma et al., 2012, Dominguez et al., 2016). In other murine models of diabetes adenoviral mediated delivery of ACE2 was reno-protective (Liu et al., 2011) and improved glycaemic control one week after intra-pancreatic injection (Bindom et al., 2010). These preclinical studies show the breadth of different pathologies that therapeutic delivery of ACE2 can treat.

1.7.4.3 Gene transfer of angiotensin receptors

The receptor members of the counter regulatory RAS are also candidates for gene therapy. Viral vectors used to overexpress the AT_2R have been effective in curbing cardiac pathologies. For example, intracardiac delivery of lenti-AT_2R decreased cardiac hypertrophy as determined by left ventricular wall thickness in the SHRSP (Metcalfe Beverly et al., 2004). The same approach inhibited hypertensive cardiac remodelling after Ang II infusion in Sprague-Dawley rats (Falcon et al., 2004). In both the aforementioned studies viral vector-mediated overexpression of the AT_2R did not affect blood pressure, suggesting it was a direct effect of preventing adverse tissue remodelling.

1.7.4.4 Gene transfer of angiotensin peptides

In order to study tissue-specific effects of angiotensin peptides rather than circulatory effects, an expression cassette was designed that encoded a fusion protein consisting of a prorenin signal peptide (to ensure secretion), a Fc portion of mouse IgG (providing molecular mass), a human prorenin prosegment (as a molecular spacer) a furin protease cleavage site and finally a cleavable angiotensin peptide (Figure 1.12A) (van Kats et al., 2001a, Methot et al., 1997).

Utilisation of this expression cassette in transgenic mice allowed for testing the effect of local expression as well as constitutive systemic exposure to raised concentrations of various angiotensin peptides (van Kats et al., 2001a, Takayanagi et al., 2012, Botelho-Santos et al., 2007).

The expression cassette was also adapted to encode the counter-regulatory peptide Ang-(1-7) (Ferreira et al., 2006, Santos et al., 2004). Transgenic expression by random integration of the construct produced a rat strain [TGR(A1–7)3292] which expressed and secreted Ang-(1-7) from the testes (Santos et al., 2004) and has been reported to raise systemic concentrations of Ang-(1-7) in order to confer cardio-protection (Ferreira et al., 2006, Senger et al., 2018). Flores-Munoz and colleagues then developed adenoviral vectors that encoded furin-cleavable Ang-(1-7) or Ang-(1-9) (Flores-Muñoz et al., 2012). Adenoviral-mediated gene transfer of Ang(1-7) or Ang-(1-9) into cardiomyocytes successfully inhibited Ang II induced hypertrophy (Flores-Muñoz et al., 2012). Subsequently AAV9-mediated delivery of Ang-(1-9) was utilised in a murine model of MI (Fattah et al., 2016). A single I.V delivery of AAV-Ang-(1-9) decreased the frequency of death by cardiac rupture one week after MI, moreover, the gene therapy improved systolic cardiac function up to eight weeks after MI (Fattah et al., 2016).

To date, no studies have assessed the effect of viral vector mediated gene transfer of Ang-(1-9) in mouse models of neointima formation. It is therefore unknown whether this peptide could be a therapeutic candidate for gene therapy in CABG. The research presented in this thesis aimed to assess the suitability of Ang-(1-9) as a candidate gene transfer approach in CABG by characterising the effect of Ang-(1-9) and adenoviral mediated delivery of Ang-(1-9) in human vascular cell *in vitro* models and murine *in vivo* models of neointima formation.

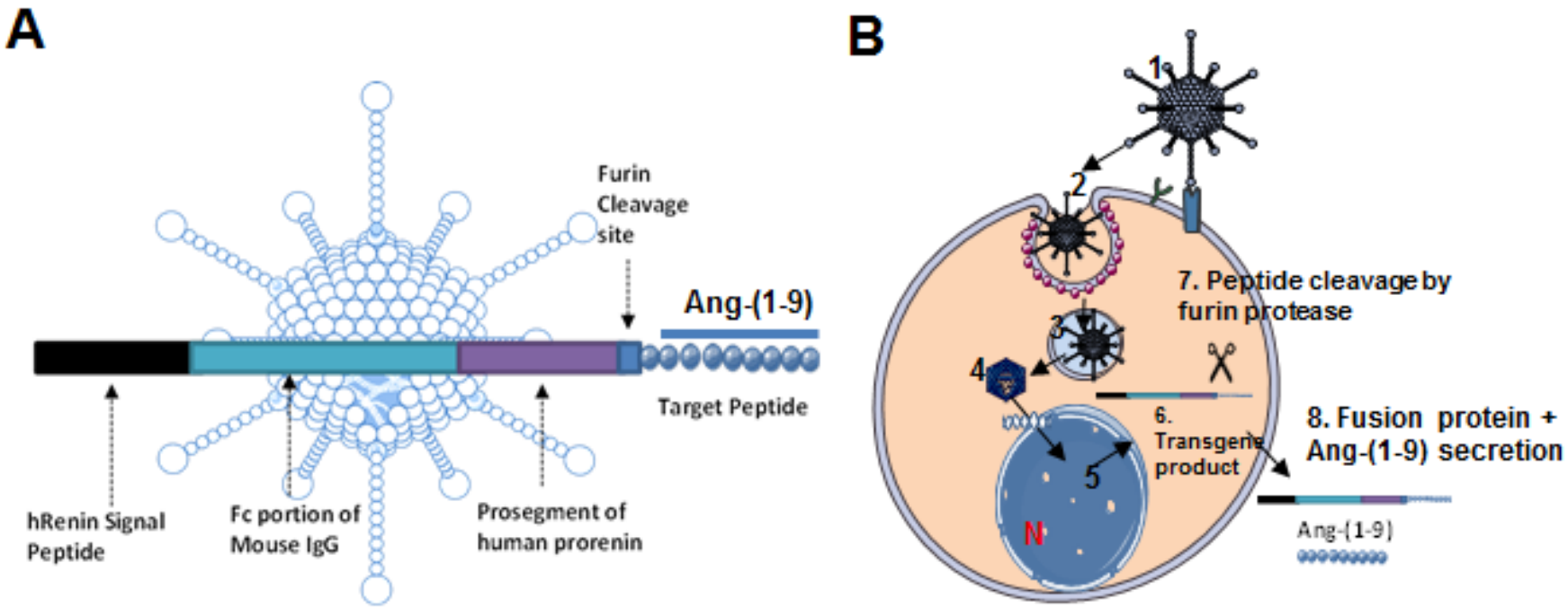

Figure 1.12: Expression cassettes to over express furin-cleavable angiotensin peptides
RAdAng-(1-9) encodes a fusion protein composed of a renin signal peptide to direct it to the secretory pathway, a mouse immunoglobulin (IgG) to provide molecular mass to prevent degradation and an Ang1-9 peptide that is cleavable by furin protease (an enzyme located at the end of the secretory pathway). (B) *In vitro*, ad5 vectors initially attach to the host cell through the fiber knob interacting with CAR. Subsequent binding of the RGD to αv integrins promotes the formation of clathrin coated pits and endocytosis[1-3]. Acidification of the endosome releases the virion[3-4]. The Ad genome then enters the host cell nucleus facilitating transcription and transgene expression5-6. In the case of cells transduced with RAdAng-(1-9), furin protease then cleaves Ang-(1-9) from the fusion protein and the products are secreted (7-8) (Flores-Muñoz et al., 2012) Image created using Servier Medical Art available at https://smart.servier.com. No permission required.

1.8 Hypothesis

Ang-(1-9) can be utilised to inhibit VSMC proliferation and migration as well as neointima formation in mice.

1.9 Aims

1. Characterise the effect of soluble Ang-(1-9) peptide infusion on arterial vascular remodelling in the murine model of neointima formation through cessation of blood flow by carotid ligation.

2. Characterise the effect of soluble Ang-(1-9) peptide on PDGF-B-mediated human saphenous vein SMC migration and proliferation *in vitro*.

3. Characterise adenoviral mediated gene transfer of Ang-(1-9) on human saphenous vein SMC and EC *in vitro*.

4. Characterise the effect of adenoviral mediated delivery of Ang-(1-9) on neointima formation in a murine wire injury model.

2 Chapter 2 Materials and Methods

2.1 Ethics statement

Vascular smooth muscle cells were isolated and maintained from the surplus human saphenous veins of male and female CABG patients. All experiments using human tissue were approved by the West of Scotland Research Ethics Committee 4 (ref: 10/S0704/60) and conducted in alignment with the principles defined in the Declaration of Helsinki.

2.2 Cell culture

2.2.1 Cell culture growth media for HSVSMC

Isolated human saphenous vein smooth muscle cells (HSVSMC) were maintained in Smooth Muscle Cell Growth Medium 2 (Cat: 39262, Promo Cell, Germany) supplemented with 10% Foetal Bovine Serum (FBS) (Cat: 10500064 Invitrogen CA, USA), L-glutamine (2mM) (Cat: 25030-024, Invitrogen CA, USA), Penicillin (100 units/mL) and Streptomycin (100 µg/mL) (Cat: P0781, Sigma Aldrich, MI, USA). Cells were incubated at 37^0C, 5% CO_2.

2.2.2 Cell culture growth media for HEK 293 and HepG2

Human Embryonic Kidney (HEK) 293 and HepG2 cells were cultivated in Minimal Essential media (MEM) (Life Technologies Cat: ZLMEM1) supplemented with 10% Foetal Bovine Serum (FBS) (Invitrogen Cat: 10500064), sodium-pyruvate (0.5mM) (Cat: S8636 Sigma Aldrich, MI, USA), L-glutamine (2 mM) (Invitrogen Cat: 25030-024), Penicillin (100 units/mL) and Streptomycin (100 µg/mL) (Cat: P0781 Sigma Aldrich, MI, USA). Cells were incubated at 37^0C, 5% CO_2.

2.2.3 Cell culture growth media for HSVEC

Endothelial Cell Medium MV (Cat: C-22020, Promocell, Germany) supplemented with 10% FBS, L-glutamine (2 mM), Penicillin (100 units/mL) and Streptomycin (100 µg/mL).

2.2.4 Cell culture media for quiescence

Prior to serum starvation, vascular cells were washed twice in warm PBS. HSVSMC and HSVEC were then place in 0% or 0.5% FBS supplemented DMEM

GLUTAMAX™ (Cat: 21885-025, Invitrogen, CA, USA), sodium-pyruvate (0.5mM) Penicillin (100 units/mL) and Streptomycin (100 µg/mL).

2.2.5 Cryopreservation

When necessary cells were placed in long term storage by cryopreservation. Cells from one confluent T75 cm^2 flask were detached through incubation in trypsin/EDTA for 5 minutes and subject to centrifugation at 460 x g for 5 minutes at RT. The cell pellet was then re-suspended in 1 mL of full culture media (cell type dependent) containing 10% dimethyl sulfoxide (DMSO) and frozen to -80 ^oC in a Mr. Frosty container (Thermo Fisher Scientific, MA, US) at a rate of -1 ^oC/minute. Frozen cells were then transferred to liquid nitrogen for long-term storage.

2.2.6 Collection of conditioned tissue culture supernatant from HepG2 cells

HepG2 ($1x10^6$ cells/well) were transduced with RAdAng-(1-9) at a range of MOIs and incubated in serum free DMEM for 48 hours (100-1000 VP/cell). Conditioned culture medium was added to 10 kDa Amicon Ultra-15 Centrifugal Filter Unit (Cat: UFC901008, Merck Millipore, MA, USA) and subject to centrifugation according to manufacturer's instructions. Conditioned culture media were then stored at 4 ^oC until use.

2.3 Proliferation Assays

2.3.1 Bromodeoxyuridine incorporation assay

HSVSMC ($1x10^4$ cells per well of a 96-well plate) were plated in complete growth medium. The next day, HSVSMC were washed twice in warm phosphate buffered saline (PBS) (Cat: 14190086 Invitrogen, CA, USA). HSVSMC were then incubated for 72 hours (37 ^0C, 5% CO_2), after which the media was removed and cells were re-stimulated with 100 µL DMEM GLUTAMAX containing human recombinant PDGF-BB [20 ng/mL] (Cat: 220-BB, R&D Systems, MN, USA) in the presence or absence of Ang-(1-9) [0.2-1 µM] (Phoenix Pharmaceuticals, Inc. CA, USA) containing 20 µL of BrdU solution (1:500). Cells were incubated for a further 48 hours (37 ^0C, 5% CO_2) after which the manufacturer's instructions were followed

exactly (Cat: 2750 Merck Millipore, MA, USA). Cells were fixed and DNA denatured by 30 minutes incubation at RT with 100 µL manufacturer's fixing agent. Each well was then washed five times using manufacturer's wash buffer (1:50). Cells were incubated at 37 ^{0}C, 5% CO_2. Next,100 µL of pre-diluted anti-BrdU monoclonal antibody was added to each well and after 1 hr the wash step described above was repeated. Next, Goat anti-Mouse IgG, Peroxidase Conjugate (1:2000) was diluted in the supplied diluent and filtered through a 0.22 µm Sterile Syringe Filter (Cat: 15206869, Fisher Scientific, NH, USA) and 100 µL/well was added and plate(s) incubated at RT for 30 minutes. Next the wash step described above was repeated followed by a final 10 second wash in distilled water and 50 µL of TMB Peroxidase substrate added and the plate protected from light for 30 minutes at RT. In this reaction BrdU positive wells turn blue and the intensity of which directly correlates with BrdU incorporation in proliferating cells. Lastly 50 µL of stop solution was added to each well. This changed the colour of wells from blue to yellow and plates were measured by exciting at 450 nM using a Victor 2 spectrometer (Perkin Elmer, MA, USA).

2.3.2 The MTS Assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Cat: G3582, Promega, WI, USA) was performed to investigate the effect of adenoviral mediated transfer of Ang-(1-9) on HSVSMC growth. Briefly, 5x10^3 cells were plated in 200 µL complete culture medium in 96 well plates and incubated over night at 37 ^{0}C, 5% CO_2. Cells were then serum starved for 48 hours, before re-stimulation by exposure to DMEM GLUTAMAX containing either 5% FCS, PDGF-BB, in the presence or absence of Ang-(1-9) (See table 2.1). This was followed by a further incubation step for 48 hours. Next, 20 µL MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was then added to total volume of 100 µL in each well as per manufacturer's instructions and read at absorbance 490 nm using a Victor 2 spectrometer.

Agonist	Stock concentration	Final concentration	Company
Angiotensin-(1-9)	1 mM	200-1000 nM	Phoenix Pharmaceuticals
Angiotensin-II	1 mM	200 nM	Sigma Aldrich
Recombinant PDGF-BB	20 µg/mL	20 ng/mL	R & D systems

Table 2.1 List of peptides and cytokines used in *in vitro* experiments.

2.4 Migration Assays

2.4.1 The Scratch Assay

The scratch assay was used to assess directional migration of HSVSMC (Liang et al., 2007). HSVSMC ($1x10^5$ cells per well) were seeded in a 12 well plate in complete SMC media and incubated overnight at 37 ^{0}C, 5% CO_2. Cells were then washed twice in PBS and serum starved in quiescence medium for 72 hours. A vertical scratch was performed down the cell monolayer using a P200 pipette tip. Cells were then washed in PBS and media replaced with serum free media (0.2% serum) containing PDGF-BB (20 ng/mL) in the presence or absence of soluble Ang-(1-9) peptide (0.2-1 μM). EVOS Cell Imaging System was used to image the scratch immediately after it was performed and 24 hours later. Images were taken at 3 points per well. Using ImageJ a horizontal line grid was set at 22350 pixels^2 per point in order to measure 10 different locations across the scratch (Figure 2.1; cyan). The migratory distance was then measured using ImageJ software and the 0 hour and 30 hour time points (post-scratch) compared and expressed as a percentage (Figure 2.1).

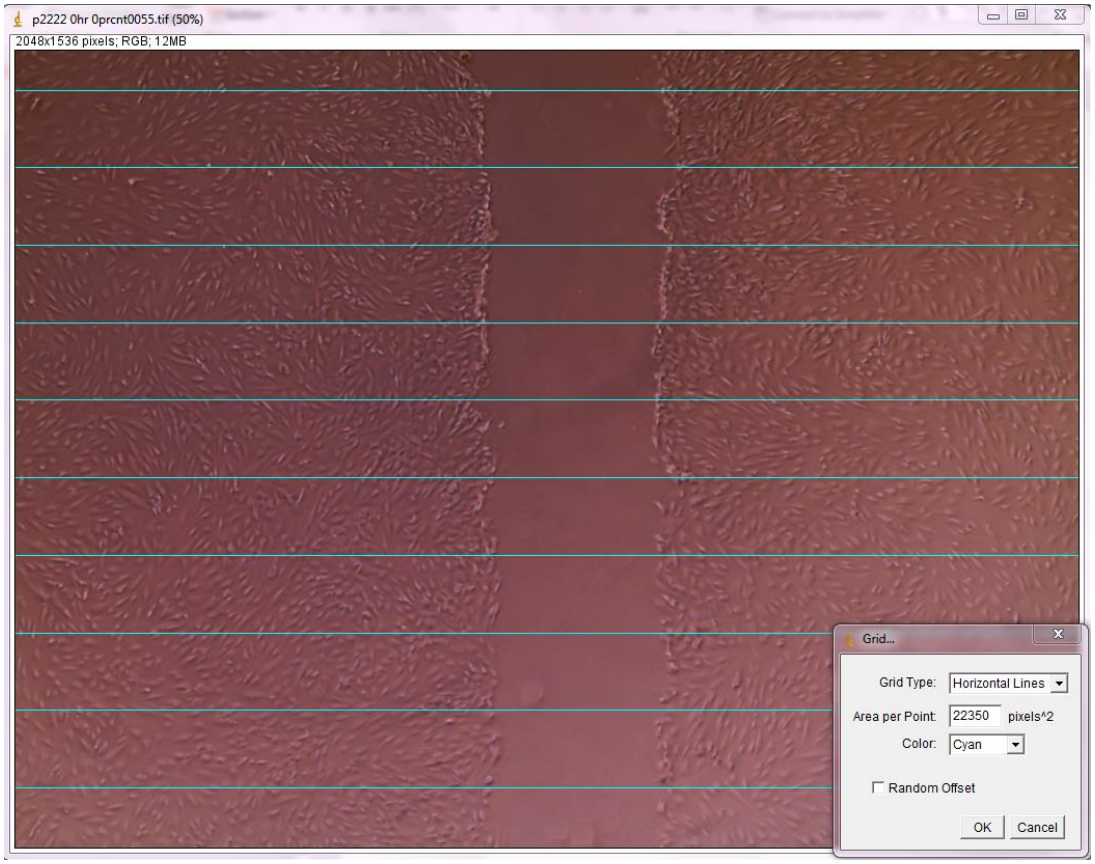

Figure 2.1 Analysis of scratch wound assay using ImageJ. Horizontal grid used to select points of measurement seen in cyan.

2.4.2 The Boyden Chamber Assay

The Boyden chamber assay was used to assess HSVSMC chemotaxis (Chen, 2005). Confluent (70%) HSVSMC in T75 cm^2 flasks were washed twice in PBS and serum starved in quiescence medium for 48 hours. At the 48 hour timepoint, 8 µm pore transwell inserts (Cat: 8734-0053 VWR, PE, USA) were equilibrated in quiescence medium. Next, $2x10^4$ HSVSMC in a volume of 500 µL serum free DMEM (+/- Ang-(1-9) [1 µM]) were plated in an 8 µm pore transwell insert in 12 well plates and 1.5 mL of culture media containing 20 ng/mL PDGF-BB in the presence or absence of Ang-(1-9) (1 µM) was added to the wells to investigate chemotaxis-induced migration. After 24 hours the Boyden chamber inserts were fixed using 4% PFA (10 minutes). At this stage the non-migrated cells on the upper side of the insert were removed using cotton wool buds. The inserts were washed in PBS three times. The membranes were carefully cut out of the inserts and mounted bottom side up (migrated cells) using ProLong™ Gold Antifade Mountant with DAPI (Cat: P36931, Thermo Fisher Scientific, MA, US). Migrated cells were then imaged by confocal microscopy. 10 images per insert were taken at random, excluding the outermost parts of the circular membrane (Figure 2.2).

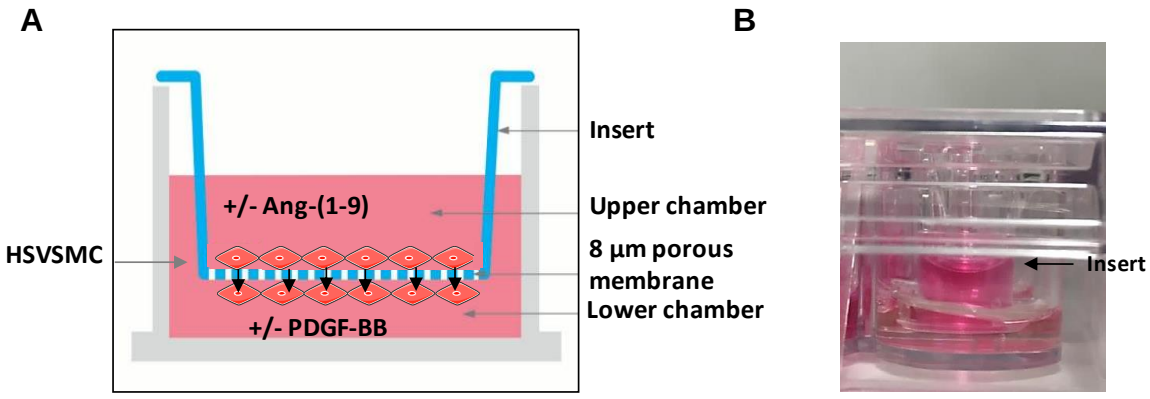

Figure 2.2 The boyden chamber assay. Schematic representation of Boyden chamber assay (Chen, 2005) as a method to investigate chemotaxis driven HSVSMC migration.

2.5 Molecular Biology

2.5.1 Extraction of RNA from HSVSMC

HSVSMC were plated at a seeding density of $3x10^5$ cells per well in 6-well plates and quiesced for 72 hours. Cells were lysed using 500 µL QIAzol (Cat: 79306 Qiagen, Netherlands) and lysates stored at -80ºC for at least 24 hours Total RNA was extracted using the miRNeasy Mini Kit according to manufacturer's instructions (Cat:217004 Qiagen, Netherlands).

2.5.2 Extraction of RNA from Mouse Aorta

The thoracic aorta was dissected from C57 BL/6 mice and snap frozen in liquid nitrogen before being stored at -80 ºC. Aortae were then lysed and disrupted in 500µL QIAzol (Cat: 79306, Qiagen, Netherlands) using TissueLyser II (Qiagen, Netherlands). Total RNA was extracted using the miRNeasy Mini Kit according to manufacturer's instructions (Cat: 217004 Qiagen, Netherlands).

2.5.3 Nucleic acid quantification

RNA and DNA were quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). 1 µL of RNA or DNA was loaded onto the lower optical pedestal. A liquid column between the upper and lower optical pedestal is created when the upper arm of the NanoDrop™ 1000 is lowered. Xenon light is passed through the sample at 260 nm. The absorption of the light through the sample is measured by a photon detector called at Charge Coupled Device (CCD). The concentration [C] of nucleic acid is then calculated using the Beer-Lambert equation: where the absorbance measured at 260 nm [A] is multiplied by the wavelength dependent extinction coefficient (ε) and divided by the pre-set distance between the two pedestals [b]. The 260/280 nm absorbance ratio was used as a measure of purity (Gallagher and Desjardins, 2006).

$$C = \frac{A \times \varepsilon}{b}$$

2.5.4 Reverse Transcriptase Polymerase Chain Reaction

First, 750 ng of murine aortic RNA was diluted in Nuclease-Free Water (Cat: 129115, Qiagen, Netherlands) in a 96 well PCR reaction plate (Cat: E1403-5200 Starlab, UK). The plate was then placed on a PTC-200 thermal cycler (Cat: 8252-30-0001 MJ Research, CA, USA) and RNA denatured for 10 minutes at 70 ºC. During this time a master mix was prepared as described in table 2.2. After 10 minutes 10 µL of master mix was added to each RNA sample. The plate was placed back onto the thermal cycler and the setting described in table 2.2 selected. Incubation at 25 ºC for 10 minutes allowed primer binding (random hexamers). Incubation for 1 hour at 42 ºC allowed promoted elongation of strands via reverse transcriptase activity. Incubation at 72 ºC for 15 minutes was used to inactivate reverse transcriptase (Table 2.3). Lastly the PCR plate was taken off the thermal cycler and reverse transcribed cDNA was diluted with a further 130 µL of nuclease free water before being stored at -20 ºC.

Reagent	Manufacturer	CAT	1 X Reaction
5X Superscript buffer (SII)	Invitrogen	18064022	4 µL
10 mM dNTPs	New England Bio labs	N0447S	1 µL
RNASEIN (RNAse inhibitors)	Promega	N2111	1 µL
Dithiothreitol (DTT)	Invitrogen	18064022	1 µL
Random Hexamers	Invitrogen	48190011	0.5 µL
SuperScript II Reverse Transcriptase	Invitrogen	18064022	1.5 µL
Nuclease Free Water	Qiagen	129115	1 µL

Table 2.2 Reagents and volumes used for reverse transcription polymerase chain reaction (RTPCR)

Step	Temperature (^{o}C)	Time (Mins)
RNA denaturation	70	10
Cooling	4	10
Primer binding	25	10
Elongation/strand transfer	42	60
Enzyme inactivation	72	17
Cooling	4	∞

Table 2.3 Thermal cycler settings used for RTPCR

2.5.5 Quantitative real time PCR

Gene expression was determined by Applied Biosystems™ TaqMan® chemistry. This method utilises forward and reverse primers specific to the target of choice as well as oligonucleotides labelled with a reporter fluorescent probe on the 5' end and a quencher probe at the 3'end. This quencher, reduces the fluorescence signal of the probe at the 5' end via fluorescence resonance energy transfer (FRET) (Cardullo et al., 1988). If the gene of interest is present in the cDNA then oligonucleotide will be at the 5' end. Next Taq polymerase will bind and cleave the fluorescent probe situated at the 5'end thus separating the reporter from the quencher probe which in turn increases the fluorescence signal. Consequently, as each PCR cycle is completed the fluorescence signal intensifies. This allows for real time analysis of the PCR product as it is generated. All TaqMan® Gene Expression Assays used 6-carboxyfluorescein (FAM) as the fluorescent reporter dye and 6 carboxytetramethylrhodamine (TAMRA) as a quencher. The endogenous housekeeping gene used was either glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for murine samples and 18S ribosomal RNA (*18S*) for human samples (see table 2.4).

To perform TaqMan® RTPCR a master mix containing the equivalent volume per well of 5 µL TaqMan Universal Master Mix II no UNG (Cat: 4440040, Invitrogen CA, USA), 2.5 µL of RNase free water and 0.5 µL of chosen probe. Next, 8 µL of master mix was pipetted into each well of a 384 well reaction plate and 2 µL of cDNA was added to each well allowing for 3 technical replicates per sample. The plate was sealed with an optical adhesive seal (Cat: 4311971, Applied Bio systems, MA, USA). Next the plate was subject to centrifugation at 1000g for 15 seconds at RT. The QuantStudio 12K Flex Real-Time PCR System (Cat: 4471087, Applied Bio Systems, CA, USA) was used to perform the quantitative real time polymerase chain reaction (qRTPCR) using the settings detailed in table 2.5 for 40 cycles. Temperatures increased or decreased by 1.6 ºC per second. Gene expression data was expressed as the delta cycle threshold (C) Higher gene expression is therefore represented as a lower ΔCt. Furthermore, with each increment the gene expression will double.

$$\Delta Ct = Ct_{(\text{test gene})} - Ct_{(\text{housekeeper})}$$

Gene	Assay ID
CCL2	Mm00441242_m1
IL1B	Mm00434228_m1
AGTR2	Mm013431373_m1
IL9	Mm00434306_m1
Col1a	Mm00801666_g1
Col3a	Mm01254476_m1
GAPDH	Mm999999915_g1
B2M	Mm0437762_m1
18S	Hs99999901_s1
PDGFRB	Hs01019589_m1

Table 2.4 List of Taqman gene expression assays

Stage	Time (Mins)	Temperature (°C)	No. of cycles
Hold Stage	2	50	1
Stage 1	10	95	1
PCR Stage 2	0.25	95	40
PCR Stage 3	1	60	40

Table 2.5 Settings used for QRTPCR The QuantStudio 12K Flex Real-Time PCR System (Cat: 4471087, Applied Biosystems, CA, USA) was used, stage 2 and 3 were repeated 40 times.

2.5.6 Preparation of protein lysates

Cell lysates were collected in 150 µL RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) Na Deoxycholate, pH 8.8] and rotated orbitally at 4ºC for 45 minutes. Samples were subjected to centrifugation at 16,100 x g at 4 ºC for 10 minutes. Finally supernatant was removed and protein concentration quantified by BCA (Bicinchoninic Acid) assay. If not used immediately, the supernatant was stored at -20ºC.

An aliquot of 5 mL volume RIPA was supplemented with ½ tablet of cOmplete™ Protease Inhibitor Cocktail (Roche Cat: 000000011697498001) and phosphatase inhibitor cocktail (1:100) (Cat:78427, Thermo Fischer Scientific, MA, USA).

2.5.7 Protein concentration determination

Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Cat: 23225, Thermo Fischer Scientific, MA, USA). This assay exploits the fact that in the presence of protein, Cu^{2+} is reduced to Cu^{1+}. Bicinchoninic acid (BCA) is used which reacts with the reduced Cu^{1+}. Chelation of two BCA molecules with one Cu^{1+} forms a purple colour, this can be measured by spectrophotometry at 590 nm.

To determine the protein concentration from cell lysates the Microplate Procedure (Sample to WR ratio = 1:8) was followed as per manufacturer's instructions. Briefly, a standard calibration curve using set dilutions of bovine serum albumin (BSA) covering a range of 2000 µg/mL to zero (blank) was generated and 25 µL of standard or cell lysate was added to independent wells of a clear 96-well microplate in duplicate. A working reagent (WR) used for the colorimetric reaction was made by adding one-part BCA Reagent B to 50 parts BCA Reagent A and 200 µL of WR was added to each well. The microplate was incubated for 30 minutes in the dark at 37 ºC. Lastly the plate was read at 590 nm using a Victor 2 spectrometer (Perkin Elmer, MA, USA).

2.5.8 SDS-Polyacrylamide Gel Electrophoresis

Samples containing 25-50 µg of protein were diluted in total volume of 40 µL nuclease free water containing 7.5 µL loading dye. Protein samples were then

denatured for 5 minutes at 95 ºC using a thermal cycler. Proteins were then separated according to their molecular mass using polyacrylamide gel electrophoresis (PAGE). A gel tank was filled with 1 L of 1X NuPAGE MES SDS running buffer (x20) (Cat: NP0002, Thermo Fisher Scientific, MA, USA) diluted in distilled water. A 4-12% Bis-Tris precast polyacrylamide gel (Thermo Fisher Scientific, MA, USA) was added to the chamber. Five µL of Amersham rainbow ladder (Cat: RPN 800E, GE Healthcare Life Sciences, UK) was loaded in the outermost wells and was used as a molecular weight marker. The full 40 µL of protein sample was then loaded into the wells. A constant voltage of 200 Volts (V) for two hours was used to separate the proteins through the gel.

2.5.9 Electrophoretic transfer of proteins from gels onto nitrocellulose membranes

Six pages of filter paper were soaked in transfer buffer and the 0.2 µm nitrocellulose membrane (GE Healthcare LifeSciences, UK) was soaked in distilled water. The transfer tank was filled with 1 L transfer buffer [24.7 mM Tris-base, 0.19 M glycine and 20% (v/v) methanol, pH 7.4]. The transfer tank cassette was removed and opened. A porous sponge followed by 3 pieces of pre-soaked filter paper were stacked on the black side of the cassette (negative). The polyacrylamide gel was removed from the plastic outer-casing and washed once in transfer buffer. Next, it was added to the stack. The 0.2 µm nitrocellulose membrane (GE Healthcare LifeSciences, UK) was added next and any air bubbles removed using a western blot roller (Cat: 84747, Thermo Fisher Scientific, MA, USA). Finally, another 3 pieces of filter paper and a sponge completed the stack. Electrophoretic transfer of proteins from gels onto nitrocellulose membranes was performed for 1.5 hours at 100 V in a cold room (4˚C).

2.5.10 Blocking of membranes and probing with primary antibodies

On completion of transfer, the nitrocellulose membranes were washed in PBS on an orbital shaker for 5 minutes (RT). Next the membranes were blocked in a 1:1 dilution of Seablock blocking buffer (Cat: 37527 Thermo Fischer Scientific, MA, USA) TBS (136.9 mM NaCl, 24.8 mM Tris-base and 2.7 mM KCl, pH 7.4) containing Tween (0.02%) [TBS-T] for 30 minutes at RT. An antibody mixture was prepared

in the same blocking solution (see table 2.6) and incubated over night at 4°C on an orbital shaker.

2.5.11 Secondary antibody incubation

The next day, nitrocellulose membranes were washed 3 times for 5 minutes each time in TBS-T. Secondary antibodies for immunodetection of proteins was performed using Western blotting and the LI-COR detection system. Secondary antibodies (see table 2.6) were prepared in a 1:1 dilution of Seablock blocking buffer (Cat: 37527, Thermo Fischer Scientific, MA, USA) TBS-T. Nitrocellulose membrane were incubated at RT for 1 hr with secondary antibodies (see figure 2.7) on an orbital shaker. Lastly, the nitrocellulose membrane was washed three times for 5 minutes each in TBS-T at RT. The membrane was then stored in TBS at 4 °C until ready to image.

2.5.12 Near-infrared fluorescent imaging

Protein bands were then imaged using the Near-infrared fluorescent Odyssey® CLx Imaging System (LI-COR , NE, USA). Both the 700 and 800 nm channels were used with the setting detailed in Table 2.7.

Target	Company	CAT	Host Species	Target Species	Dilution
αSMA	Pierce	MA5-11547	Mouse	MS	1 in 200
αSMA	Abcam	ab5694	Rabbit	Mouse	1 in 200
PCNA	Abcam	ab18197	Rabbit	Mouse	1 in 500
MCP-1	BioRad	AAM43	Rat	Mouse	1 in 50
β-GALACTOSIDASE	MP-BIO	O559762	Rabbit	E.coli	1 in 208
GAPDH	Abcam	ab8245	Mouse	Human	1 in 7500

Table 2.6 List of primary antibodies used for immunoblotting and immunofluorescence.

Target	Company	CAT	Host Species	Conjugate	Dilution
Mouse IgG	LI-COR	926-32210	Goat	IRdye 800CW	1 in 15000
Rabbit IgG	LI-COR	926-32221	Goat	IRDye 680CW	1 in 15000
Mouse IgG	Life Technologies	110001	Goat	Alexa fluor 488	1 in 500
Rabbit IgG	Life Technologies	110081	Goat	Alexa fluor 543	1 in 500

Table 2.7 List of secondary antibodies used for immunoblotting and immunofluorescence.

2.5.13 Pathscan intracellular signalling array

HSVSMC were exposed to PDGF-BB (20 ng/mL) in the presence of absence of 1 μM of Ang-(1-9) or left unstimulated. HSVSMC were incubated for 5 minutes before protein lysates were collected in 140 μL RIPA buffer and rotated orbitally at 4 ºC for 45 minutes. Samples were subjected to centrifugation 16,100 x g at 4 ºC for 10 minutes. Finally, supernatant was removed and protein concentration quantified by BCA (Bicinchoninic Acid) assay and stored at -20 ºC.PathScan® Intracellular Signalling Array Kit (Fluorescent Readout) (Cat:#7744, Cell Signalling Technology, MA, USA) was then performed according to manufacturer's protocols. This array probed for the detection of: ERK1/2, Stat1, Stat3, Akt (Thr308), Akt (Ser473), AMPKa, S6, mTOR, HSP27, Bad, p70 S6 KinasePRAS40, p53, p38 MAPK, SAPK/JNK, Cleaved PARP, Cleaved Caspase-3, GSK-3β pathway activation (Table 2.8). Briefly, each well containing nitrocellulose pads was blocked using manufacturer's blocking buffer for 15 minutes. Next, 0.5 mg/mL cell lysate was added to each well and incubated at room temperature (RT) for 2 hours on an orbital shaker. Next each array was washed for 4 x 5 minutes using manufacturer's array wash buffer on an orbital shaker. A detection antibody cocktail was added and incubated with the array for 1 hour at RT. The array was then washed as described above and probed using a DyLight 680TM-linked streptavidin antibody (1:10 dilution Cell signalling technology) for 30 minutes at RT. The wash step described earlier was repeated. A final wash in deionized water for 10 seconds completed the protocol. The whole slide was allowed to completely dry. The slide was then imaged on the Odyssey® CLx (LI-COR) imaging system exciting at 680 nm with detection at 700 nm on medium quality and resolution 21 μm.

Spot	Target	Phosphorylation Site	Modification
1	Pos. Control	N/A	N/A
2	Neg. Control	N/A	N/A
3	ERK	Thr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Thr308	Phosphorylation
7	Akt	Thr473	Phosphorylation
8	AMPK	Thr172	Phosphorylation
9	S6	Ser235/Ser236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70S6 Kinase	Thr389	Phosphorylation
14	PRAS40	Thr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Thr180/Tyr182	Phosphorylation
17	JNK	Thr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK3b	Ser9	Phosphorylation

Table 2.8 Pathscan Intracellular Signalling Array
Table of cleavage and phosphorylation sites detected by PathScan® Intracellular Signalling Array Kit (Fluorescent Readout) #7744.

A

Spot	Target
1	Pos. Control
2	Neg. Control
3	ERK
4	Stat1
5	Stat3
6	Akt
7	Akt
8	AMPK
9	S6
10	mTOR
11	HSP27
12	Bad
13	p70S6 Kinase
14	PRAS40
15	p53
16	p38
17	JNK
18	PARP
19	Caspase-3
20	GSK3b

B

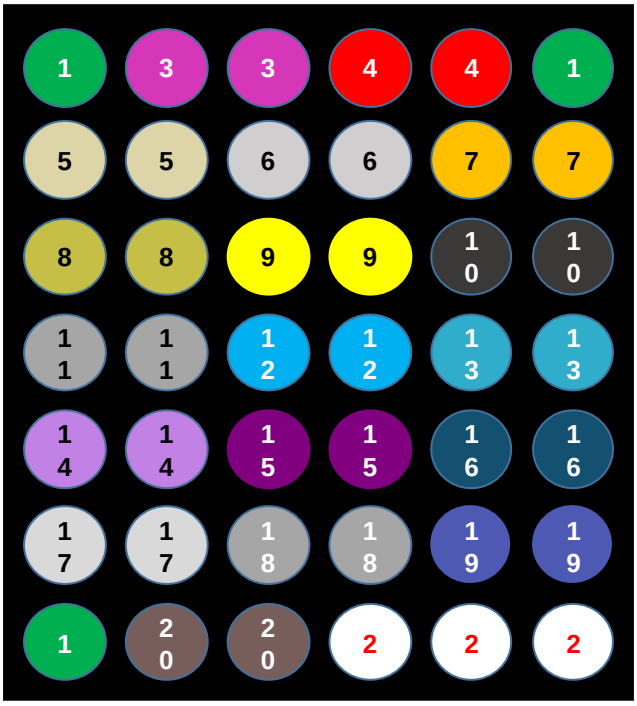

Figure 2.3 Pathscan Intracellular Signalling Array
Accompanying target map of the PathScan® Intracellular Signalling Array Kit (Fluorescent Readout) #7744.

2.5.14 Enzyme-linked immunosorbent assay (ELISA)

Two different Ang-(1-9) enzyme-linked immunosorbent assay (ELISA) were purchased (Cat:Abx575059, Abbexa, UK) and (Cat:CET844Mi, Cloud Clone Corp, HO, USA). Both detection types were colorimetric and the style of ELISA was competitive. 50 µL of sample (serum or conditioned culture media [section 2.2.6]) was used, alongside samples spiked with commercially purchased Ang-(1-9) at concentrations within the detection range (10-1000 pg/mL). Murine serum samples taken from mice that had been infused with Ang-(1-9) by osmotic mini pump (section 2.7.5) were also analysed. Otherwise the manufacturer's protocol was followed exactly. Neither ELISA was able to effectively measure commercially purchased or adenoviral-derived Ang-(1-9) peptide and therefore were uninformative (Figure 2.4). Data discussed in *Chapter 5*.

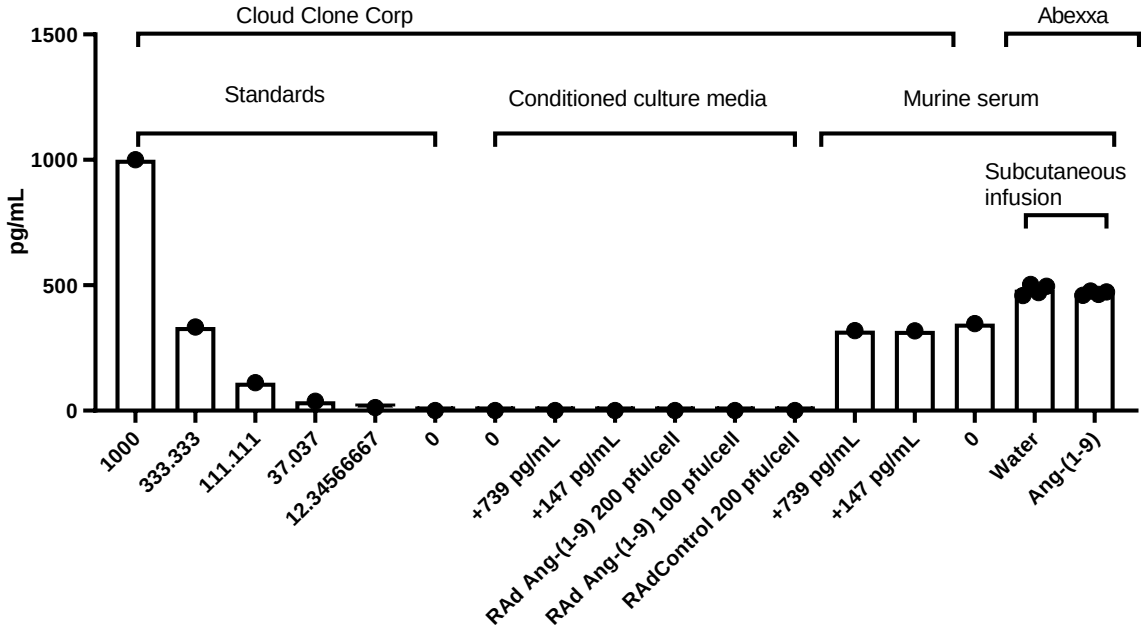

Figure 2.4 Ang-(1-9) enzyme linked immunosorbent assay

Conditioned media from untreated HepG2 cells was spiked with commercially produced Ang-(1-9) at concentrations within the detection limit (177 pg/mL + 739 pg/mL). Secreted Ang-(1-9) in the culture media collected from RAdAng-(1-9) transduced HepG2 cells 2 days after viral transduction was quantified with the Cloud Clone Corp ELISA kit. Murine serum was left untreated or spiked with commercially bought peptide at known concentrations and tested by the Cloud Clone Corp ELISA n=1. The Abexxa Ang-(1-9) ELISA tested serum that was collected from C57BL6/J mice subcutaneously infused with Ang-(1-9) or water via implantation of osmotic mini pumps n=4 mice.

2.6 Recombinant Adenoviral vectors

2.6.1 Adenoviral vector propagation

All adenoviral vectors utilised within this report were propagated on a large-scale using replication permissive HEK 293 cells (Graham et al., 1977). Twenty T150 cm^2 culture flasks of 90% confluent HEK 293 cells were transduced with crude vector stocks and incubated at 37 ^{0}C, 5% CO_2 until the cytopathic effect (CPE) was complete (approx. 5 days). Media containing virus and cellular debris was combined in 50 mL centrifuge tubes and subjected to centrifugation at 300 x g for 10 minutes at RT after which the supernatant was discarded and the pellet was re-suspended in 6 mL PBS. If adenoviral vector extraction was not performed immediately the re-suspended pellet was frozen at -80^{o}C. To extract adenoviral vectors the re-suspended pellet had an equal volume of 1,1,2-Trichloro-1,2,2-trifluoroethane (ArkloneP) added. The mixture was then inverted repeatedly at a slow pace for 30 seconds, followed by rapid inverting for 30 seconds. These steps were repeated three times. The adenoviral suspension was subjected to centrifugation to purify the viral particles from the media and cellular debris at 800 x g at RT for 10 minutes. The top aqueous layer was removed taking care not to disturb the interface containing cellular protein and kept for further purification.

2.6.2 Caesium chloride gradient ultracentrifugation

In order to prepare the caesium chloride gradients first Ultra Clear ultracentrifuge tubes (14x19mm) (Cat: 344059, Beckman Coulter, CA, USA) were sterilized using 70% ethanol followed by rinsing in sterile water. Next, 2.5 mL of 1.25 g/mL Caesium Chloride was pipetted into the UC tubes. Next 2.5 mL of 1.40 g/mL Caesium Chloride was carefully aspirated directly to the bottom of the UC tube creating a gradient. Following this the virus/ArkloneP solution was slowly dripped onto the top of the gradient. The UC tubes were then filled to 1 mm from the top with sterile PBS. UC tubes were then subject to ultracentrifugation for 1.5 hours at 90,000 x g at 20 ^{o}C, with a deceleration speed of 9 (Beckham Coulter, CA, USA).

Following ultracentrifugation, the viral particle rich band was carefully collected using a 23 g needle and a 1 mL syringe (Figure 2.5). The adenoviral solution was then inserted into a Slide-A-Lyzer Dialysis Cassettes,10,000 molecular weight cut off, 0.5–3.0 mL capacity (Cat: 66380, Pierce Protein Research Products, Thermo Scientific, Rockford, IL, USA).and subjected to three rounds of dialysis. While the first and second round where performed in 3 L of 1 x TE (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) for 2 hours. In the third round, the cassette was dialysed in 1 x TE 10% glycerol for 2 hours. Lastly the virus was removed from the cassette and aliquoted at 5 µL before being stored at -80^{o}C. Adenoviral vector stocks were only used twice, i.e. only 1 additionally freeze thaw was permitted.

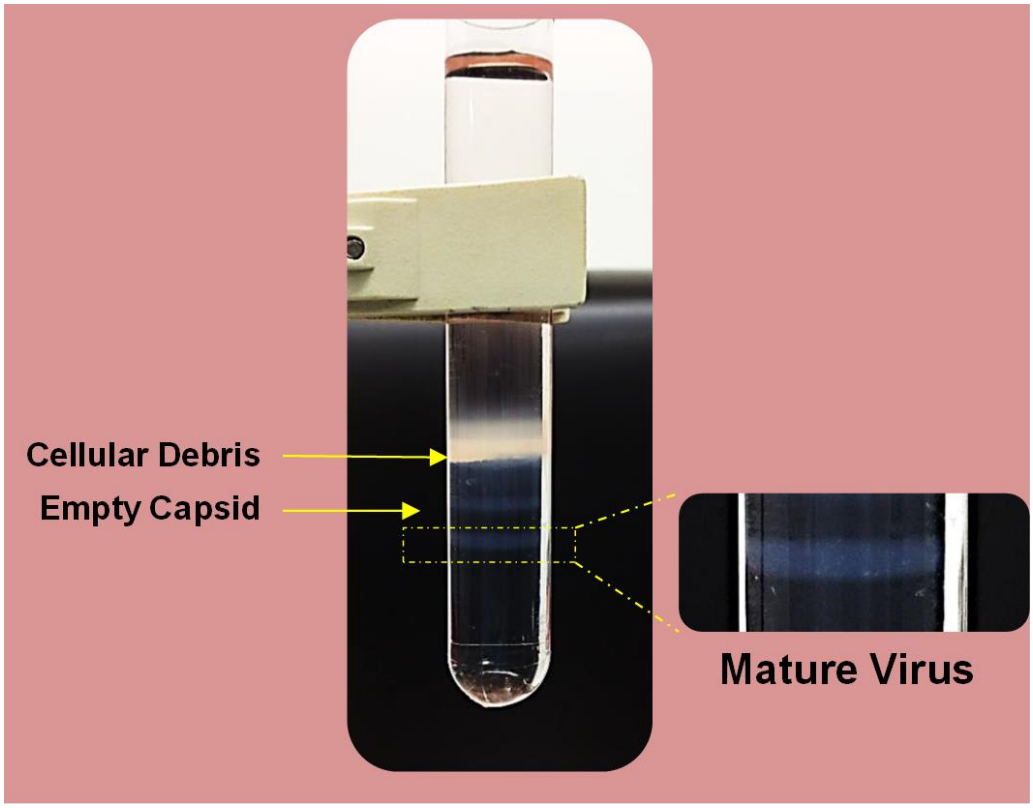

Figure 2.5 Purification of adenoviral vectors by caesium chloride density gradients.

2.6.3 Adenovirus titration

The physical titer (virus particles/mL; VP/mL) of pure adenoviral vector stocks was then determined by Micro BCA™ Protein Assay Reagent Kit (Cat: 23235, Thermo Fischer Scientific, MA, USA).

In order to determine the infectious titer of adenoviral vector stocks (plaque forming units or PFU/mL) a T150 cm^2 flasks of HEK293 cells was passaged and re-suspended in 25 mL complete HEK293 media (section 2.2.2). Three mL of the cell suspension was further diluted into 17.5 mL MEM. Next, 200 µL of cell suspension was added to each well. Plates were incubated overnight at 37 ^{0}C, 5% CO_2.

A serial dilution of the adenoviral vector preparation was made as described (Table 2.9). The culture media from the titer plate and 100 µL of fresh MEM was added to each well on the rows. Next the serial dilutions were added working from the bottom of the plate upwards replacing the tips each time. The culture media was replaced the next day and every 3 days until day number 8. At this point the number of wells with visible plaques were counted and the PFU/cell calculated using the calculation as previously described (Alba et al., 2012). The number of wells with CPE effect was counted. The proportionate distance was calculated as described below.

$$\text{Proportionate distance } = \frac{(\%\ CPE\ positive\ above\ 50\% - 50\%)}{(\%\ Positive\ above\ 50\% - \%\ Positive\ below\ 50\%)}$$

Next, the Log Infectivity dose (ID_{50}) was calculated using the equation below.

$$ID_{50}\ = Log\ dilution\ above\ 50\% + (Proportionat\ distance\ \times -1)$$

Subsequently the tissue culture infectivity dose ($TCID_{50}$) was calculated using the formulae below.

$$TCID_{50}\ = \frac{1}{ID_{50}}$$

The $TCID_{50}$ value was multiplied by the dilution factor to produce a concentration (per mL), in this case 10 (i.e. 100 µL was added). Finally, this concentration was multiplied by 0.7 to convert this value to a concentration in PFU/mL. According to the notion that 1 $TCID_{50}$ = 0.7 PFU (Alba et al., 2012).

$$TCID_{50} \times 10 = TCID_{50}\ per\ mL$$

$$TCID_{50}\ per\ mL \times 0.7 = Titer\ in\ PFU\ per\ mL$$

	Serial Dilutions
1	30 µL virus into 3mL media (MEM) -2
2	50 µL of –2 diluted into 5 mL media -4
3	50 µL of –4 diluted into 5 mL media -6
4	500 µL of –6 diluted into 4.5 mL media -7
5	500 µL of –7 diluted into 4.5 mL media -8
6	500 µL of –8 diluted into 4.5 mL media -9
7	500 µL of –9 diluted into 4.5 mL media -10
8	500 µL of –10 diluted into 4.5 mL media -11
9	500 µL media alone

Table 2.9 List of serial dilutions for PFU assay

2.7 In Vivo

2.7.1 Ethics Statement

All animal experiments were performed under an approved UK Home Office project licence according to the regulations of the UK Home Office Scientific Procedures Act (1986). All studies were reviewed and approved by the Named Animal Care and Welfare Officer (NACWO) at the University of Glasgow. Animal experimentation detailed in Chapter 3 was conducted under the project licence 70/8572. The viral gene transfer studies were conducted under the project licence 60/4429. Male C57 BL/6 mice (age 8-10 weeks) were purchased from Charles River Laboratories (Charles River Laboratories, MA, USA). Mice were maintained on a standard chow diet with free access to water and a 12-hour light/dark cycle by Biological Services staff at the Central Research Facility (University of Glasgow). All studies were blinded by an independent researcher prior to processing of tissues as described in section 2.8.

2.7.2 Carotid ligation surgery

All mice were anaesthetised and maintained under isoflurane (2-3.5%) before having their necks shaved and eyes lubricated. An incision was then made down the midline proximal to ribcage. Blunt dissection allowed exposure and liberation of the left carotid artery (LCAR) from the vagus nerve and surrounding vessels. A braided silk suture (size 6, F.S.T, UK) was tied in a double knot around the proximal branch of the exposed carotid. The wound was then closed by continuous suturing and mice were recovered in incubation cages.

2.7.3 Wire injury surgery

A modified version of the carotid artery wire injury model (Linder et al., 1993) originally adapted by Tennant et al., was used in order to mechanically denude the EC layer of the left common carotid artery (Tennant et al., 2008).

Male C57 BL/6 mice (age 8-10 weeks) were purchased from Charles River Laboratories. Like as described above (section 2.7.2), all mice were anaesthetised and maintained under isoflurane (2-3.5%). Next, the neck of each mouse was shaved and the eyes lubricated. The left carotid artery was exposed and dissected away from the vagus nerve and surrounding vessels by blunt dissection. A braided silk suture (size 6, F.S.T, UK) was tied in a double knot around the proximal branch of the exposed carotid (Figure 2.6A). A temporary vessel clamp was placed at the distal end to prevent blood flow (Figure 2.6B) before an incision was made in the stretch of vessel between clamp and suture (Figure 2.6C). A piece of nylon line was then inserted through the incision and a loose suture tightened around the vessel/wire (Figure 2.6D). The distally located clamp was then removed and the wire passed down the carotid artery to the aortic arch; mechanically removing the endothelium. This movement was repeated 3 times (Figure 2.6E). After which the clamp was replaced to block blood flow while the distal suture was loosened, and wire removed (Figure 2.6F). The top suture was then permanently tied slightly distal to the incision in a double knot (Figure 2.6G). The clamp was then removed, and the surgical wound closed by suturing. Mice were allowed to recover in incubation cages with free access to food and water supplemented with aspirin (300 mg/L).

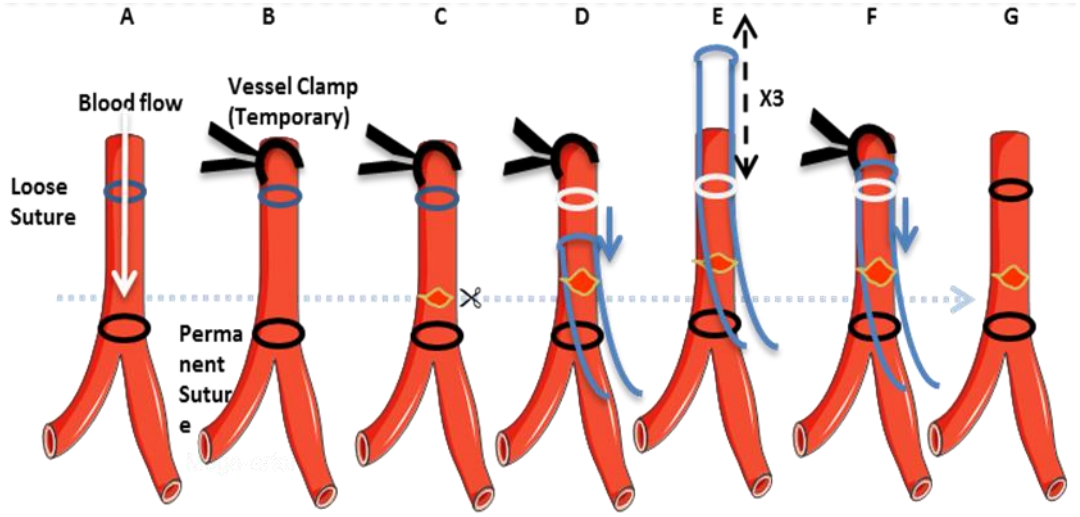

Figure 2.6 Schematic of the modified wire injury procedure. (A) The carotid artery was exposed and liberated from the vagus nerve before a permanent ligation was made distal to the bifurcation in the common carotid artery. A second suture was loosely fixed distal to the first. (B) a vessel clamp was placed and (C) an incision made between the two ligations. (D) A modified piece of flexible nylon fishing line was inserted within the carotid through the site of incision and passed through the distal loose suture to the permanent vessel clamp. (E) The distal ligation was tightened around the carotid artery and flexible wire. The vessel clamp was then removed. The whole site was lubricated with sterile saline solution and the wire passed downward until a tension point indicated the wire had reached the aortic arch. This was repeated three time. (F) Next the vessel clamp was re-attached, and the distal ligation loosened to allow the removal of the wire. (G) Lastly, the distal ligation tightened, and a second knot made to perform a secure permanent ligation proximal to the incision site.

2.7.4 Administration of adenoviral vectors

Mice were placed in a warming box or incubator set at 30ºC for 15 minutes before being restrained and $1x10^{11}$ viral particles (VP) (total volume of 100 µL) administered by tail vein injection.

2.7.5 Osmotic mini-pump implantation

Osmotic mini pumps (Model 2002, Alzet CA, USA) were used to deliver Ang-(1-9) at a constant rate of 0.5 µL/hour, at a concentration of 48 µg/kg/hour. Mini pumps containing sterile RNase-free water (Qiagen, Netherlands) were used as a control.

Immediately after carotid artery ligation surgery, mice were re-positioned facing down and an incision site prepared using betadine iodine solution and sterile cotton buds. An incision was then made down the sagittal plane. Blunt surgical scissors (Cat: 14000-20 F.S.T, CA, USA) were passed through the incision site and blunt dissection downward to the hind legs allowed for the creation of a subcutaneous pouch in which to place a mini pump. Mini pumps were then implanted, and the wound closed by suturing (4-0 Ethicon).

2.7.6 Termination procedure and tissue harvesting

Mice were culled at 7, 14 or 28 days according to the experimental objective. Transcardial perfusion with 10 mL PBS using a 23-gauge needle was performed to clear the viscera and vasculature of blood. All major organs, aorta and carotid arteries were isolated, washed in PBS and either snap frozen in liquid nitrogen or fixed for 24 hours in 4% PFA. Snap frozen tissue was then stored at -80 ºC and fixed tissue transferred to 70% ethanol for long-term storage.

2.7.7 5-ethynyl-2'-deoxyuridine (EdU) administration

Five hours prior to termination and tissue harvesting mice that had undergone carotid ligation surgery were administered 100 µL of 25 mg/mL EdU (prepared in PBS) (Cat: A10044, Thermo Fischer Scientific, MA, USA) via intraperitoneal injection.

2.7.8 Serum Collection

Following the termination of procedure (section 2.7.6), approximately 0.5 - 1 mL of peripheral blood was harvested from C57 BL/6 mice by cardiac puncture using a 23-gauge needle and placed in a BD Microtainer® SSTTM Tube (Cat:365968, BD Biosciences, CA, USA). Samples were inverted 5 times and incubated at RT for 30 minutes before being subjected to centrifugation at 10,000 g for 1.5 minutes at 4 ^{o}C in order to separate the serum from the peripheral blood. Serum samples were then stored at -80^{o}C.

2.7.9 Luminex analyses

In order to determine the levels of 23 different cytokines (Table 2.10) the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Cat:m60009rdpd, BioRad, CA, USA) was performed according to manufacturer's instructions with one exception; the magnetic beads provided were diluted by half in order to simultaneously run two assays.

First the lyophilised standard was reconstituted in 500 µL of standard diluent, vortexed briefly and incubated on ice for 30 minutes. During this incubation period murine serum samples were thawed and diluted 1 in 4 i.e. 30 µL of serum was added to 90 µL of the manufacturer's sample diluent. These sample dilutions were prepared in a spare clear plastic 96 well plate (Figure 2.7).

Following the 30-minute incubation described above, the standards were prepared by serial dilution in 5 mL Falcon® Round-Bottom Polypropylene Tubes (Cat:352053, Corning Inc NY, USA).

Next the magnetic beads were vortexed for 30 seconds and prepared by diluting 575 µL in 10,350 µL assay buffer. The beads were protected from light and brought to RT before use. Next the magnetic beads were vortexed once more and 50 µL added to each well in the black 96 well plate provided. Using a magnetic plate, the beads were washed twice in 100 µL BioPlex wash buffer. Standards and samples were then vortexed briefly and 50 µL was added to the wells as designated by the user. The plate was then protected from light and incubated at 4 ^{o}C overnight on an orbital shaker at 850 RPM.

The next day, the detection antibodies were vortexed and prepared in FACS tubes by diluting 300 µL in 2700 µL detection Ab diluent. The plates were washed three time in 100 µL wash buffer and 25 µL of detection antibody was added to each well and incubated at RT for 30 minutes on an orbital shaker at 850 RPM. The 100X streptavidin-PE (SA-PE) was subjected to centrifugation for 30 seconds at 8000g. In a FACs tube, 60 µL of SA-PE was added to 5940 µL assay buffer diluent and protected from light. The plates were washed again as described above. Next 50 µL of 1X SA-PE was added to each well and incubated at RT for 10 minutes on an orbital shaker at 850 RPM. The plates were washed again as described above. Lastly, 125 µL assay buffer was pipetted into each well and shaken for 30 seconds on 850 RPM before reading the plate on the BioPlex® 200 system (Cat: 171000201 Bio-Rad, CA, USA).

Cytokine	Basic immunological function	Examples of effects in vascular pathologies	Reference
G-CSF (Granulocyte-colony stimulating factor)	Proinflammatory colony stimulating factor. Stimulates haematopoiesis. Growth and differentiation of immune cells.	Reduced neointima formation in a rabbit angioplasty model. Inhibited neointima formation after wire injury in mice by enhancing EC regrowth.	(Hasegawa et al., 2006) (Yoshioka et al., 2006)
GM-CSF (Granulocyte macrophage-colony stimulating factor)	Proinflammatory colony stimulating factor. Growth and differentiation of monocytes, dendritic cells, granulocytes, macrophages.	Inhibited neointima formation following radiation injury in rabbits by enhancing EC regrowth.	(Cho et al., 2003)
IFN-γ (Interferon gamma)	Proinflammatory interferon. Host defence, viral and pathogen elimination. MHC-I expression. Cytotoxic T cell response.	IFN-γ blockade inhibited neointima formation after balloon injury in rats.	(Kusaba et al., 2007)
KC (Keratinocyte Derived Chemokine)	Proinflammatory chemokine. Chemotaxis of neutrophils and eosinophils.	Blockade of KC via monoclonal antibody prevented EC regeneration and prompted neointima formation in ApoE-deficient mice.	(Liehn et al., 2004)
MCP-1 (Monocyte chemotactic protein 1) [*CCL2*]	Proinflammatory chemokine. Chemotaxis of monocytes.	MCP-1 promoted neointima formation after vein grafting and femoral artery cuff placement in mice. CCR2 KO inhibited vein graft and femoral artery neointima formation in mice.	(Fu et al., 2012) (Egashira et al., 2002)
MIP-1α (Macrophage Inflammatory Protein -1α) [CCL3]	Proinflammatory chemokine. Chemotaxis of monocytes and T cells.	MIP-1α deficiency had no effect on neointima formation in the carotid ligation model. Leucocyte specific MIP-1α deficiency reduced atherosclerotic lesion formation.	(Zhang et al., 2007) (de Jager et al., 2013)
MIP-1β (Macrophage Inflammatory Protein-1β) [CCL4]	Proinflammatory chemokine. Chemotaxis of monocytes and T cells.	Increased EC oxidative stress.	(Tatara et al., 2009)
RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) [CCL5]	Proinflammatory chemokine. Chemotaxis of leukocytes.	CCL5 deficiency reduced neointima formation in ApoE deficient mice. Blocking RANTES mediated signalling through receptor antagonism inhibited neointima formation in ApoE deficient mice.	(Czepluch et al., 2016) (Schober et al., 2002)
Eotaxin [CCL11]	Proinflammatory chemokine. Chemotaxis of eosinophils. Roles in autoimmunity and asthma.	Induces VSMC migration.	(Kodali et al., 2004)

TNF-α (Tumor necrosis factor-α)	Proinflammatory necrosis factor. Associated with tumor cell death. Upregulates cytokine expression in macrophage.	Promoted vein graft remodelling in mice. Promoted VSMC proliferation and migration.	(Zhang et al., 2004) (Davis et al., 2012)
IL-1α (Interleukin-1α)	Proinflammatory interleukin. T, B and natural killer (NK) cell activator.	Promoted neointima formation	(Rectenwald et al., 2000)
IL-1β	Proinflammatory interleukin. T, B and NK cell activator.	Carotid ligation mediated neointima formation was inhibited in transgenic IL-1B and IL-1R null mice.	(Chamberlain et al., 2006)
IL-2	Proinflammatory interleukin. Promotes proliferation of activated B and T cells.	Promoted proliferation and migration of VSMCs.	(Arumugam et al., 2019)
IL-3	Proinflammatory interleukin. Stimulates haematopoiesis, growth of progenitor cells and histamine release by mast cells.	Promoted VSMC proliferation and migration.	(Brizzi et al., 2001)
IL-4	Anti-inflammatory interleukin. Differentiation of B and T cell to anti-inflammatory phenotype.	Atherosclerotic plaque development was reduced in transgenic IL-4 null mice.	(Davenport and Tipping, 2003)
IL-5	Proinflammatory interleukin. Proliferation and differentiation of B cells.	Macrophage specific overexpression of CCL5 inhibited atherosclerotic lesion development.	(Zhao et al., 2015)
IL-6	Proinflammatory interleukin. Activation of T, B, VSMC and ECs.	Promoted VSMC migration. Inhibition of IL-6/STAT signalling inhibited neointima formation in a rat balloon injury model.	(Wang et al., 2007)
IL-9	Pleotropic interleukin. T cell differentiation. Promotes mast cell proliferation.	Promoted chronic kidney disease mediated VGF Promoted atherosclerotic lesion development in ApoE deficient mice.	(Zhang et al., 2017) (Zhang et al., 2015)
IL-10	Anti-inflammatory interleukin. Promotes regulatory T cells proliferation. Inhibits proinflammatory cytokine production.	Inhibited neointima formation after stent deployment in hypercholesterolemic rabbits. Inhibited neointima formation by prompting re-endothelialisation in mice.	(Feldman et al., 2000) (Verma et al., 2016)
IL-12 (p40)	Proinflammatory interleukin. Enhances NK and cytotoxic T cell activity.	Ang II induced AAA was exacerbated in transgenic IL-12(p40) null mice.	(Sharma et al., 2019)
IL-12 (p70)	Proinflammatory interleukin. Enhances NK and cytotoxic T cell activity.	Promoted T cell infiltration into atherosclerotic plaque. Atherosclerotic plaque development reduced in transgenic IL-4 null mice	(Arumugam et al., 2019) (Davenport and Tipping, 2003)
IL-13	Anti-inflammatory interleukin. Modulates macrophage phenotype	Atheroprotective in mice and atherosclerosis was exacerbated in transgenic IL-13 KO mice.	(Cardilo-Reis et al., 2012)

IL-17A	Proinflammatory interleukin. Induction of other proinflammatory cytokines by VSMC, fibroblasts, epithelial cells and ECs.	IL-17A upregulated expression of proinflammatory cytokine by VSMC with no direct effect on proliferation. Promoted murine carotid artery VSMC migration via upregulation of MMP9.	(Pietrowski et al., 2011) (Cheng et al., 2009)

Table 2.10: A list of cytokines measured by immuno-multiplexing. Brief details of each cytokine, their basic immunological function (Reviewed in Sprague and Khalil, 2009) and role in vascular remodelling.

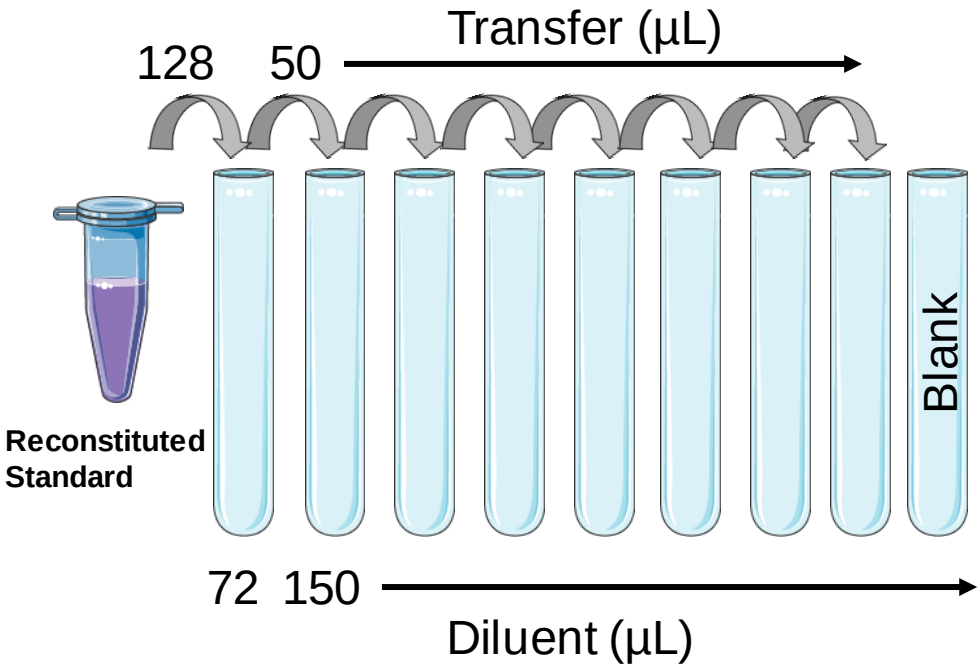

Figure 2.7 Dilution series used for Luminex assay.

The lyophilised standard was re-suspended in in 500uL standard diluent. The solution was then vortexed for 30 seconds and incubated on ice for 30 minutes. The standards were then serial diluted in standard diluent. Image created using Servier Medical Art available at https://smart.servier.com. No permission required**.**

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2.8 Histology

2.8.1 Tissue fixation and processing

All tissues were fixed overnight in 4% PFA. The next day tissues were placed in Ethanol (70% v/v) and then processed using the Shandon Excelsior Tissue Processor (Thermo Fischer Scientific, MA, USA). The carotid arteries, hearts, lungs, livers and kidneys of study mice were dehydrated using a concentration gradient of ethanol in combination with final steps of Xylene treatment (Table 2.11). The next day tissues were embedded in paraffin wax blocks (vertically) using Shandon Histocentre 3 (Thermo Fischer Scientific, MA, USA).

Solution	Time (minutes)
70% ETOH	30
95% ETOH	30
100% ETOH	30
100% ETOH	30
100% ETOH	45
100% ETOH	45
100% ETOH	60
Xylene	30
Xylene	30
Xylene	30
Paraffin wax	30
Paraffin wax	45
Paraffin wax	45

Table 2.11 Solutions and incubation steps used for the Shandon Excelsior Tissue Processor. ETOH (Ethanol).

2.8.2 Haematoxylin and Eosin Staining (H&E)

(H&E) staining was used to visualise nuclei (blue) and cytoplasm (red) of murine left carotid arteries. First, 5 µm tissue sections were deparaffinised by incubating the sections in histoclear for 5 minutes. This step was repeated once before the sections were rehydrated by passing them through an ethanol gradient (5-minute washes: 100%, 95%, 70%) and then placed in distilled water for 7 minutes. Tissue sections were then stained with Harris haematoxylin (Cell Path, UK) for 2 minutes. Tissue sections were then washed in running water for 5 minutes. Next, tissues sections were briefly dehydrated in 70% ethanol for 30 seconds before being immediately stained with Eosin Y (Cell Path, UK) for 3 minutes. Slides were then dehydrated through an alcohol concentration gradient. Tissue sections were placed in 90% ethanol for 30 seconds, twice. This was followed by 1 minute in 100% ethanol before being placed in fresh ethanol for 7 minutes. Finally, two 5-minute immersions in Histoclear completed the protocol before mounting with DPX slide mountant.

Elastic Tissue Fibers - Elastin Van Gieson (EVG)

EVG staining was used to visualise elastic lamina (black), collagen (pink) and other cellular component (yellow). Sections of carotid arteries were cleared and rehydrated to distilled water as described above. Hydrated sections were then oxidised in potassium permanganate (0.5% w/v) for 10 minutes, followed by a 3-minute wash in running tap water. Oxalic acid (1% w/v) was used to differentiate for 1 minute before the sections were returned to running tap water. Next, tissues sections were briefly dehydrated in 70% Ethanol for 30 seconds before being immediately stained with Millers Elastin stain overnight.

2.8.3 Picrosirius red staining

In order to image collagen (Red) and cytoplasmic content (yellow) picrosirius red staining was used. All samples were dewaxed and rehydrated as described in section 2.8.2. Sections of carotid artery were stained with 0.1% Picrosirius red solution (Sirius Red F 3B in saturated picric acid) for 60 minutes protected from light. All sections were then washed in 0.01M HCl for 5 minutes. The acidified water was then replaced and sections washed for a further 5 minutes. Next the

sections were dehydrated in 100% ethanol for 5 minutes. This was followed by two 5-minute washes in Histoclear. Finally coverslips were then mounted using DPX Mountant (Cat: 44581 Sigma Aldrich, MI, USA).

2.8.4 Picrosirius red quantification

Stained carotid arteries were imaged using light microscopy on an EVOS microscope (X10 magnification). Positively stained areas were then quantified using ImageJ. For each image a pixel intensity threshold was set that appropriately highlighted only picrosirius red positive staining (red). The area of pixels was automatically quantified. Next a new threshold was selected that highlighted the entire vessel, the area of which was quantified. Finally, a percentage of the red positive area to total area of the vessel was calculated.

2.8.5 Morphometric analysis of vessel

The researcher was blinded to treatment groups before the sections were cut and during imaging and analysis.

The area of the lumen, intima and media was measured from 4 cross-sections at 5 regions within the vessel separated by at least 200 μm. The mean of each value was then calculated and neointima and media area presented here.

2.8.6 Whole tissue X-Gal staining

Following a schedule one procedure, C57 BL/6 mice were perfused with PBS. Livers were then fixed in 2% PFA overnight (4ºC). The next day, the fixative was removed and tissues were washed in PBS twice and 5 mL X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) stain [0.1 M PBS, 1.3 mM $MgCl_2$, 3 mM $K_3F_3(CN)_6$, 3 mM $K_4Fe(CN_6)_6$, 1 mg/mL X-gal pH 7.3] was added to the tissue samples and incubated at 37 ºC overnight.

2.8.7 Immuno-histo-fluorescence

Sections were deparaffinised and rehydrated before performing a heat and pH-mediated antigen retrieval using Citrate Buffer (0.01 M sodium citrate, pH 6). Sections were permeabilised using Triton-X-100 (0.05% in TBS) and unspecific

binding blocked with 15% Goat Serum (Cat: G9023 Sigma Aldrich, MI, USA). Sections were then incubated overnight at 4 ºC with primary antibodies according to the experimental objective. Appropriate IgG controls were set up in all cases. The following day sections were washed in TBS (3x5 mins) in the dark. All sections were incubated in the following secondary antibodies for 3 hours at RT. Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:500 TBS-T) (Cat 11001, Life Technologies, CA, USA). Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 543 (1:500 w/ TBS-T) (Cat: 110081 Life Technologies, CA, USA). After removing excess unbound secondary antibodies by washing in TBS-T three times (5 minutes each wash), 0.01% Sudan black was used to reduce lipofuscin mediated auto-fluorescence. All sections were then counterstained with DAPI (Cat: D1306, Life Technologies, CA, USA) at 100 µg/mL (1:1000 dilution with TBS) for 10 minutes at RT. Lastly, Prolong Gold (Life Technologies, CA, USA) was used to mount coverslips and slides cured over night at RT.

2.8.8 Click-IT™ Edu

Edu incorporation was assessed using the Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit (Cat: C10337 Thermo Fischer Scientific, MA, USA) and fluorescence microscopy. First, 5 µm sections were prepared as in section 2.8.1 and then slides were de-waxed, dehydrated and subjected to the same antigen retrieval step as described in section 2.8.7. Carotid artery sections were washed in running tap water for 2 minutes. A hydrophobic barrier was drawn around each section (3 per slide) using a wax pen. Next, sections were permeabilised in a Triton-X-100 (0.1% in PBS) for 10 minutes at RT. This was followed by 2 wash steps in 100 µL Bovine Serum Albumin solution (Cat: A2153 Sigma Aldrich, MI, USA) [3% in PBS]. Click-iT cocktail was prepared exactly as described in the manufacturer's protocol and 30 µL was added to all sections followed by incubation at RT for 30 minutes, protected from light, in a hybridisation chamber. Next, the wash step described above was repeated. DNA was then stained for 10 minutes at RT using the provided Hoechst solution (1 in 1000). Lastly, the sections were washed twice in PBS for 5 minutes and coverslips mounted using ProLong™ Gold Antifade Mountant with DAPI (Cat: P36931 Thermo Fischer Scientific, MA, USA).

2.8.9 Quantification of nuclear co-localisation

ImageJ was used to identify nuclei and measure the percentage of those nuclei that were stained positive for either PCNA or EdU. Firstly, each image was exported as a Tagged Image File Format (.Tiff). These images were then opened on ImageJ. The Red, Blue and Green (RGB) channels were split. Next the channels were stacked. The Blue (DAPI) channel was selected and the image had a threshold applied to highlight all of the nuclei (EdU: 100 PCNA: 130). The area of the highlighted nuclei was then measured. Then a "selection" was created which drew a region of interest around all nuclei. The channel of interest (Edu: Green, PCNA: Red) was opened and the image had a threshold applied to highlight the positive areas (Edu 100, PCNA 60). The area of the positive signal within the highlighted regions (nuclei) was the measured. The two measurements were expressed as a percentage of area (Figure 2.8).

2.9 Statistical analysis

Data is presented as the mean ± the standard error mean (S.E.M). *In vitro* experiments were repeated at least three times using cells isolated from independent patients (unless stated otherwise). Within each biological repeat, a minimum of three technical repeats were performed.

Based on previous literature the minimum number of biological replicates for *in vivo* experiments needed to achieve 95% confidence intervals was 8 (Zimmerman et al., 2004). This was calculated using StatMate software (Graph Pad Inc. CA, USA) by Dr Claire McKinney (formerly University of Glasgow). It determined that when using 8 animals there would be an 80% probability of finding a difference in neointimal area (7.2×10^3 μm^2 with standard deviation of 3.55×10^3 μm^2) between treated and untreated groups. Therefore, with the exception of sham operated animals (n=6), the number of biological repeats for *in vivo* experiments was set at 8. Post-operative mortality accounted for approximately 5% of mice not reaching the study end point. Therefore, there are instances where only seven mice have been analysed.

Prism 6 Software (Graph Pad Inc. CA, USA) was used to statistically analyse data. The difference between two data sets was calculated by an unpaired student's *t*

test. The differences between three or more groups were calculated using a one-way analysis of variance (ANOVA) and Tukey's post-hoc multiple comparison. Significance was set at $p<0.05$. A normality test was not performed to determine the distribution of data.

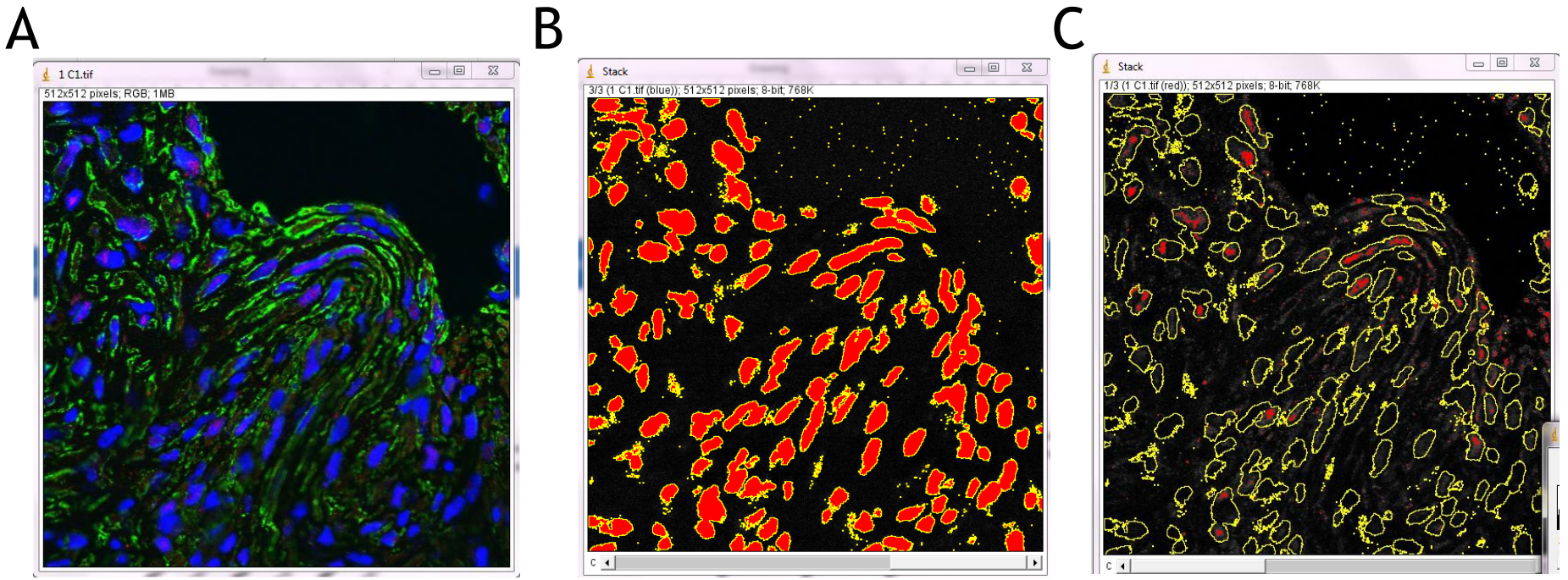

Figure 2.8: Key steps taken to quantify the nuclear presence of PCNA in murine carotid arteries 14 days after ligation.

(A) Screen shot of a representative image (B) the DAPI channel was selected and a pixel threshold of 130 was chosen in order to select all nuclei. A measurement of total area was taken and the nuclei selected as a region of interest (ROI). (C) The green channel was selected and using the same ROI the image was then thresholded to 60 to highlight only PCNA positive area. A measurement was then taken and the area of PCNA signal within the nuclei was presented as a percentage.

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3 Chapter 3 An investigation into the functional role of Ang-(1-9) in the blood flow cessation model of neointima formation

3.1 Introduction

Patients with advanced coronary heart disease are regularly prescribed revascularisation surgeries in order to alleviate symptoms. The choice of surgery is largely dependent on the disease state of the patient. In patients with left main coronary artery disease (with reduced ejection fraction) and patients with multi-vessel disease, the gold standard procedure is CABG surgery (Yahagi et al., 2016). In these patients, CABG (as compared to PCI) is associated with reduced risk of cardiovascular events after one year (Serruys et al., 2009). Despite this, the patency of vein grafts is poorer when compared to arterial grafts (Goldman et al., 2004). With 40% of vein grafts failing between 10-20 years after engraftment, there is a clear unmet medical need to improve the long-term outcomes of vein graft procedures (Fitzgibbon et al., 1996). Because of this need, prosthetic grafts have been developed that are composed of synthetic materials including poly(ethylene terephthalate) (PET, Dacron) and expanded poly(tetrafluoroethylene) (ePTFE, Gore-Tex). These have found some success in peripheral graft procedures where the lumen diameter is larger (>6 mm) (Kapfer et al., 2006). However, complications arise in smaller diameter (<4 mm) grafts that would be required for CABG. For example, the patency of small diameter ePTFE grafts in CABG was only 32% after two years as compared to around 90% patency in SV grafts (Chard et al., 1987, Hehrlein et al., 1984). For this reason, the need to improve the patency of grafted autologous blood vessels is great.

The key process underlying vein graft disease is the development of an expanding and occlusive neointimal lesion (Yahagi et al., 2016). During CABG, the vein graft will experience loss or damage of the endothelium. This can occur in the preparation steps preceding grafting. When vein graft is inflated to check for leakages, the vessel is subjected to distension and increased pressure which can lead to EC apoptosis (Osgood et al., 2014). The vein graft will also be exposed to a period of ischaemia following harvesting and reperfusion injury following engraftment. The damaged endothelium is less capable of producing vasodilatory effects. For example, EC NO production can become impaired leading to increased oxidative stress (Sugimoto et al., 2009).This sets in motion a plethora of cellular and immunogenic events leading to a mitogenic process known as intimal hyperplasia. Intimal hyperplasia describes the migration of VSMCs from the media to the intima where differentiation from a contractile

phenotype to a synthetic phenotype leads to increased proliferation and synthesis of ECM (Wan et al., 2012, Hu et al., 2002, Kalra and Miller, 2000). During this phenotype switch, medial VSMC lose classical contractile genes such as those encoding myosin heavy chain, alpha smooth muscle actin and myocardin (Shankman et al., 2015).

In response to acute injury, ECs and VSMCs in grafted blood vessels upregulate expression of different growth factors and cytokines including but not limited to PDGF-B and MCP-1, which are important mediators of neointimal hyperplasia (Sterpetti et al., 1996, Fu et al., 2012a). Although some remodelling is needed for the vascular graft to have patency, in many cases this leads to the creation of a hyper-cellular and fibrotic lesion which becomes increasingly susceptible to atherosclerosis and can lead to the total occlusion of the vessel (Reviewed in de Vries et al., 2016).

Preclinical models clearly demonstrate that inhibiting VSMC proliferation and migration as well as curbing inflammation aid the prevention of intimal hyperplasia and vein graft disease (Reviewed in de Vries et al., 2016). Such preclinical models include ligation and wire injury of the carotid/femoral artery as well as various vein graft models including the vena cava interposition graft (Abarbanell et al., 2010).

Changes in blood flow contribute to the pathophysiology of vein graft disease (Kohler et al., 1991, Geary et al., 1994, Bassiouny et al., 1992). The effects of blood flow alterations on VSMC can be modelled using murine microsurgical techniques (Nam et al., 2009, Kumar and Lindner, 1997). Ligation of the left carotid artery below the bifurcation results in cessation of blood flow, reduced shear stress, increased arterial-wall tension and delayed thrombus formation at the site of ligation. The remaining section of carotid artery experiences pulsatile blood flow (Kumar and Lindner, 1997). Following ligation, blood flow becomes turbulent and oscillatory, rather than laminar and this is linked to EC damage and apoptosis (Heo et al., 2011). This can lead to neutrophil accumulation and platelets interact with monocytes which can infiltrate within the vessel wall (Franck et al., 2017). Deposition of fibrin (an insoluble protein formed from fibrinogen) is localised around the infiltrated monocytes (Kawasaki et al., 2001). VSMC can then use the deposited fibrin as a matrix to migrate across (Kawasaki

et al., 2001).The onset of rapid VSMC proliferation follows, leading to constrictive vascular remodelling (Kumar and Lindner, 1997).

The carotid ligation model of vascular injury is one of the most commonly used *in vivo* surgical procedures due to the simplicity of the procedure. This arterial vascular injury model does not exactly mimic the setting of a vein graft procedure. Nevertheless, the cellular and molecular events that follow carotid ligation are similar to those that arise during pathological vein graft remodelling e.g. leukocyte infiltration, VSMC proliferation, collagen deposition and increased expression of matrix metalloproteinases (MMPs) (Godin et al., 2000). Moreover, vascular lesions have been associated with sites of low shear stress such as seen at the bifurcation of the carotid artery in humans (Ku et al., 1985)

The origin of the cells that construct the neointimal lesion in response to acute vascular injury has been an issue of contention. For example, in atherosclerotic coronary arteries, resident neointimal foam cells have been reported to express the classical SMC marker aSMA (Allahverdian et al., 2014). Whilst in preclinical models of atherosclerosis a proportion of neointimal VSMCs express macrophage markers (Feil et al., 2014). In order to better identify the origin of these neointimal cells, transgenic fate tracking mice have been designed to fluorescently label VSMCs (Wirth et al., 2007, Gomez et al., 2013). This is made possible by tamoxifen inducible cre-loxP recombination allowing inducible expression of a fluorescent protein under promoter control of a VSMC specific gene such as *MYH11* (Chappell et al., 2016, Gomez et al., 2013). In this scenario, all progeny of *MYH11* positive VSMC will be fluorescently labelled, allowing lineage tracking. Using lineage tracing mice. Chappell et al., demonstrated that the subsequent neointima is composed of a small proportion of VSMCs that have undergone extensive proliferation and described the importance of a unique subset of stem cell antigen-1 (sca1) positive VSMCs that exist in healthy murine carotid arteries (Chappell et al., 2016). After carotid artery ligation, these neointimal VSMCs undergo de-differentiation leading to clonal expansion (Dobnikar et al., 2018). Taken together, the carotid ligation model possesses distinct differences to other models of neointima formation and is a technically simple and high-throughput model that has the capacity to elucidate some of the complex processes underlying neointima formation.

The RAS has substantial influence over VSMC behaviour and EC function via its main effector peptide, Ang II. Manipulation of the RAS in VGF is of great interest from a therapeutic perspective (Min et al., 2005). Excessive Ang II signalling is linked to the pathophysiology of vein graft disease. Ang II induces VSMC proliferation and migration *in vitro* and Ang II infusion accelerates vascular remodelling processes in models of neointima formation (van Kleef et al., 1996, Lee et al., 2018). Gene expression of the AT_1R is increased after vascular injury in rats (Viswanathan et al., 1992). Furthermore, local Ang II production is upregulated in stenotic coronary arteries via upregulation of ACE in neointimal VSMCs and macrophage (Ohishi et al., 1997). Exposure to Ang II leads to the upregulation of chemokines such as MCP-1 in VSMCs and ECs which further contribute to excessive vascular remodelling (Chen et al., 1998, Castineiras-Landeira et al., 2016).

Since its introduction in the 1990s, inhibition of Ang II/AT_1R signalling through the use of ARBs and ACE inhibitors has been immensely successful in treating cardiovascular diseases (Lonn et al., 1994, Burnier and Brunner, 2000). Distribution of ACE inhibitors has been prolific. In 2009, approximately 163 million prescriptions of ACE inhibitors were dispensed in the USA alone (Bian et al., 2010). Despite this, clinical trials evaluating the therapeutic effects of ACE inhibitors in CABG and PCI have not seen the same success. The MERCATOR (Multicentre European Research Trial with Cilazapril after Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis) trial investigated the effect of the ACE inhibitor cilazapril (Vascace) following PCI (MERCATOR, 1992). In this trial, patients received cilazapril twice daily (5 mg) for 6 months following surgery. The study end point was determined by measurement of lumen diameter by angiography. Adverse cardiovascular events following PCI were also recorded, these included death, MI, requirement for further surgery and recurrent angina. Cilazapril did not reduce coronary stenosis following PCI, nor did it significantly reduce any of the adverse clinical events (MERCATOR, 1992).

The Ischemia Management with Accupril post–bypass Graft via Inhibition of the coNverting Enzyme (IMAGINE) trial investigated the effect of treatment with the ACE inhibitor accupril after CABG (Rouleau et al., 2008). In this study, 2553 low risk patients were recruited and adverse cardiovascular events were quantified

after 3 years. These included death, non-fatal MI/stroke, revascularisation and recurrent angina. When compared to patients treated with a placebo, accupril did not improve these outcomes after 3 years (13.7% vs 12.2%). For unknown reasons, ACE inhibition increased incidence of adverse cardiovascular events after 3 months (Rouleau et al., 2008). Therefore, alternative strategies to inhibit the local renin angiotensin system in the context of vascular disease may be a powerful new approach.

Targeting counter-regulatory RAS effectors such as ACE2 which acts to hydrolyse Ang II to Ang-(1-7) may represent a promising new way to inhibit actions of Ang II. Overexpression of ACE2 reverses experimental hypertension in preclinical models (Rentzsch et al., 2008, Yamazato et al., 2007), whilst in the setting of acute vascular injury, genetic deletion of ACE2 exacerbates neointima formation induced by mechanical injury (Sahara et al., 2014). In preclinical models Ang-(1-7) has been shown to prevent onset of experimental hypertension (Shi et al., 2015). Notably, a vasodilatory effect has also been reported in humans. Intra-arterial delivery of Ang-(1-7) increased forearm blood circulation in both healthy and hypertensive patients (Sasaki et al., 2001). Ang-(1-7) has been reported to inhibit basal VSMC proliferation as well as proliferation induced by serum, Ang II and PDGF-BB (Freeman et al., 1996). In a rat model of restenosis, Ang-(1-7) infusion inhibited neointima formation (Langeveld et al., 2005). Similarly, Ang-(1-7) inhibited vein graft neointima formation in a rat jugular interposition model of disease (Wu et al., 2011). A phase II clinical trial assessing the effect of Ang-(1-7) on cognitive impairment following CABG was approved however has been terminated due to funding (*ClinicalTrials.gov*: NCT03252093). This is unfortunate as it would have assessed the safety of Ang-(1-7) administration in CABG patients. Furthermore, secondary follow up analyses of graft patency could have been insightful.

Ang-(1-9) is a another novel angiotensin peptide that has been reported to confer cardio-protection by antagonising Ang II/AT_1R signalling via the AT_2R (Flores-Muñoz et al., 2011). The role of Ang-(1-9) in neointima formation is beginning to be unravelled. Chronic infusion of Ang-(1-9) inhibited neointima formation after mechanical arterial injury (McKinney, Robertson et al., in preparation). In these studies, Ang-(1-9) administration also inhibited neointima

formation after interpositional grafting of the vena cava into the carotid artery (McKinney, Robertson et al., in preparation). Furthermore, Ang-(1-9) significantly inhibited Ang II induced migration of human saphenous vein VSMC (HSVSMC) through a pathway blocked by the AT_2R antagonist PD123,319 (McKinney et al., 2015). These data suggest Ang-(1-9) is a potential therapeutic target to inhibit intimal hyperplasia in the setting of vascular injury.

However, the pathophysiology of VGF is complex, dependent on pulsatile pressure, ischemia, mechanical damage to the endothelium, inflammation and activation of VSMCs (Reviewed in de Vries et al., 2016). For this reason, it is important to study the effect of Ang-(1-9) in different animal models that are able to recapitulate different features of pathology relevant to vein graft disease. Local and systemic inflammation an important step in the multifaceted process that leads to the development of vein graft disease. Shortly after revascularisation platelets, neutrophils, monocytes and leukocytes are activated and recruited to the site of injury (Serrano et al., 1997, Welt et al., 2000). After adherence or infiltration to the site of injury, production of growth factors and cytokines such as PDGF-BB can promote intimal hyperplasia (Jawien et al., 1992). VSMCs and ECs subsequently also upregulate expression of endogenous cytokines, adhesion factors, and growth factors (Rectenwald et al., 2000, Herring et al., 2017). Gene expression of proinflammatory cytokines in remodelled venous grafts have been reported as up to 30 times higher than in atherosclerotic coronary arteries (Christiansen et al., 2004).

Preclinically, induction of systemic inflammation through administration of lipopolysaccharide (LPS) has been shown to exacerbate neointima formation in a rabbit model of restenosis (Danenberg Haim et al., 2002). Inhibition of inflammation through administration of recombinant IL-10 or anti-IL-1B antibodies has been reported to attenuate neointima formation in various models on vascular injury (Feldman Laurent et al., 2000). Systemic elevations in certain cytokines also have prognostic value as they can be used to detect complications following revascularisation (de Lemos et al., 2007, Buffon et al., 1999).

The present study aimed to analyse the effect of Ang-(1-9) infusion on neointima formation triggered by cessation of blood flow using the carotid ligation model

(Kumar and Lindner, 1997). This was investigated at an early (Day 7) and at an intermediate time point (Day 14) in order to study three key processes in vascular remodelling (1) inflammation, (2) VSMC proliferation and (3) fibrosis.

3.1.1 Aims

- Investigate the effect of Ang-(1-9) on inflammation in the setting of vascular injury by quantification of 23 circulating chemokines and cytokines including those established as key players in the progression of vein graft disease, such as MCP-1 and IL-1B.

- Investigate the effect of subcutaneous delivery of Ang-(1-9) on VSMC proliferation in a carotid artery ligation model of neointima formation.

- Investigate the effect of subcutaneous delivery of Ang-(1-9) on arterial fibrosis after carotid ligation.

3.2 Results

3.2.1 The effect of subcutaneous delivery of Ang-(1-9) on the inflammatory prolife of serum in healthy and injured mice

The first studies assessed the effects of osmotic mini pump-mediated delivery of Ang-(1-9) at 48 µg/kg/hour in normal healthy mice with the aim of establishing a benchmark as to any effects on serum cytokine profiles. Two groups of healthy mice were compared which had either continuous subcutaneous infusion of Ang-(1-9) at 48 µg/kg/hour or ultra-pure water (control) for seven days. Serum was isolated from whole blood obtained via cardiac puncture. Analytes were then detected using a color-coded bead-based immuno-multiplexed assay (Bio-Plex™). The following analytes were either undetected or detected at low concentrations within the 1-10 pg/mL range (mean concentrations). These were largely interleukins; IL-1A, IL-1B, IL-2, IL-3, IL-4, IL-5, IL-6, IL7, IL-9, IL-13, IL-17. (Figure 3.2). Cytokines detected in this range included granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-g) and macrophage inflammatory protein 1 alpha (MIP-1a) (Figure 3.2). The analytes detected in the low to mid-range (mean 20-50 pg/mL) were IL-10, IL 12 (p70), keratinocyte chemoattractant (KC) [also known as C-X-C motif chemokine ligand (CXCL-1), macrophage inflammatory protein 1 beta (MIP-1b) and TNF-a (Figure 3.3). Mean concentrations of RANTES (CCL5), EOTAXIN (CCL11), MCP-1, G-CSF and IL-12 (p40) were detected between 50-750 pg/mL (Figure 3.4). In non-injured animals Ang-(1-9) infusion did not significantly alter the circulating concentrations of any cytokine or interleukin.

3.2.1.1 Sera inflammatory prolife seven days after carotid artery ligation

Next, mice were subjected to carotid ligation (CL) surgery and separated into groups that were implanted subcutaneously with an osmotic mini pump infusing either Ang-(1-9) or water for 7 days. Again, serum cytokine profiling revealed no significant differences between treatment groups, with all analytes in the same ranges as described above (Figure 3.1). IL-9 was detected in a number of healthy mice infused with water (2.63 pg/mL) or Ang-(1-9) (2.88 pg/mL), or in control water infused mice that had undergone carotid ligation (1.325 pg/mL) but was undetectable in any mice infused with Ang-(1-9) (Figure 3.2). However, these

levels were at the lower end of the detection limit so these data may be negligible.

Lastly, the effect of carotid ligation was assessed by comparing the levels of cytokines from healthy and ligated mice. Carotid ligation in control water infused mice minorly altered two cytokines at the seven-day time point. Systemic concentrations of IL-5 (Water 1.3±0.121 vs Water + CL 9.25±1.178 pg/mL $p<0.001$) and KC (Water 11.799±2.011 vs Water + CL 31.21±5.718 pg/mL $p<0.01$) were significantly increased in mice treated with water (Figure 3.2-3.3).

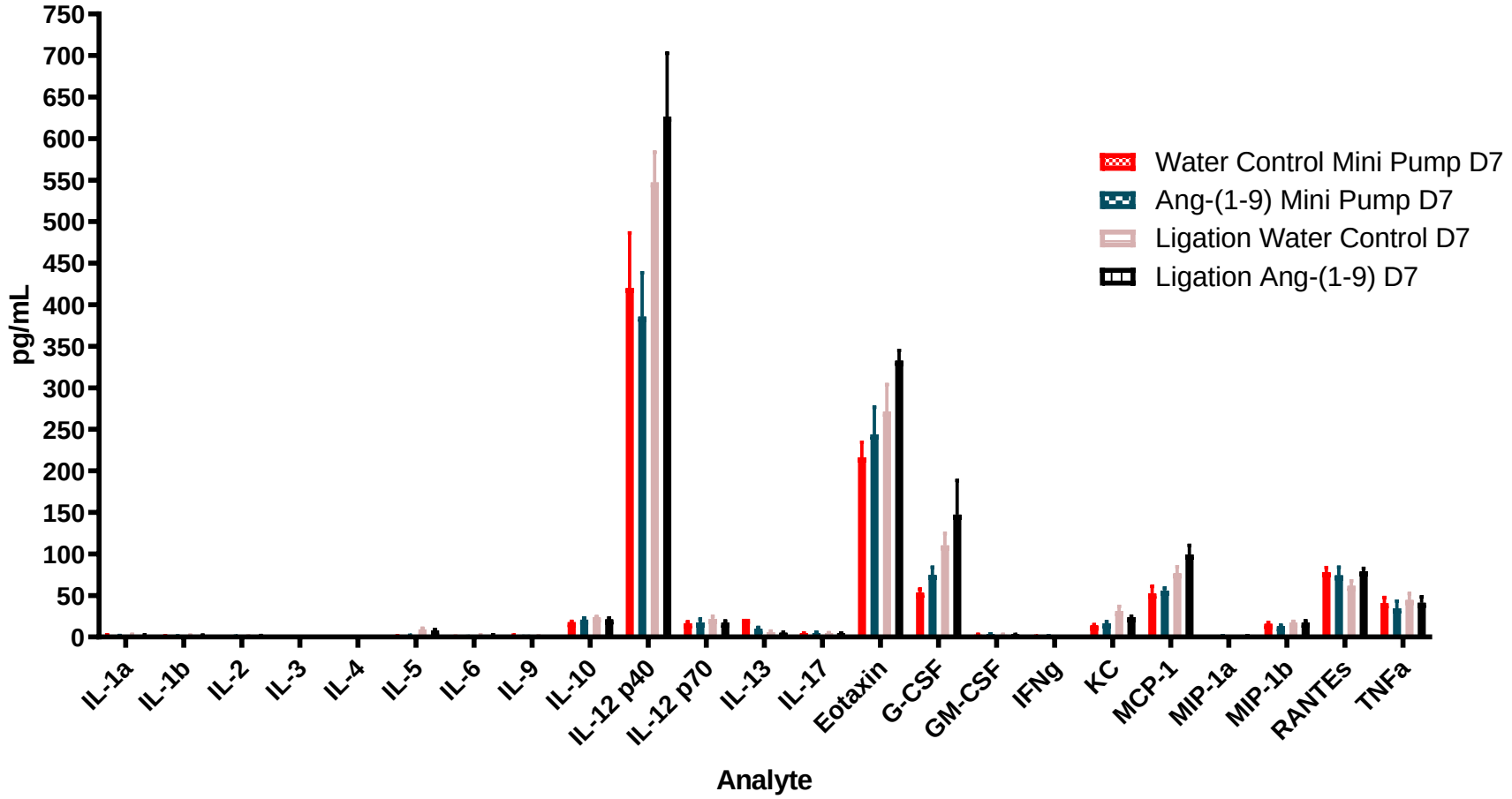

Figure 3.1 The inflammatory serum profile after Ang-(1-9) infusion and/or carotid ligation at the 7 day timepoint following injury.

C57 BL6/J mice were infused with water or Ang-(1-9) at 48 µg/kg/hour and either left uninjured or were subject to carotid ligation surgery. Seven days later concentrations of 23 circulating cytokine and chemokines were determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay. Observed concentrations of each analysed detected in serum in mice that had been systemically infused with water (red) [control] or Ang-(1-9) (teal) for 7 days [n=7]. Sera from mice that had undergone carotid ligation and treated with water (pink) or Ang-(1-9) (black) [n=8].

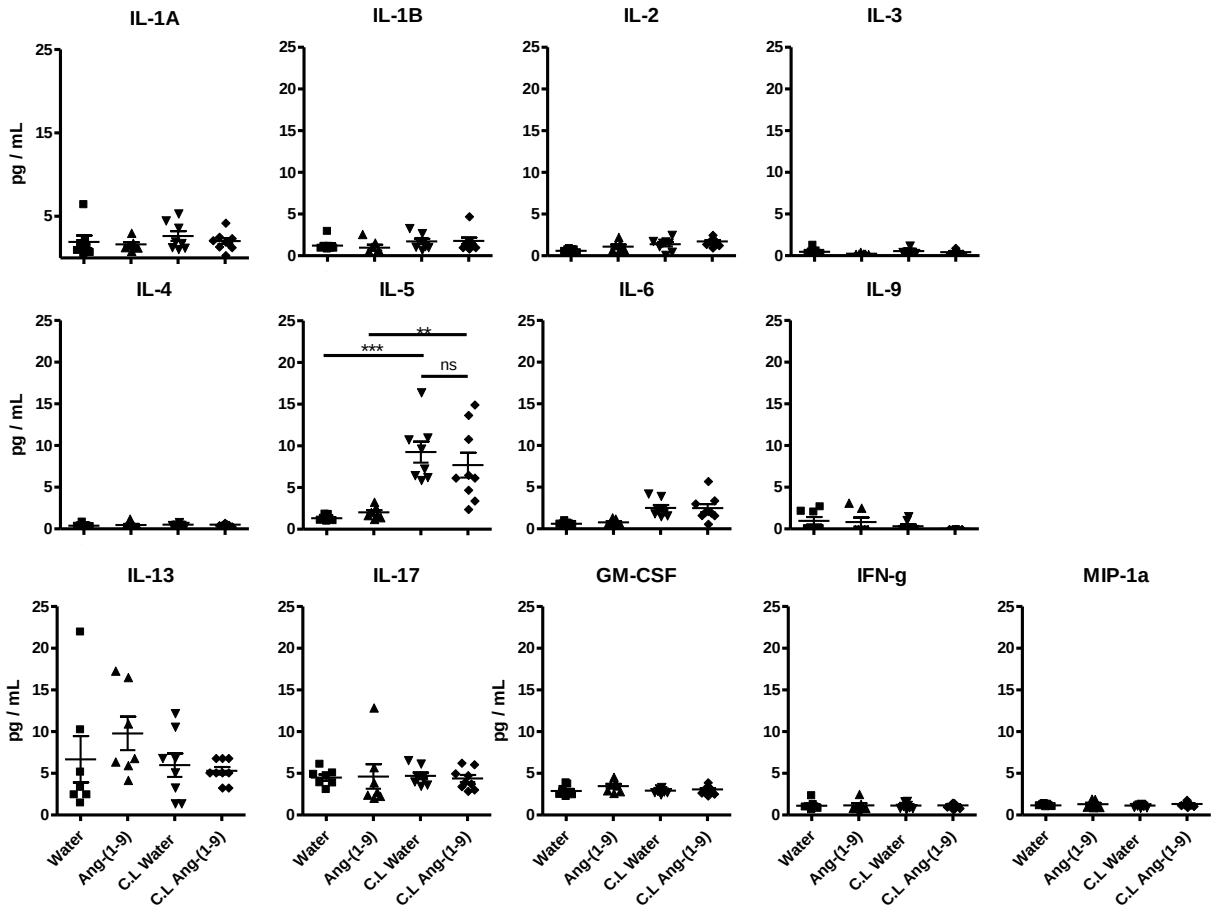

Figure 3.2 Analytes that were undetectable or detected in the low range (1-10 pg/mL) in murine serum 7 days after ligation injury. C57 BL6/J mice were infused with water or Ang-(1-9) [n=7] at 48 µg/kg/hour and either left uninjured or were subject to carotid ligation surgery [n=8]. Seven days later concentrations of 23 circulating cytokine and chemokines were determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay Observed concentrations of analytes either undetected or detected in the low range of (1- 10 pg/mL). One-way ANOVA with Tukey's multiple comparison post hoc test. ** = $p<0.01$ ***=$p<0.001$

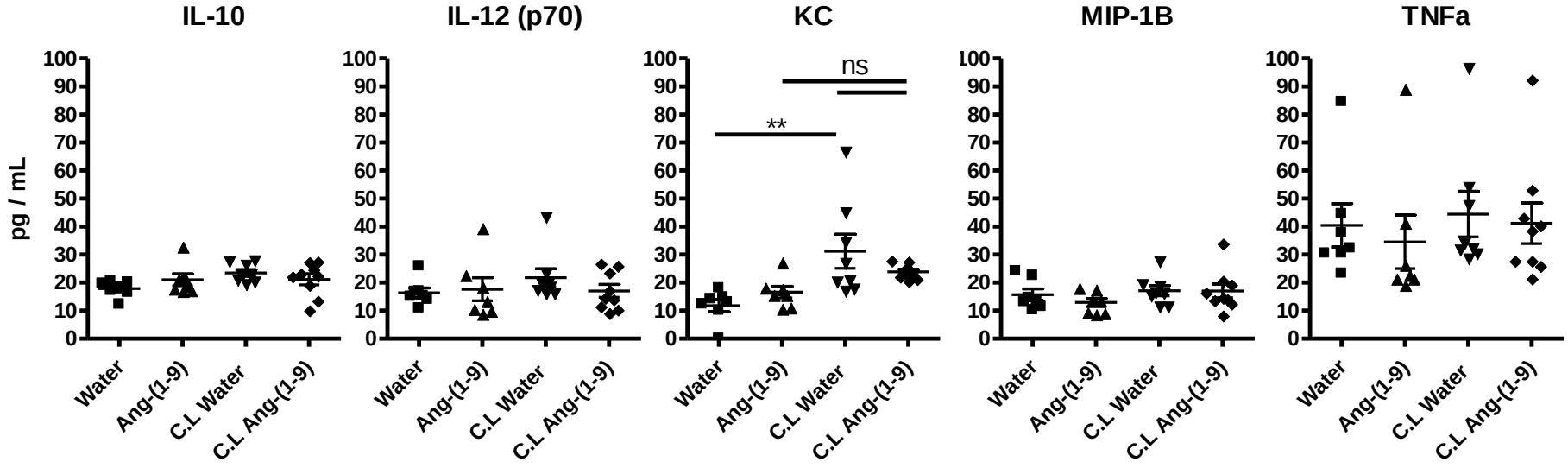

Figure 3.3 Analytes that were detected in the low- mid range (10-50 pg/mL) in murine serum 7 days after ligation injury.

C57 BL6/J mice were infused with water or Ang-(1-9) [n=7] at 48 µg/kg/hour and either left uninjured or were subject to carotid ligation surgery [n=8]. Seven days later concentrations of 23 circulating cytokine and chemokines were determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay Observed concentrations of analytes either undetected or detected in the low range of (1- 10 pg/mL). One-way ANOVA with Tukey's multiple comparison post hoc test. ** = $p<0.01$

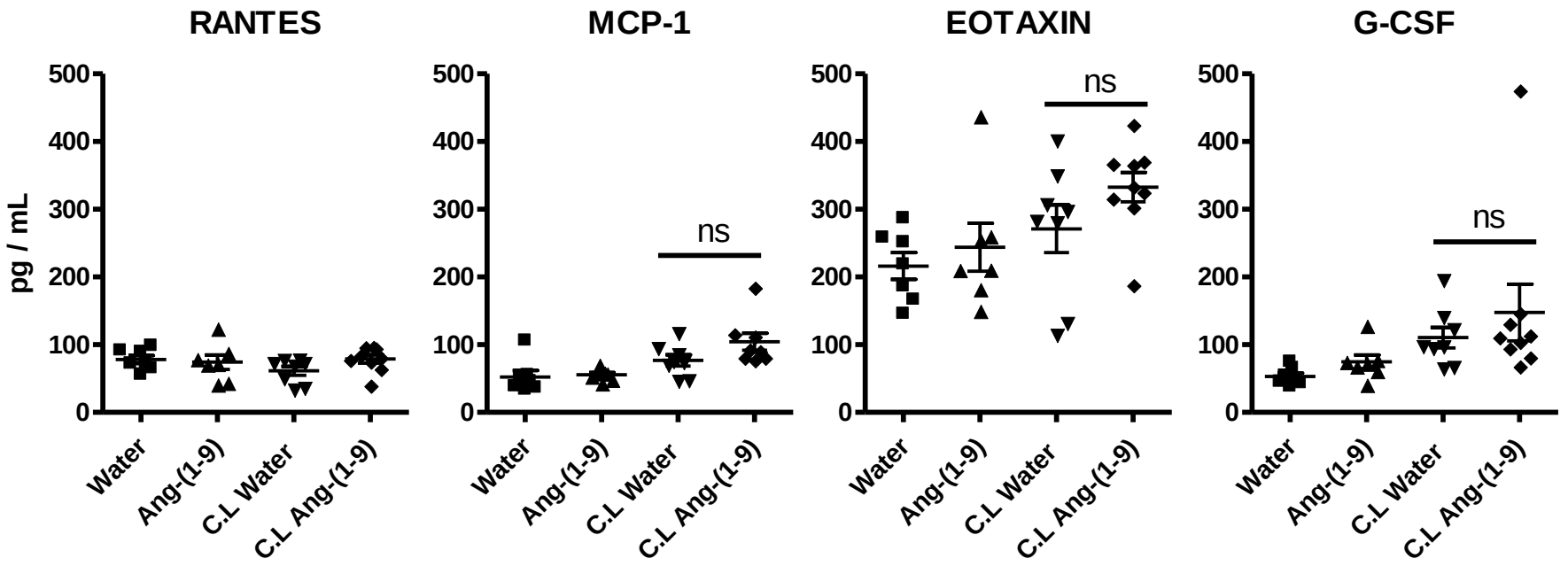

Figure 3.4 Analytes detected at a mid to high range concentration (>50 pg/mL) in murine serum 7 days after ligation injury.

C57 BL6/J mice were infused with water or Ang-(1-9) [n=7] at 48 µg/kg/hour and either left uninjured or were subject to carotid ligation surgery [n=8]. Seven days later concentrations of 23 circulating cytokine and chemokines were determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay Observed concentrations of analytes either undetected or detected in the low range of (1- 10 pg/mL). One-way ANOVA with Tukey's multiple comparison post hoc test. ns = not significant p>0.05

3.2.2 The effect of subcutaneous delivery of Ang-(1-9) on aortic gene expression

The data previously described utilised multiplexing technology to measure cytokine levels in serum at the 7-day timepoint following carotid ligation injury. This might not be sensitive enough to detect local vascular changes in inflammatory genes. Subsequently, it was decided to employ quantitative real time polymerase chain reaction (QRTPCR) to investigate whether systemic infusion of Ang-(1-9) altered the mRNA transcript levels of pro-inflammatory cytokines in murine arteries. Since the entire left carotid arteries were taken for histological analysis, the thoracic aortae were snap frozen in liquid nitrogen for analysis of target gene expression. First, it was confirmed that murine aortae from both healthy and CL mice infused with either water or Ang-(1-9) expressed the AT_2R (*AGTR2*) at similar levels (Figure 3.5 first panel).

Murine aortae from healthy mice infused with water expressed *AGTR2* [Water control ΔCt 16.82±0.190] and this was not altered by infusion of Ang-(1-9) [Ang-(1-9) ΔCt 16.77±0.197 $p>0.05$] (Figure 3.5). MCP-1 otherwise known as C-C motif chemokine ligand 2 (*CCL2*) and IL-1B were assessed due to well established roles in vascular remodelling and the pathophysiology of VGF (Reviewed in de Vries et al., 2016). While IL-9 was chosen based on being identified as promising in section 3.2.5. In healthy mice the target genes chosen differed in expression levels in thoracic aortae with *CCL2* being the most highly expressed gene [Water Control ΔCt 13.11 ±0.131] but Ang-(1-9) infusion did not affect expression levels [Ang-(1-9) ΔCt 13.10 ±0.156 $p>0.05$]. Similarly, IL-1B expression was evident and again Ang-(1-9) infusion did not affect expression levels [Water Control ΔCt 15.83 ±0.318 *vs* Ang-(1-9) 15.97±0.202 $p>0.05$]. However, IL-9 mRNA transcripts were detected in mice infused with water [ΔCt 18.44±1.22] but completely undetected in the aortic tissue taken from mice infused with Ang-(1-9) for 7 days (Figure 3.5).

3.2.2.1 Aortic gene expression seven days after carotid artery ligation

Next, the thoracic aortae from mice that had undergone carotid ligation and infused with water or Ang-(1-9) were assessed for expression of the same target genes. *AGTR2* [ΔCt 16.938±0.41 Water Control] was expressed and this was not

altered by infusion of Ang-(1-9) [ΔCt 16.805±0.217 Ang-(1-9) $p>0.05$] (Figure 3.6). IL-1B was expressed [ΔCt 14.735±0.236 Water Control] but Ang-(1-9) did not affect expression levels [ΔCt 15.171±0.329 Ang-(1-9) $p>0.05$]. While *IL-9* was largely undetected (Figure 3.6). However, *CCL2* expression was significantly decreased (approx. 43%) in Ang-(1-9) infused mice compared to water infused mice (ΔCt 10.917±0.13 Water Control vs 11.340±0.159 Ang-(1-9) $p<0.05$) (Figure 3.6).

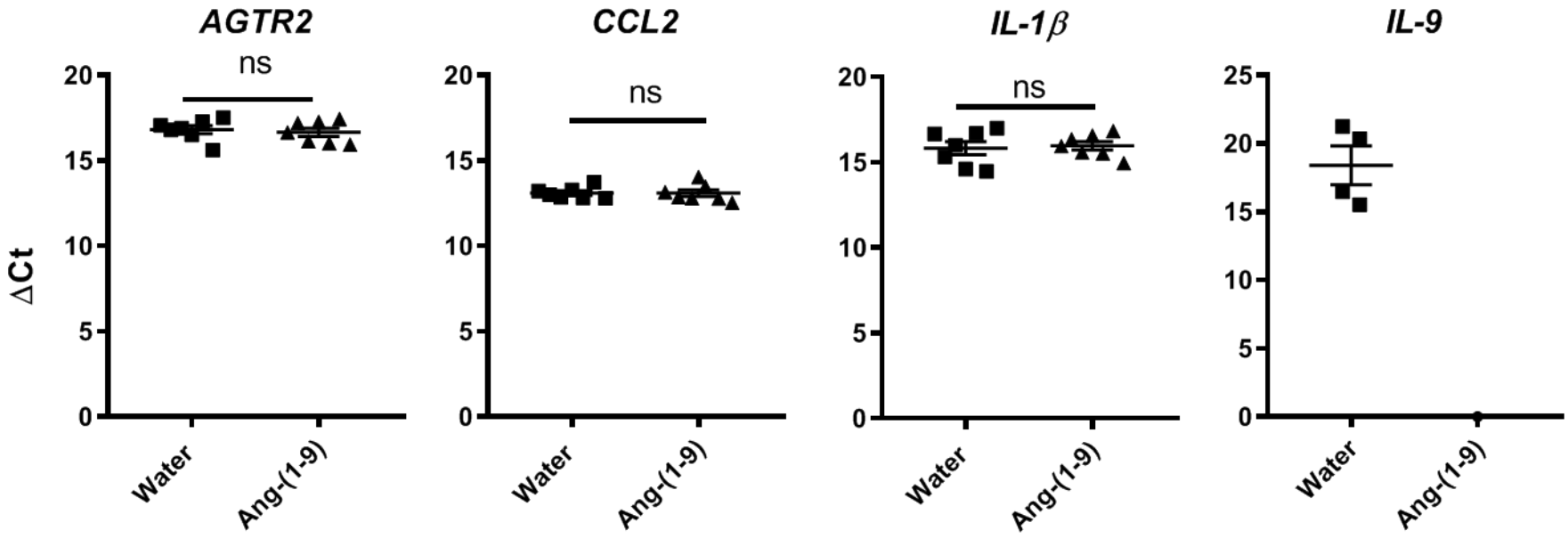

Figure 3.5 Effects of subcutaneous delivery of Ang-(1-9) on murine aortic gene expression in healthy mice.

QRTPCR was used to determine gene levels of *CCL2, IL-1B*, *AGTR2* and *IL-9* after mice were infused with water or Ang-(1-9) for 7 days (n=7) Students *t* test was applied to this data. ns= non-significant (p>0.05.)

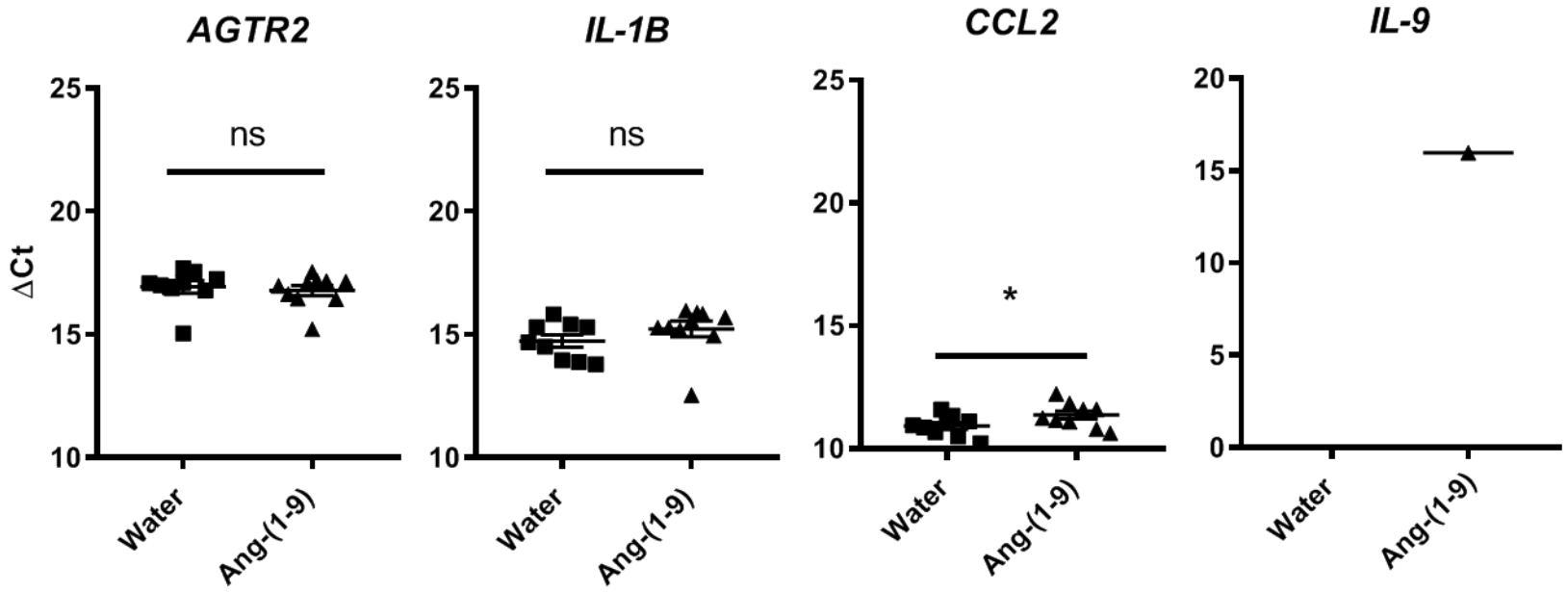

Figure 3.6 Effects of subcutaneous delivery of Ang-(1-9) on murine aortic gene expression seven days after carotid ligation.

QRTPCR was used to determine gene levels of *CCL2, IL-1B*, *AGTR2* and *IL-9* after mice were infused with water or Ang-(1-9) for 7 days after carotid ligation surgery (n=9) Values were normalised to *GAPDH* and represent the mean delta Ct ± SEM. A student's t test was applied to compare treatment groups. *p<0.05 ns>0.05.

3.2.3 The effect of subcutaneous delivery of Ang-(1-9) on early vascular remodelling in the murine carotid ligation model of neointima formation (day 7)

Previous studies have demonstrated that ligating the murine left carotid artery at the distal bifurcation leads to neointimal hyperplasia as a consequence of cessation of blood flow, leukocyte infiltration, VSMC proliferation and endothelial dysfunction (Kumar and Linder, 1997, Zhang et al., 2008). This process takes between 7-28 days (Herring et al., 2014). C57 BL/6 mice underwent carotid ligation surgery in order to cause an immediate cessation of blood flow. This was followed by implantation of an osmotic mini pump containing either water or Ang-(1-9) [48 μg/kg/hour]. Mice were recovered and the effect on vascular remodelling assessed 7 days later by standard histological methods (H/E staining) in order to visualise vessel morphology (Figure 3.7 A). Vascular remodelling was detected by the presence of cell layers at the lumen side of the internal elastic lamina (Figure 3.7A). Upon quantification, Ang-(1-9) did not significantly alter of the area of the intima [6989.1±1507 μm^2 Water Control vs 8229.73±859.6 μm^2 Ang-(1-9) $p>0.05$] (Figure 3.7C). Similarly, the mean media area was unaffected by Ang-(1-9) infusion at this time point [32701±2614 μm^2 Water Control vs. 40568±2221.3 μm^2 Ang-(1-9) $p>0.05$] (Figure 3.7D). Furthermore, the differences between ratio of the intima and media (NI:M) were also statistically insignificant [0.195±0.04 Water Control vs 0.207±0.03 Ang-(1-9) $p>0.05$]. (Figure 2.7E). Overall, Ang-(1-9) had no detectable effect on vessel morphology 7 days after carotid ligation.

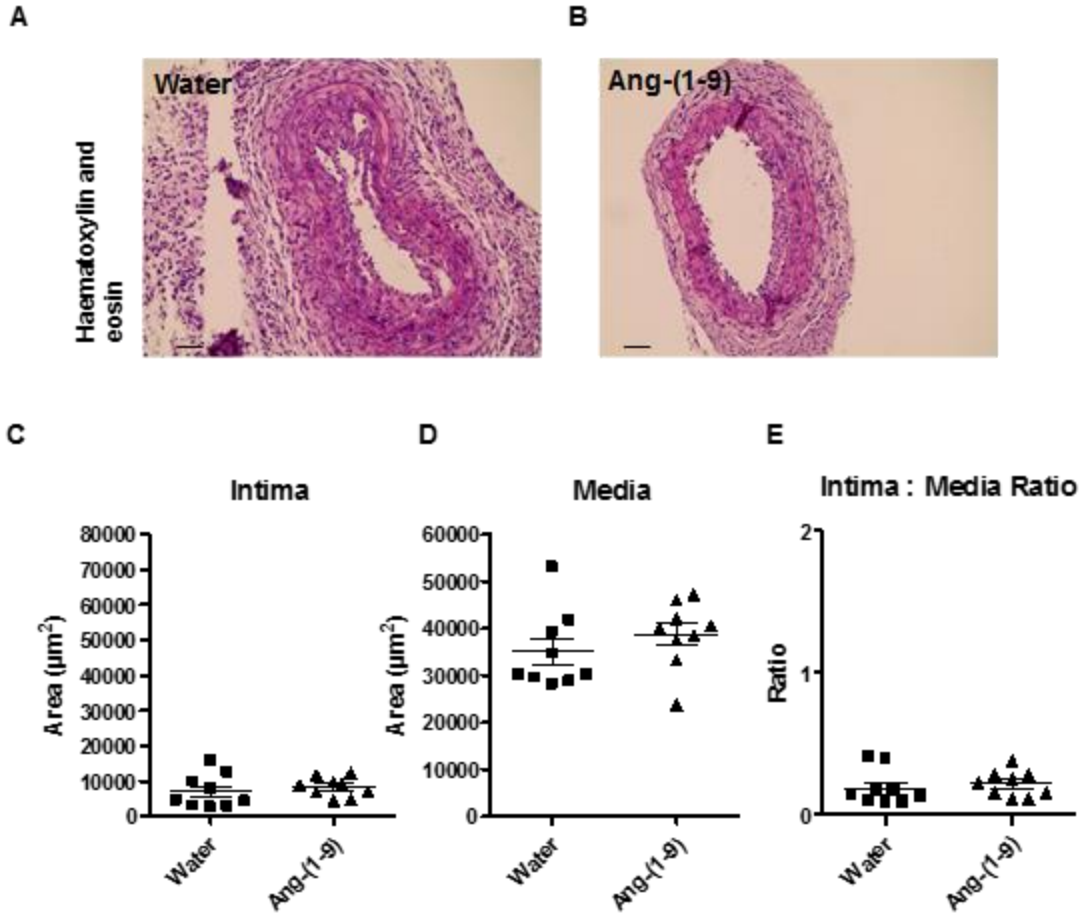

Figure 3.7 The effect of subcutaneous delivery of Ang-(1-9) in a mouse model of blood flow cessation induced intimal hyperplasia

(A/B) Histological staining (H/E) of ligated carotid arteries 7 days after injury allowed visualisation of vessel morphology. (C-E) To assess the effect of Ang-(1-9) infusion on vascular remodelling: the areas of the intima and media (μm^2) were calculated and is presented also as a ratio of the intima and media area. These mean values were used to compare each data set by applying a student's *t* test. 4μm cross sections were cut from 3 separate locations of injured murine carotid arteries in this study. Per loci, the morphology of 3 sections were analysed. Values represent the means ± SEM**.** Scale bar 100μm.

3.2.4 The effect of Ang-(1-9) on 5-Ethynyl-2´-deoxyuridine (EdU) incorporation 7 days after ligation

Seven days after carotid ligation the neointimal lesion in the carotid artery is reported to be still developing (Kumar and Lindner, 1997). It was therefore decided that it was an appropriate time point to assess early events such as VSMC proliferation that lead to the progression of intimal hyperplasia. C57 BL/6 mice were administered with EdU via IP injection 5 hours before they were humanely killed. *In vivo* EdU labelling was performed to identify proliferating cells in ligated arteries. EdU incorporation in the nuclei of cells of vascular cells gave a highly sensitive "snap shot" of cells that were actively replicating their DNA at this time point. Seven days after carotid ligation, VSMC in the intima but also the media were actively proliferating (Figure 3.8A). Region of interest (ROI) image analysis was used to quantify the percentage area of EdU (green) positive signal within all the nuclei (blue) of the whole cross section of the carotid artery. Using this as a read out for nuclear co-localisation no statistically significant differences were determined [Water Control 4.916±2.01% vs Ang-(1-9) 1.93±0.41% p=0.18) (Figure 3.8B). Next, the number of EdU positive nuclei in the intima, media and adventitia were counted manually with the aid of ImageJ analysis software. Upon quantification, the average percentage of EdU positive cells in the intima of water infused mice was higher than in Ang-(1-9) infused mice [Water Control 3±1.29% vs Ang-(1-9) 0.33±0.316% p>0.05]. However, when a student's *t* test was applied the p value was only approaching significance (p=0.083) (Figure 3.9A). Similarly, there was no statistical differences in Edu positive nuclei in the media [Water Control 1.25±0.765% vs Ang-(1-9) 2.02±0.87% p>0.05] or the adventitia [Water Control 0.91±0.43% vs Ang-(1-9) 2.27±1.52% p>0.05]. (Figure 3.9B/C)

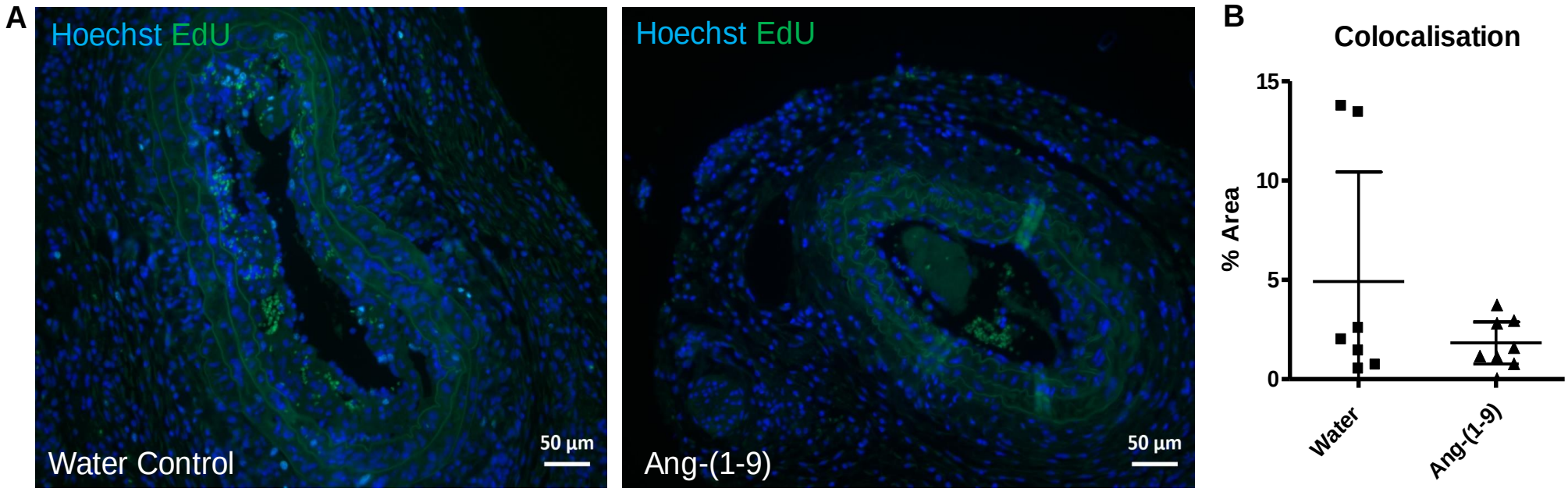

Figure 3.8 *In vivo* EdU incorporation 7 days after ligation.

(A) Fluorescence microscopy was used to detect EdU positive regions (green) that co-localised within the nuclei (blue) that were visualised using Hoechst dye. (B) ImageJ analysis determined the of percentage area of EdU positive signal within nuclei within the whole vessel. Scale Bar 50µm Representative images. One cross section of carotid artery per mouse. n=7-8.

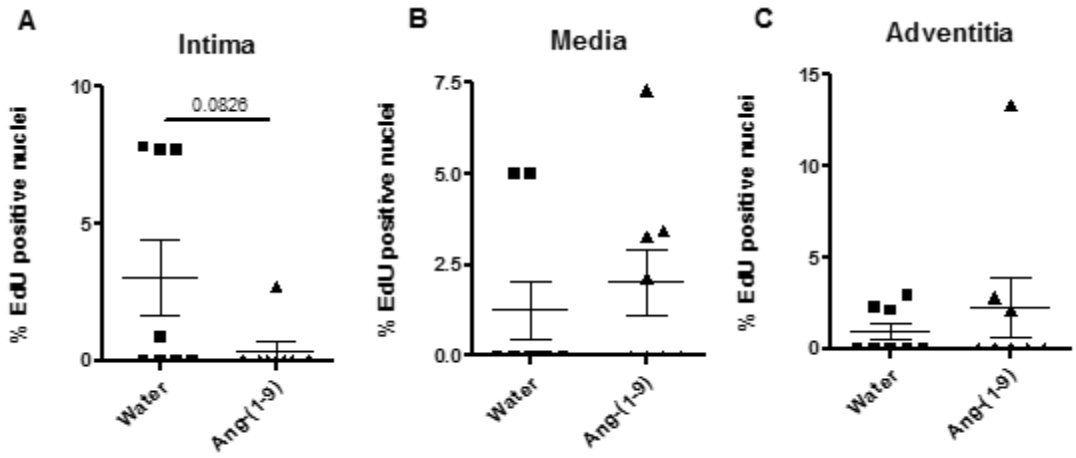

Figure 3.9 *In vivo* EdU incorporation 7 days after ligation.

Manual cell counts determined the percentage of EdU positive nuclei with different anatomical regions of vessel including the (A) intima (B) media and (C) adventitia.

3.2.5 Ang-(1-9) infusion affects the cytokine profile of murine serum 14 days after injury

Next, the sera from individual mice infused with water or Ang-(1-9) for 14 days after carotid ligation in comparison to sham-operated mice were analysed by the bead-based Bio-Plex™ assay (Figure 3.10). The following analytes were either undetected or detected at low concentrations within the 1-10 pg/mL range (mean concentrations). These were IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, IFN-g, MIP-1a and MIP-1b (Figure 3.11). Analytes detected at the low to intermediate range (10-60 pg/mL) were IL-1A, IL-5, IL-10, IL-12 (p70),IL-13, IL-17, EOTAXIN, G-CSF, KC and RANTES (Figure 3.12A).Cytokines detected at the highest range of concentrations (100-350 pg/mL were IL-12 (p40) and TNF-α (Figure 3.12B).

At this timepoint, systemic concentrations of IL-1A ($p<0.05$) and RANTES ($p<0.05$) were significantly lower in mice that had underwent carotid ligation surgery (Figure 3.13). Circulating concentrations of IL-1B were increased after carotid ligation as compared to sham-operated animals [Sham 62.23±2.66 pg/mL vs Water Control 105.98±14.6 $p<0.05$] (Figure 3.14). This effect was not present when mice had been implanted with an Ang-(1-9) containing osmotic mini pump.

Subcutaneous delivery of Ang-(1-9) only significantly altered the circulating levels of one cytokine, MCP-1. Systemic levels of MCP-1 was decreased by 27.4% [Water Control 53.4±4.7 pg/mL vs Ang-(1-9) 38.76 1.7± pg/mL: $p<0.05$ via Students T test] (Figure 3.13). IL-9 was undetectable in all mice treated with Ang-(1-9) (Figure 3.13). The levels of the anti-inflammatory cytokines IL-10 or IL-13 were unaffected by Ang-(1-9) infusion (Figure 3.13A)

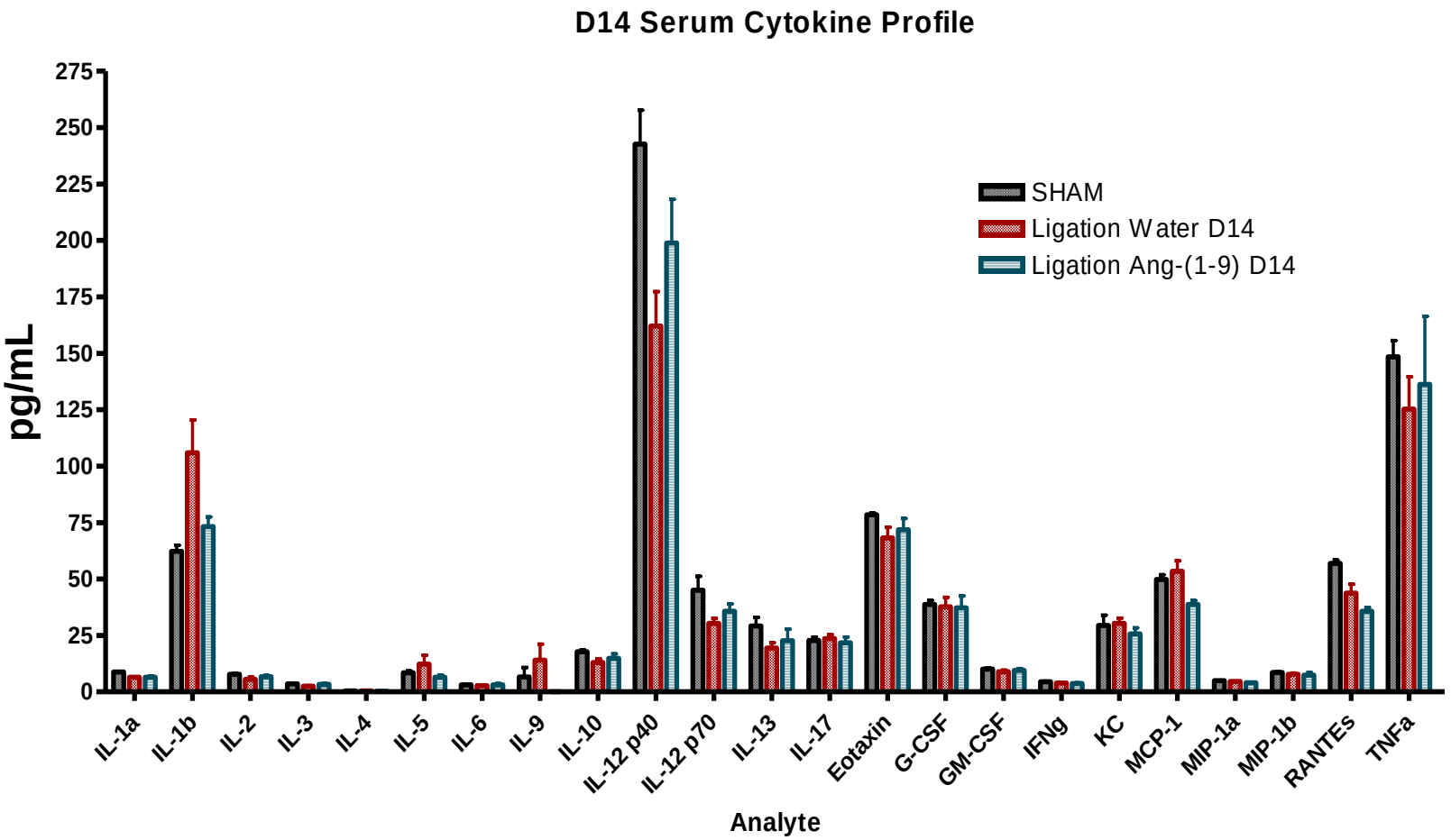

Figure 3.10 Ang-(1-9) alters the inflammatory prolife in serum 14 days after ligation. Serum concentrations of (A) 23 circulating cytokine and chemokines as determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay Cytokine concentrations in mice that had undergone carotid ligation surgery and treated with water or Ang-(1-9) by subcutaneous osmotic mini pump infusion. Sera from independent mice n=7.

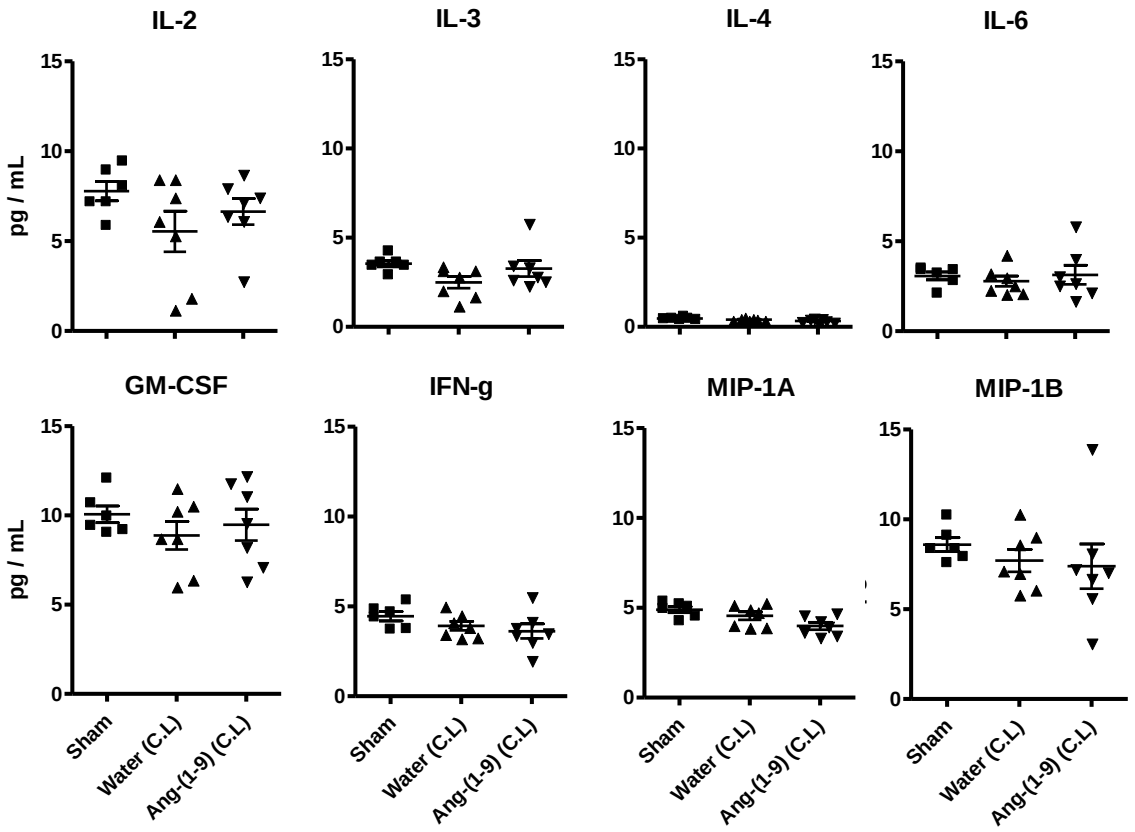

Figure 3.11 Inflammatory cytokines and chemokines detected at low levels (1-10 pg/mL) in mouse serum 14 days after carotid artery ligation. Serum concentrations of 23 circulating cytokine and chemokines as determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay. Dot plot of concentration levels to MCP-1, IL-1B and IL-9 in mice that had undergone carotid ligation surgery and treated with water or Ang-(1-9) by subcutaneous osmotic mini pump infusion. Sera from independent mice n=7. Values represent the means ± S.E.M. A One-way ANOVA was performed with Tukey's multiple comparison post hoc test to compare the effects of Ang-(1-9) to water infusion in the carotid ligation model...

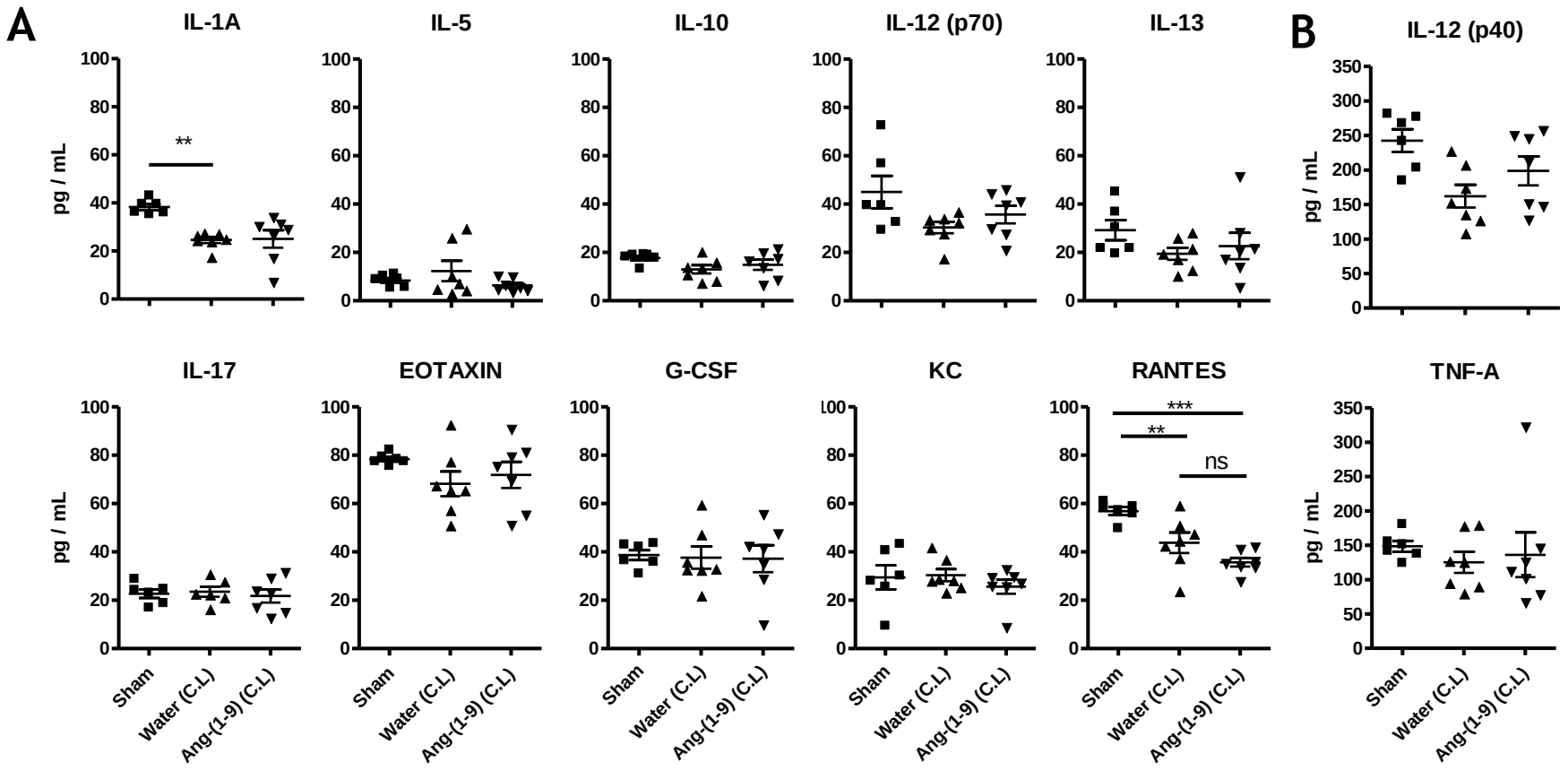

Figure 3.12 Inflammatory cytokines and chemokines detected at mid-range (10-100 pg/mL) and high levels (100-300 pg/mL) in mouse serum 14 days after carotid artery ligation Serum concentrations of (A) Circulating cytokine and chemokines detected within the range of 10-100 pg/mL as determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay**. (B)** Cytokines detected above 100 pg/mL. Sera from independent mice n=7. Values represent the means ± SEM. A One-way ANOVA was performed with Tukey's multiple comparison post hoc test to compare the effects of Ang-(1-9) and water infusion in the carotid ligation model *p<0.05.*p<0.05 ***p<0.001.

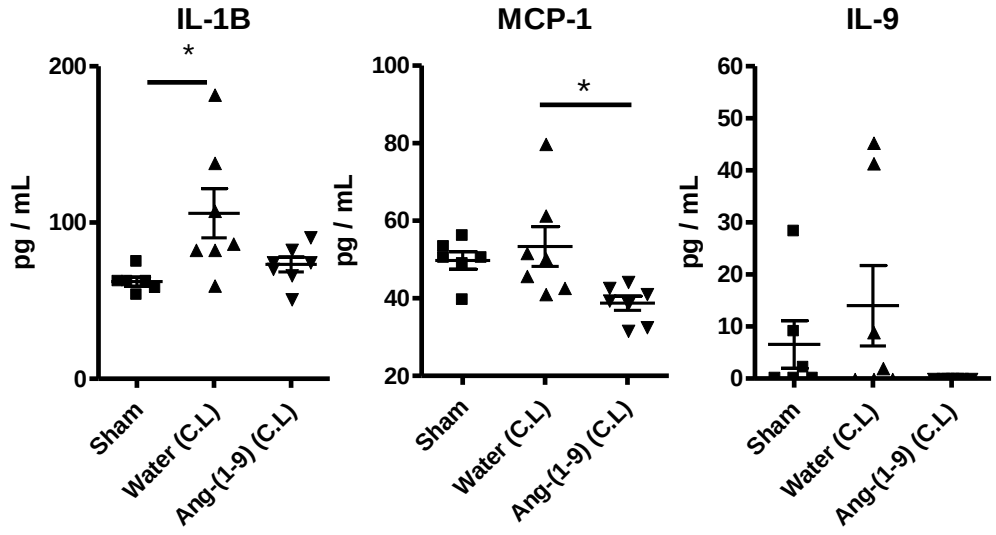

Figure 3.13 Inflammatory analytes detected in serum at varying concentrations across treatment groups 14 days after carotid artery ligation

Serum concentrations of 23 circulating cytokine and chemokines as determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay. Dot plot of concentration levels to MCP-1, IL-1B and IL-9 in mice that had undergone carotid ligation surgery and treated with water or Ang-(1-9) by subcutaneous osmotic mini pump infusion. Sera from independent mice n=7. Values represent the means ± SEM. A One-way ANOVA was performed with Tukey's multiple comparison post hoc test to compare the effects of Ang-(1-9) and water infusion in the carotid ligation model *p<0.05.

3.2.6 Aortic mRNA expression in water and Ang-(1-9) peptide infused mice

It was important to assess whether changes in the concentrations of circulating cytokines were also present in arterial blood vessels. Gene expression of *CCL2* (MCP-1), *IL-1B, IL-9* and *AGTR2* (AT_2R) were analysed in the aortae of sham-operated, mini pump infused and ligated mice. *CCL2* [ΔCt 13.9±0.38], *AGTR2* [ΔCt 18.362±0.36] and *IL-1B* [ΔCt 16.877± 0.22] were all detected in C57 BL/6 aortae while *IL-9* was undetectable (Data not shown). Carotid ligation led to a significant increase in aortic *IL-1B* gene expression as compared to sham-operated animals (vs ligation: ΔCt 15.380±0.42 $p<0.01$) (Figure 3.14 middle panel). Similarly, *AGTR2* mRNA levels were significantly increased in the aortae of Ang-(1-9) infused mice as compared to sham-operated mice [Sham ΔCt 18.362±0.36 vs Ang-(1-9) 16.561±0.58 $p<0.05$] but were not different to control (water infused) carotid ligation mice [Water Control ΔCt 17.130±0.346 vs Ang-(1-9) 16.561±0.58 $p<0.05$] (Figure 3.14 right panel).

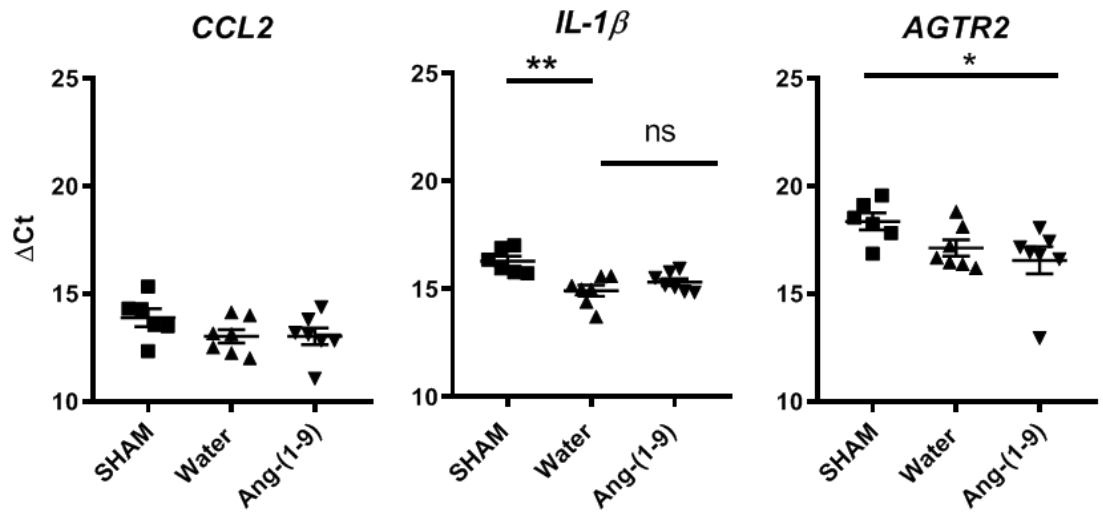

Figure 3.14 Effects of subcutaneous delivery of Ang-(1-9) on murine aortic gene expression 14 days after ligation injury

QRTPCR was used to determine gene expression levels of *CCL2, IL-1B*, *AGTR2* and *IL-9* (undetected) in the aortae of mice infused water or Ang-(1-9) 14 days after sham-operation or carotid ligation surgery (n=6-8). The data presented is expressed as delta cycle threshold (ΔCt), where the higher ΔCt value translates as lower expression. The endogenous control used was GAPDH. A One-way ANOVA was performed with Tukey's multiple comparison post hoc test *p<0.05 **p<0.01

3.2.7 The effect of subcutaneous delivery of Ang-(1-9) on vascular remodelling 14 days after carotid ligation

Fourteen days after ligation the morphology of the left carotid arteries was analysed by H/E staining (Figure 3.15A-C). The carotid arteries from mice that had undergone ligation surgery exhibited an increase in vascular remodelling as determined by an increase in mean media area as compared to sham procedures [Sham 24554.1±1550.3 μm^2 vs Water Control 45714.2±4435.9 μm^2 $p<0.01$] (Figure 3.15D).

Developing neointimal lesions were present in mice that had undergone ligation surgery and absent sham-operated mice (Figure 3.15A/B). Neointima formation was quantified as an increased intimal area or intima to media ratio (Figure 3.15E/F). The mean intimal area was increased by over 200-fold in mice that had undergone carotid ligation however this difference was not statistically significant, due to the large variance between individual animals in the injured group [Sham 123.4±33.48 μm^2 vs Water Control 20672.9±6808.46 μm^2]. Similarly, the intima to media ratio was not significantly altered following ligation [Ratio: Sham 0.005±0.001 vs Water Control 0.46±0.13]. The mean average intima [Ang-(1-9) 3808.4±765.95 μm^2] and intima/media ratio [Ang-(1-9) 0.1±0.02 μm^2] were lowered after Ang-(1-9) infusion by up to 70% (as compared to water infused control mice). Despite this no statistical significance was found following a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test (Figure 3.15E-F). Medial hypertrophy was not inhibited by Ang-(1-9) infusion [Ang-(1-9) 35571.9±2396.7 μm^2 vs Water Control 45714.2±4435.9 μm^2].

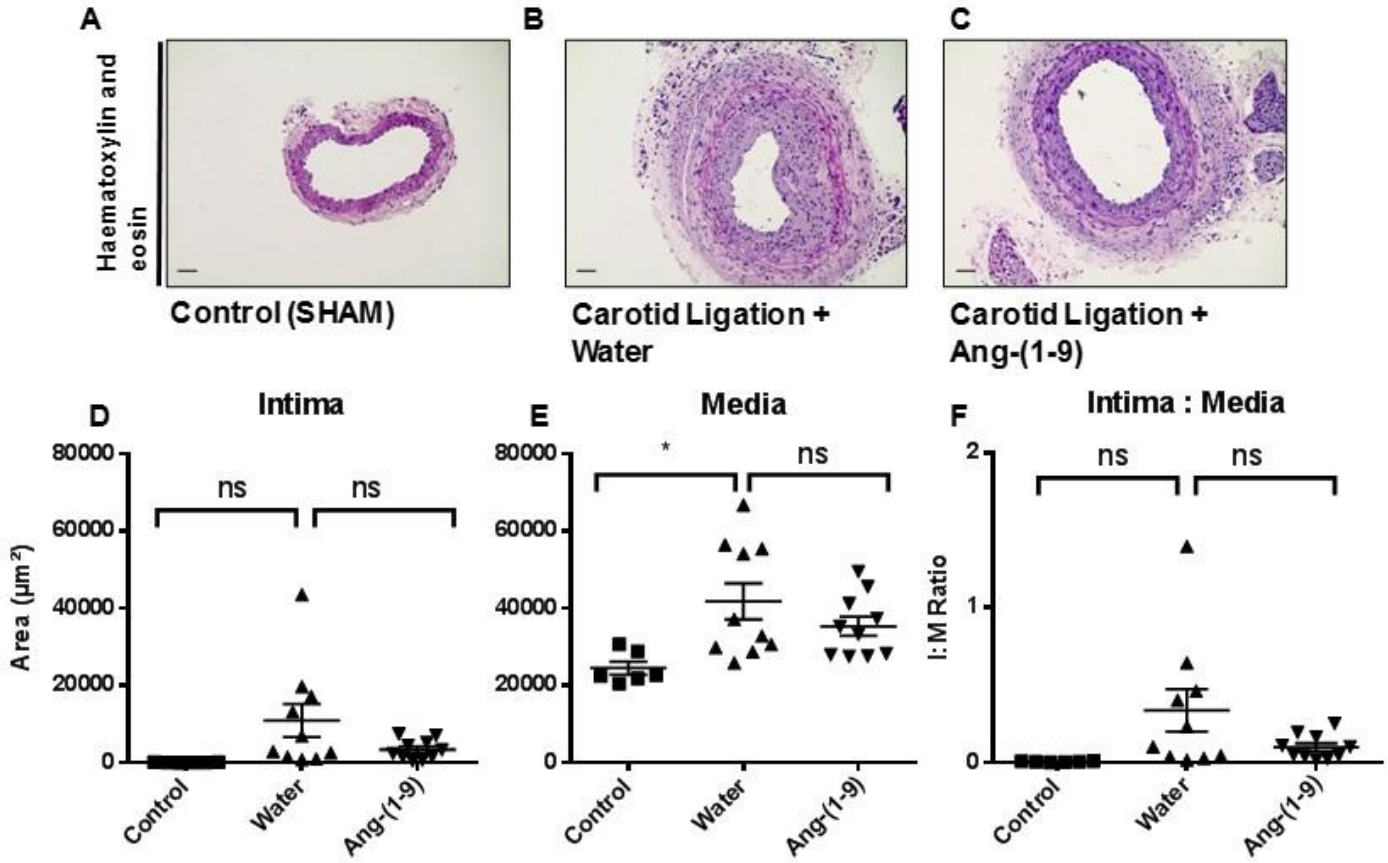

Figure 3.15 The effect of subcutaneous delivery of Ang-(1-9) on vascular remodelling 14 days after carotid ligation

(A-C) Histological staining (H/E) of carotid arteries from mice that had undergone sham-operation (n=6) and carotid ligation surgery (n=9) (D-F) To assess the effect of Ang-(1-9) infusion on vascular remodelling: the areas of the intima and media (μm^2) were calculated and is presented also as a ratio of the intima and media area. These mean values were used to compare each data set by applying a one-way ANOVA with tukey's multiple comparison. 4µm cross sections were cut from 3 separate locations of injured murine carotid arteries in this study. Per loci, the morphology of 3 sections were analysed. The mean of each value is presented here. Values represent the means ± SEM**.** (*$p<0.05$). Scale bar 100µm

3.2.8 Ang-(1-9) inhibits VSMC proliferation 14 days after carotid artery ligation

The consequence of systemic infusion of Ang-(1-9) on VSMC proliferation after carotid ligation was assessed by immunofluorescence detection of a classical SMC marker (aSMA) and a classical marker of proliferation; proliferating cell nuclear antigen (PCNA). Confocal microscopy detected distinct aSMA positive regions that composed the media and intima (Figure 3.16). First, the number of DAPI stained nuclei in aSMA positive regions (media + intima) was quantified and presented the mean total number per field of view. Water infused control mice which had undergone carotid ligation exhibited a significantly higher number of nuclei in the media and intima as compared to Ang-(1-9) infused mice which had undergone carotid ligation [Water Control 51.8±6.66 vs Ang-(1-9) 31.8±3.05 $p<0.05$] (Figure 3.17A). PCNA nuclear translocation was present in the ligated carotid arteries in water infused control mice, however Ang-(1-9) infusion significantly reduced PCNA nuclear translocation in aSMA positive regions 76.7% [Water Control 23.9±6.1 vs Ang-(1-9) 5.57±2.05 $p<0.05$] (Figure 3.17B). Region of interest image analysis detected a significantly increase (5.2-fold) in percentage of co-localisation in the whole cross section of vessel ($p<0.05$) (Figure 3.17D).

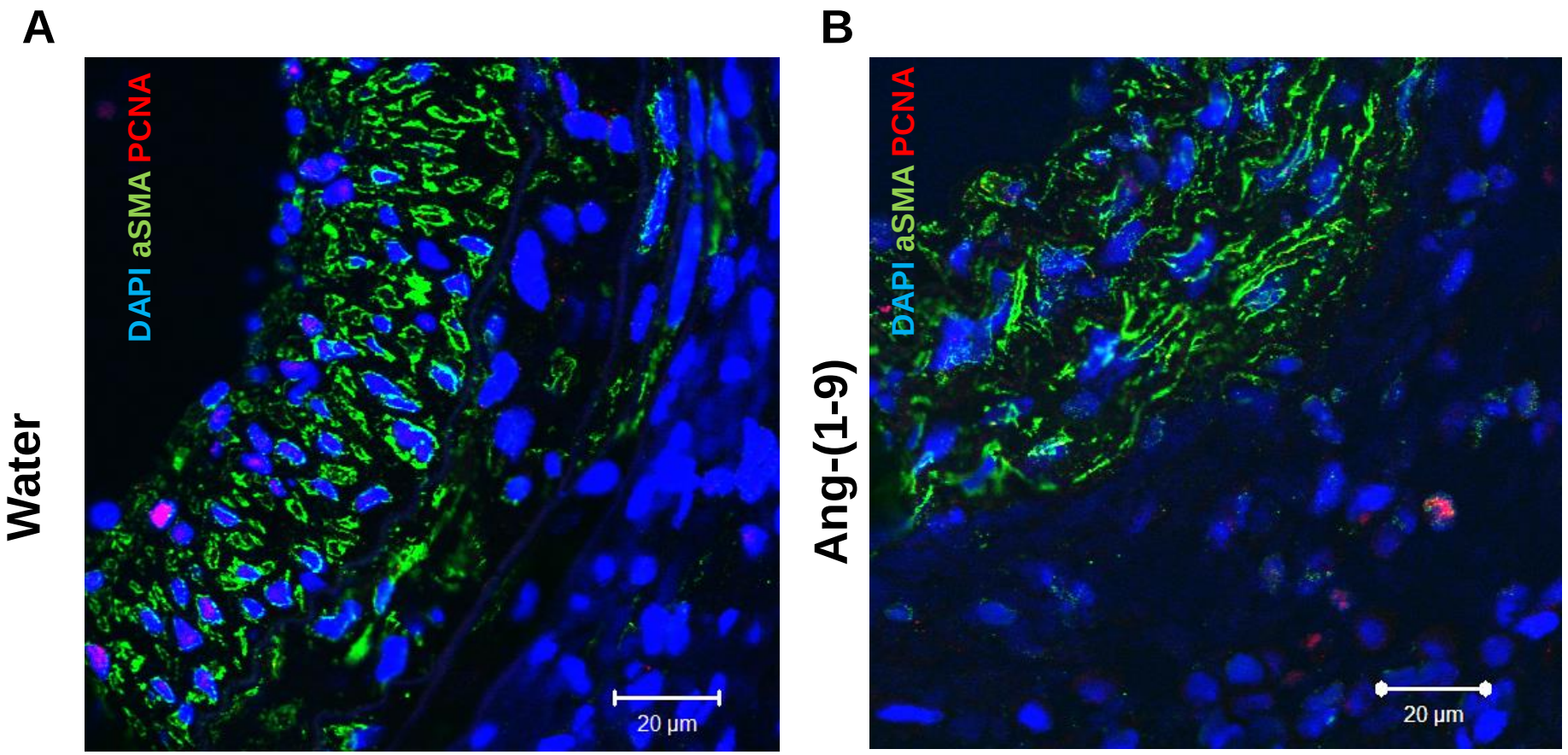

Figure 3.16 Subcutaneous delivery of Ang-(1-9) markedly reduces PCNA nuclear co-localisation after carotid ligation

(A-B) Immunofluorescence and confocal microscopy detected PCNA (red) nuclear (Blue) co-localisation within aSMA (green) positive regions. Three images were taken per section in order to approximately image the whole vessel (x40). One 4µm cross sections from 2-3 separate sections with ligated arteries were imaged. Representative images. n=6 mice Objective X40. Scale bar 20µm..

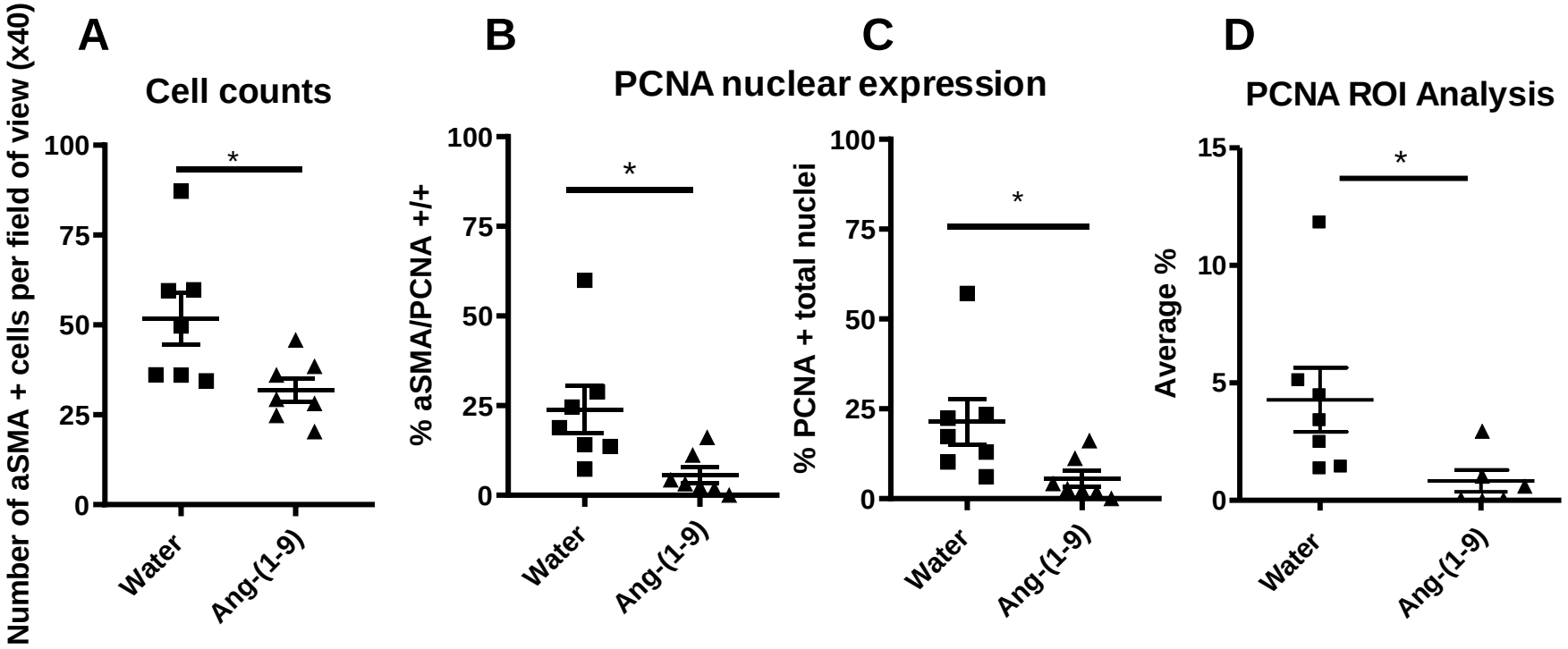

Figure 3.17 Subcutaneous delivery of Ang-(1-9) markedly reduces PCNA nuclear co-localisation after carotid ligation

(A) The number of nuclei within aSMA positive regions (intima + media) were manually counted. Next, PCNA positive nuclei in (B) aSMA positive regions and (C) the whole vessel was counted (D) Region of interest analyse determined the PCNA percentage positive area within DAPI stained nuclei. The mean average percentage compared between Ang-(1-9) and water treated control mice by applying a student's t test.4µm cross sections from 2-3 separate sections with ligated arteries were imaged by confocal microscopy. Three images were taken per section in order to approximately image the whole vessel (x40) (*$p<0.05$). Values represent the means ± SEM. n=6 mice per treatment.

3.2.9 Ang-(1-9) infusion does not affect carotid artery fibrosis after ligation

Fibrosis plays an important role in vascular repair and contributes to the expansion of the neointima at later stages of the remodelling process. It was therefore important to quantify the effect of Ang-(1-9) infusion on large vessel fibrosis after carotid ligation. Picrosirius red staining was performed to assess total vessel fibrosis after vascular injury 7 and 14 days after ligation (Figure 3.18). Collagen I/III (red) was detected primarily in the adventitia and surrounding tissue (Figure 3.18B and D). Image analysis determined no significant effect of Ang-(1-9) infusion on total vessel fibrosis 7 (58.25±5.13% vs Ang-(1-9) 61.41±3.79% $p>0.05$) (Figure 3.18A) or 14 days after ligation (Water Control 48.54±3.16% vs Ang-(1-9) 43.88±4.37% $p>0.05$) (Figure 3.18C).

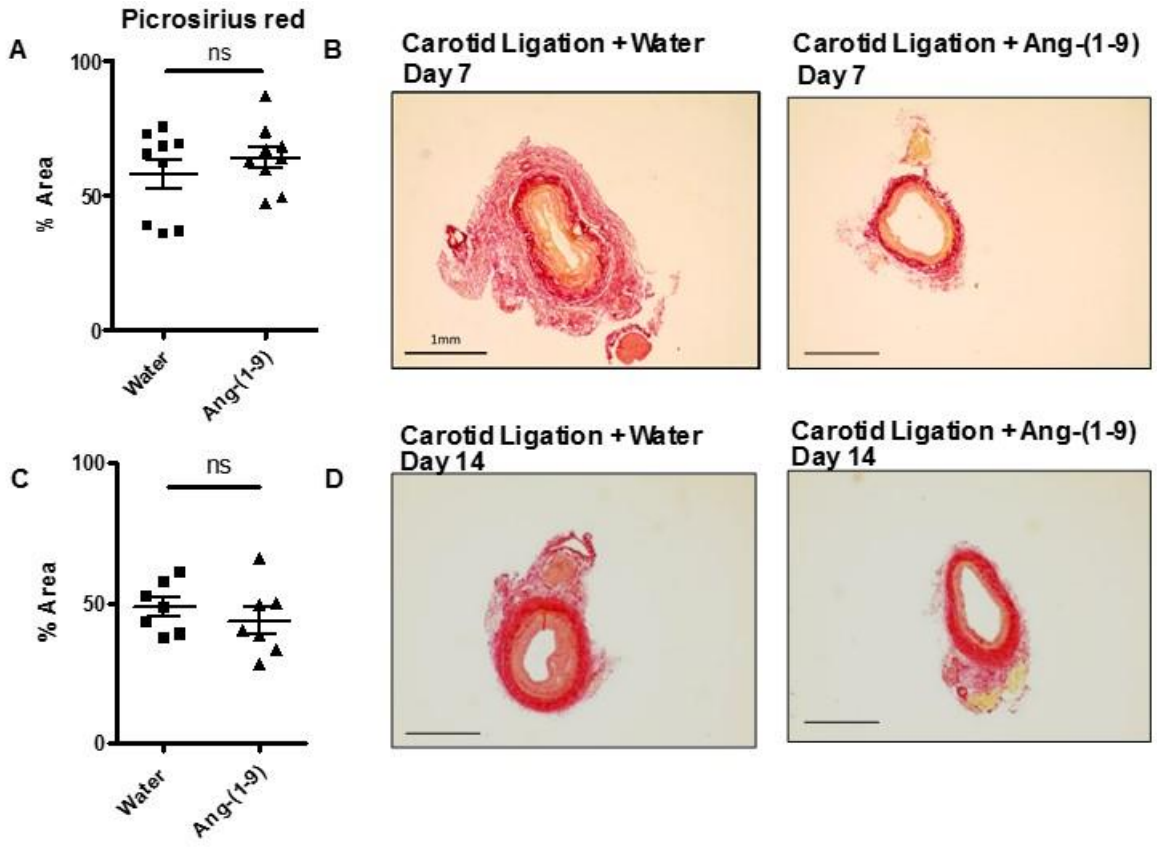

Figure 3.18 Picrosirius red staining of left ligated carotid arteries. Carotid ligation surgery was performed on C57 BL6/J mice.

(A/B) 7 days and (n=9) (C/D) 14 days post operation vessel fibrosis was assessed by picrosirius red staining (n=7) (A/C) Quantitative analysis of vessel fibrosis reported as the percentage area positive (red) staining of the whole vessel. Values represent the means ± SEM (B/D) Representative images of picrosirius red staining (X10 Scale bar 1mm). Three 4µm sections from one location on the carotid artery was analysed per mouse (n=9/7). Students *t* test was used to compare data sets. ns = non-significant.

3.2.10 Aortic fibrosis after carotid ligation as determined by qRTPCR

Intimal resident VSMC develop a synthetic profile, upregulating expression of deposition of ECM contents such as collagens which contribute to intimal thickening (Jiang et al., 2009). Thoracic aortae were utilised in lieu of carotid artery tissue in order to detect any changes in arterial fibrosis following carotid ligation and Ang-(1-9) infusion. Arterial fibrosis after carotid ligation was assessed at the gene expression level by qRTPCR. As expected, collagen 1a (*Col1a*) and collagen 3a (*Col3a*) was expressed in the thoracic aortae from healthy mice (Figure 3.19A). However, expression levels of *Col1a* in non-injured mice were unchanged after 7 days of infusion with Ang-(1-9) (Figure 3.19A). Similarly, 7 days after ligation *Col1a* expression was unaffected by Ang-(1-9) infusion (Figure 3.19B). Interestingly, *Col1a* was upregulated in aortae of mice that had previously undergone carotid ligation surgery as compared to sham procedure animals [Sham ΔCt 3.201±0.54 vs Water Control1.499±0.31 $p<0.05$] (Figure 3.20 left panel). Ang-(1-9) infusion did not reverse this effect at this time point [Ang-(1-9) ΔCt 1.8±0.2] (Figure 3.20 left panel). The same effect on *Col3a* gene expression was observed after ligation. *Col3a* was significantly upregulated in aorta from ligated mice as compared to sham-operated mice [Sham ΔCt 5.902±0.34 vs Water Control 4.26±0.25 $p<0.001$] (Figure 3.20, right panel). This effect was not seen in Ang-(1-9) infused mice. However, no significant differences were determined in aortic *Col3a* expression between water and Ang-(1-9)-infused injured mice [Ang-(1-9) ΔCt 5.07±0.17 $p>0.05$] (Figure 3.20, right panel).

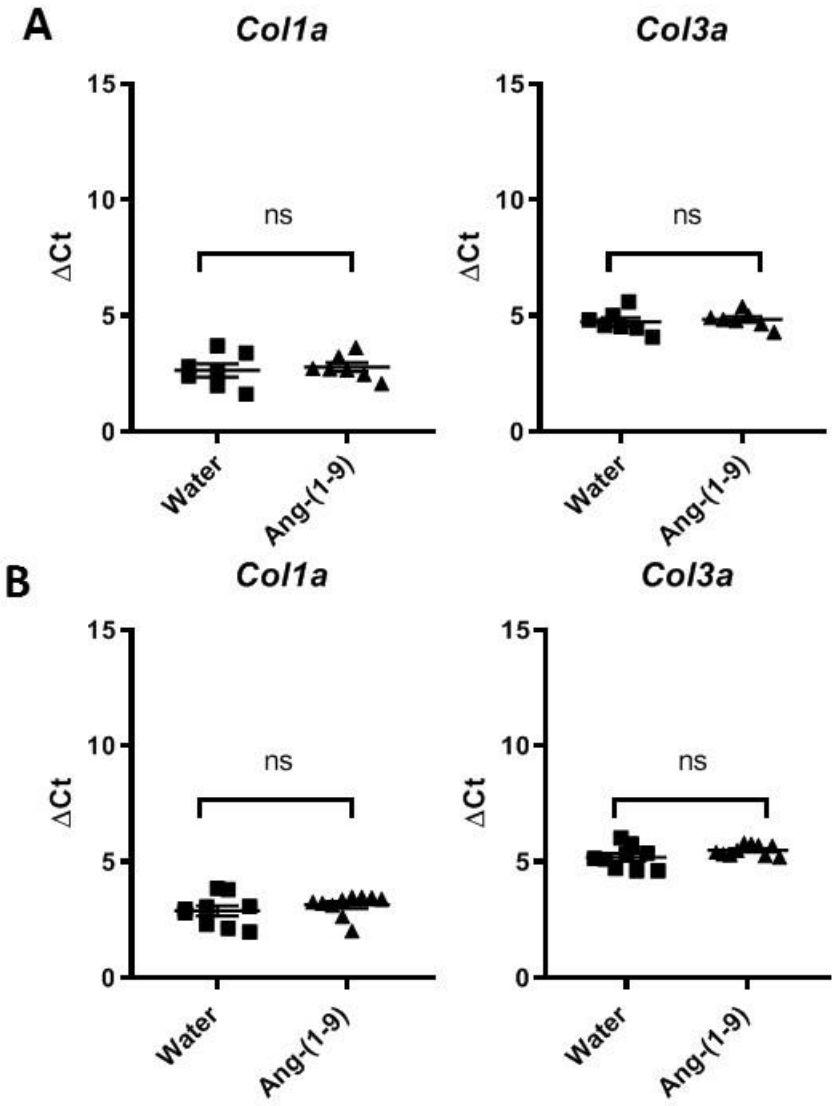

Figure 3.19 Effects of subcutaneous delivery of Ang-(1-9) on murine aortic gene expression (A) QRTPCR was used to determine gene levels of *Col1a* and *Col3a* after water or Ang-(1-9) infused mice (A) 7 days infusion in uninjured mice (n=8) (B) 7 days infusion after carotid ligation (n=9) Values were normalised to *GAPDH* and represent the mean delta Ct ± SEM. A One-way ANOVA was performed with tukey's multiple comparison post hoc test *p<0.05 ***p<0.001 ns>0.05

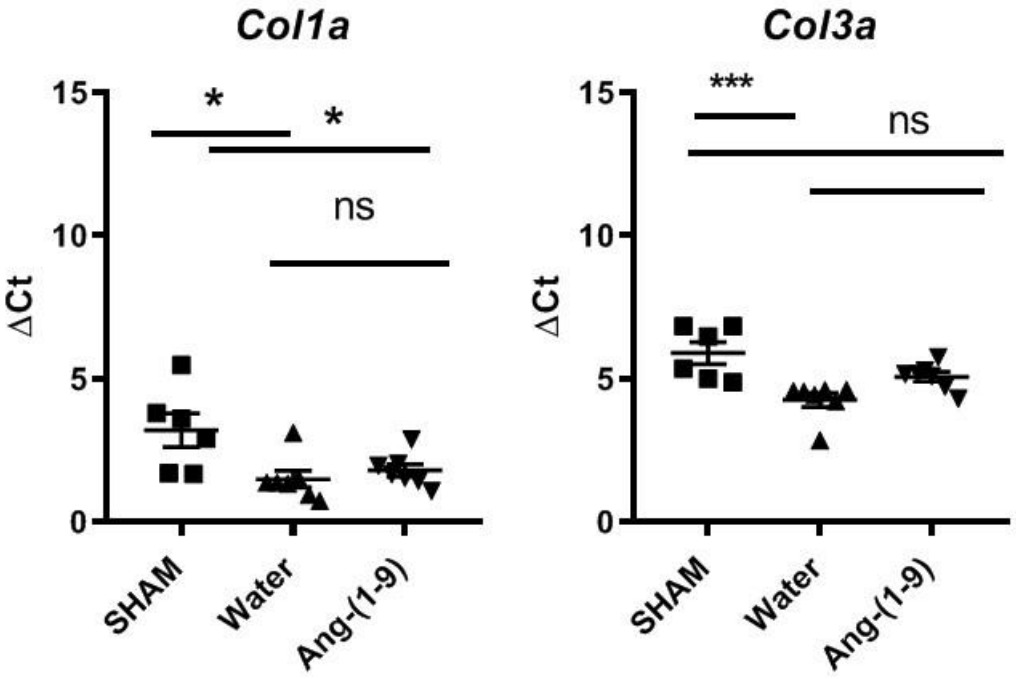

Figure 3.20 Effects of subcutaneous delivery of Ang-(1-9) on murine aortic gene expression 14 days after carotid ligation surgery QRTPCR was used to determine gene levels of *Col1a* and *Col3a* after water or Ang-(1-9) infused mice 14 days after sham or carotid ligation surgery (n=6-8). Values were normalised to *GAPDH* and represent the mean delta Ct ± SEM. A One-way ANOVA was performed with Tukey's multiple comparison post hoc test *p<0.05 ***p<0.001 ns>0.05

3.3 Discussion

The healing response that leads to the development of vein graft disease can be divided into three main stages. Initially the response is inflammatory (hours to days), which leads to a proliferative response (days to weeks) and later vascular fibrosis (weeks to months) (Jiang et al., 2009b). Inhibition of all three processes have been recognised as legitimate therapeutic strategies to prevent neointima formation after vascular injury. Here, the effect of subcutaneous infusion of Ang-(1-9) on inflammation, VSMC proliferation and fibrosis was assessed at two time points (day 7 and day 14) in the carotid ligation model of neointimal formation. In the present study, subcutaneous infusion of Ang-(1-9) did not affect the serum inflammatory profile of healthy or injured mice. With the exception of one analyte. Systemic concentrations of MCP-1 was decreased in Ang-(1-9) infused mice 14 days after carotid ligation. Ang-(1-9) infusion did not impact upon arterial fibrosis nor did it affect neointimal formation at 7 or 14 days. Despite this, nuclear expression of a proliferation marker (PCNA) was markedly decreased in aSMA positive cells 14 days following Ang-(1-9) infusion (Figure 3.21).

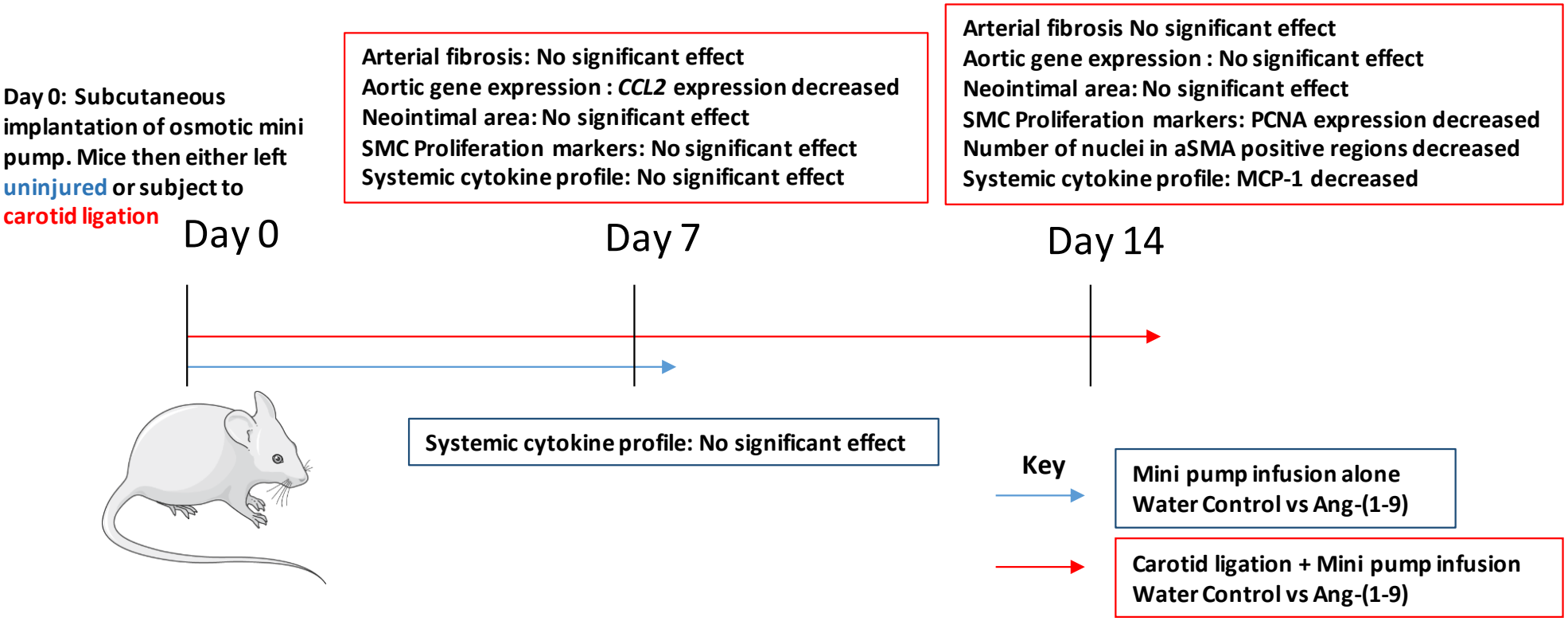

Figure 3.21 Schematic representation of the effect of Ang-(1-9) infusion [Ang-(1-9) vs Control Water] in uninjured mice (blue) and in the carotid artery ligation model (Red). Mice were implanted with osmotic mini pumps in order to continuously infuse Ang-(1-9) or water (control) subcutaneously. Mice were culled 7 or 14 days later. The carotid arteries were isolated and assessed by histology and immunohistofluorescence in order to analyse VSMC proliferation, neointima formation and fibrosis. Serum was purified from whole blood to investigate the effect of Ang-(1-9) infusion of the systemic cytokine profile. The ascending aorta was taken to investigate the effect of Ang-(1-9) infusion on cytokine gene expression in a vascular bed.

3.3.1 Inflammation

Members of the counterregulatory RAS have been reported to curb chronic inflammation (Rodrigues Prestes et al., 2017). For example the Ang-(1-7)/Mas receptor pathway is therapeutic in experimental models of arthritis (da Silveira et al., 2010), asthma (Magalhaes et al., 2015), atherosclerosis (Skiba et al., 2017) and renal injury (Barroso et al., 2012). And recently it has been reported that both Ang-(1-7) and alamandine elicit an anti-inflammatory macrophage response both *in vitro* and *in vivo*. Both counter-regulatory peptides decreased macrophage expression of *CCL2* and *IL-1B in vitro* while *in vivo,* infusion of the peptides decreased the number of neutrophils and classically activated macrophages (M1) following LPS administration (de Carvalho Santuchi et al., 2019)

In this study, Ang-(1-9) was delivered systemically. Therefore, the effect of Ang-(1-9) on circulating levels of inflammatory cytokines was investigated. Immuno-multiplexing technology was employed to analyse the levels of 23 different cytokines in the serum of water (control) and Ang-(1-9) treated mice. In the absence of carotid ligation, the serum cytokine profile was unchanged after seven days of continuous infusion of Ang-(1-9). Similarly, seven days after carotid ligation: Ang-(1-9) infusion appeared to have little impact on systemic inflammation as determined by the concentrations of circulating cytokines. Frequently an earlier time point of 6 hours to 3 days has been used to assess inflammation before any neointima is present (Herring et al., 2014, Herring et al., 2017). This may be a more appropriate time point to assess inflammation, however, inflammation in models of vascular injury is a dynamic process. For example, in $ApoE^{-/-}$ mice, injury-induced infiltration of monocytes into the carotid artery decreased between 7-14 days, whilst the infiltration of leukocytes remained constant (Alberts-Grill et al., 2012). This group also showed that gene expression of pro-inflammatory cytokines markers peaked at 7 or 14 days depending on the target (Alberts-Grill et al., 2012).

Interestingly, 14 days after carotid ligation, circulating concentrations of IL-1B were significantly increased in mice infused with water that had undergone carotid ligation surgery whereas no significant increase was detected in mice that had undergone carotid ligation and infused with Ang-(1-9).

IL-9 was detected sporadically in the serum taken from sham-operated mice and control animals that had undergone carotid artery ligation in combination with systemic infusion of water. Strikingly, IL-9 was undetected in all Ang-(1-9) treated mice at 7 and 14 days after carotid ligation. IL-9 is relatively understudied in the field of vascular remodelling however is clinically relevant with systemic concentrations elevated in heart failure patients (Cappuzzello et al., 2011). In pre-clinical studies, IL-9 has been reported to mediate chronic kidney disease-mediated neointima formation in the vein graft interposition model in mice and potentiate atherosclerosis in ApoE-/- mice (Zhang et al., 2017b, Zhang et al., 2015). In the same studies an IL-9-neutralizing IgG inhibited vein graft neointima formation (Zhang et al., 2017b). It is unknown how Ang-(1-9) mediated the down regulation of systemic IL-9 concentrations reported here or whether this is a biologically relevant finding given the near-detection limit concentrations of IL-9 observed.

Here, Ang-(1-9) infusion significantly lowered the concentration of circulating MCP-1 in the serum of mice 14 days after carotid ligation. This finding strengthens the evidence that Ang-(1-9) counter-regulates the RAS because MCP-1 is a key effector of Ang II via NFkB activation (Chen et al., 1998). Similarly, MCP-1 levels are elevated in AT_2R knock out mice (Koitka et al., 2010). Previously, the oral AT_2R agonist compound 21 (C21) (Wan et al., 2004b) attenuated MCP-1 mRNA expression in the femoral artery 7 days after cuff placement (Kukida et al., 2016). In a preclinical pulmonary hypertension study, MCP-1 has also been reported to be upregulated in monocrotaline treated mice whilst this was prevented by chronic administration of Ang-(1-9) (Cha et al., 2018). The findings presented in this chapter are clinically relevant because systemic levels of MCP-1 are higher in patients suffering from restenosis (Cipollone et al., 2001). Elevations in MCP-1 is a marker of prognosis after cardiac events (de Lemos et al., 2007). Kervin et al., associated an increase in serum MCP-1 levels with an increase in necessity for revascularisation surgery in patients who had presented acute coronary syndromes (Kervinen et al., 2004).

It is known that in response to injury VSMC produce MCP-1 and through activation of CCR2 promote intimal hyperplasia in murine and non-human primate models of vascular injury (Egashira et al., 2002). Furthermore, in an

epigastric vein graft model in rats, local *CCL2* mRNA expression has been reported to increase by over 100-fold after 7 days (Stark et al., 1997). Arguably the importance of this pathway is illustrated most clearly by the high levels of MCP-1 expression that are observed in atheroma from human and non-human primates (Nelken et al., 1991, Yu et al., 1992). Consequently, MCP-1 inhibitors have now been extensively studied in vein grafting models and also utilised by gene therapy successfully in preclinical models. Plasmid mediated delivery of 7ND-MCP-1 (a CCL2R inhibitor) is non-toxic in non-human primates and has been reported to reduce neointima formation by 50% in a mouse model of vein grafting (Kitamoto et al., 2004, Schepers et al., 2006). *Ex vivo* transduction of autologous vein grafts following adenoviral delivery of 7ND attenuated neointima formation in dogs (Tatewaki et al., 2007).

It was therefore relevant to assess expression of MCP-1 (*CCL2*) in a vascular bed taken from injured and Ang-(1-9) infused mice. Quantitative real time PCR determined that aortic mRNA levels of *CCL2* changed throughout the injury process. Higher aortic expression was observed 7 days after injury, suggesting it may be a more inflammatory time point (as compared to day 14). At this time point Ang-(1-9) infusion was associated with a modest but significantly decreased level of *CCL2* mRNA however such as small decrease may not be biologically relevant. Moreover, Ang-(1-9) infusion had no effect of aortic *CCL2* expression at 14 days or in non-injured mice. A more pronounced effect might be observed 3 days after carotid ligation, which is a time point typically used to assess early stages of inflammation (Cercek et al., 1997, Kovacic et al., 2010).. Therefore, some of the findings presented here agree with the previous literature that Ang-(1-9) can reduce MCP-1 expression (Gonzalez et al., 2018, Cha et al., 2018, Ocaranza et al., 2014, McKinney et al., 2015). However, it is important to note than Ang-(1-9) did not affect systemic concentrations of MCP-1 in healthy mice or 7 days following carotid ligation.

3.3.2 VSMC proliferation and neointima formation

The effect of Ang-(1-9) in the carotid ligation model had not been previously investigated. In these studies, Ang-(1-9) was subcutaneously delivered via osmotic mini pump immediately after carotid ligation in C57 BL/6 mice. After 7 and 14 days the morphology of the carotid arteries were assessed. No significant

differences in vessel morphology were determined between Ang-(1-9) and water infused mice 7 days after ligation. This was not an unexpected result as this is early in the vascular remodelling process. The 7 day time point is however a relevant for assessing markers of proliferation, intracellular signalling events and inflammation (Kumar and Lindner, 1997). Here, *in vivo* incorporation of EdU was utilised to label cells actively replicating their genome 7 days after ligation. In water infused mice, EdU incorporation was present in intimal and medial vascular cells 7 days after carotid ligation. This is in agreement with the original research article that characterised this model (Kumar and Lindner, 1997). The authors utilised BrdU incorporation to report that medial VSMCs are proliferating 5 days after ligation, while this number drops at 8 days. However intimal VSMCs continue to rapidly proliferate until as long as 14 days after cessation of blood flow (Kumar and Lindner, 1997).

Next the effect of Ang-(1-9) infusion was assessed. No statistically significant differences were detected when the levels of EdU incorporation in water and Ang-(1-9) infused mice were compared. It should be noted here that only one section per vessel was quantified, therefore a more extensive analysis of EdU incorporation at multiple levels through the injured vessel could be used to verify this result. While EdU incorporation is a sensitive and direct method to detect replication of DNA it only provides a snapshot of cells actively undergoing DNA replication (Salic and Mitchison, 2008). Analyses of proliferation markers such as PCNA and Ki67 are a common method to visualise a broader representation of VMSC phenotype with relation to quiescence or hyperplasia. Previous studies have quantified PCNA expression in carotid arteries 3, 7 and 28 days after ligation and have reported that PCNA expression peaked at the 7 day time point (Moura et al., 2007). Therefore, quantification of PCNA expression may be useful in the present study.

At the 14-day time point, carotid ligation surgery significantly increased vascular remodelling in left carotid arteries as determined by an increase in media area (as compared to sham-operated mice). No significant increase in intimal area was found between sham-operated and carotid artery ligated mice. This is a key reason why proliferation markers were assessed later.

Likewise no statistically significant differences in intima area were observed between water and Ang-(1-9) infused mice 14 days after ligation. In the original research article intimal area is nearly twice as high as what was found here (Kumar and Lindner, 1997). Since then, the intimal area reported for wild type mice varies and mean values similar to those presented here have been reported in peer reviewed journals (Sommerville et al., 2008, Yu et al., 2015).

To elucidate the effect of Ang-(1-9) on VSMC proliferation at the 14-day time point, nuclear co-localisation of a proliferation marker (PCNA) was quantified. PCNA is a cofactor of DNA polymerase and is an important orchestrator of cell cycle control (Moldovan et al., 2007). Here, PCNA was expressed and located in the nucleus of vascular cells in ligated carotid arteries as in good agreement with previous studies (Li et al., 2011b). This indicated that VSMCs at this time point were still proliferating and contributing to an evolving neointima as originally reported by Kumar and Linder (Kumar and Lindner, 1997).

Subcutaneous infusion of Ang-(1-9) significantly decreased PCNA nuclear co-localisation in carotid arteries 14 days after carotid ligation. This is promising as PCNA is a well-established marker of proliferation and antisense oligonucleotides targeting PCNA and other cell cycle factors have led to sustained inhibition of neointima formation in preclinical models (Morishita et al., 1993). Moreover, when the number of nuclei in aSMA positive regions were quantified; Ang-(1-9) infusion was associated with a significantly lower cell count as compared to water infused mice. Taken together this might be indicative that Ang-(1-9) partially inhibited carotid artery VSMC proliferation after cessation of blood flow. The caveat of this finding being that it was not also detected the earlier time point (day 7).

3.3.3 Fibrosis

Fibrosis is an important process for vein graft arterialisation or adaption to an arterial environment. Later in the vascular remodelling process a VSMC phenotype change occurs and the neointimal lesion becomes less hyper-cellular and more fibrotic (Zhang et al., 1999). However excessive deposition of ECM is a key process in the later stages of vein graft disease that enables the neointima lesions to continue to increase in area (Jiang et al., 2009a). Locally, aldosterone

acts through the mineralocorticoid-receptor in HSVSMCs leading to inflammation and fibrosis (Bafford et al., 2011). Inhibiting the MR using spironolactone has been reported to inhibit vascular remodelling in the vena cava interposition vein-grafting model in mice (Ehsan et al., 2013). Therefore, targeting fibrosis may be a therapeutic strategy to improve outcomes of CABG procedure.

Here Ang-(1-9) infusion had no effect on arterial fibrosis as determined by picrosirius red staining of carotid arteries. Interestingly, 14 days after carotid ligation there was increased levels of *Col1a* and *Col3* gene expression in murine aortae indicating that ligation of the left carotid artery led to increased vascular fibrotic pathway activation in the aorta. Also of note *Col3a* mRNA levels were significantly increased in mice that had undergone carotid ligation surgery (water infused) as compared to sham-operated mice. While the difference between Ang-(1-9) and water infused mice aortic *Col3a* expression was not statistically significant.

The counter-regulatory peptide Ang-(1-7) has been reported as safe in humans (Savage et al., 2016b, Petty et al., 2009) and is in early clinical trials for treatment-refractory sarcoma (Savage et al., 2016b). The delivery mechanism in these Ang-(1-7) studies is subcutaneous injection. It is therefore possible that that subcutaneous administration of Ang-(1-9) could be utilised before and in weeks after CABG surgery. Because these peptides naturally exist in the circulation it may be likely that subcutaneous injection of Ang-(1-9) would be tolerated. However, due to the rapid half-life of these peptides this may not be the optimal delivery platform for CABG surgeries, therefore novel ways to utilise Ang-(1-9) should be investigated. This could be via gene therapy approaches or by combination therapy. Combining Ang-(1-9) treatment with ACE inhibitors may improve the half-life of systemically administered Ang-(1-9). Evidence for this has been seen in human plasma samples treated with recombinant ACE2 and ACE inhibitors (Basu et al., 2017). Similarly, new formulations of angiotensin peptides have been created (Durik et al., 2012). For example, insertion of a thioether bridge to cyclise Ang-(1-7) dramatically increased the half-life whilst retaining its cardio-protective action post MI (Sevá Pessôa et al., 2015).

3.4 Conclusion

Here, morphometric analyses of carotid arteries detected an increase in media area following ligation but failed to determine an increase in neointimal area. Subcutaneous delivery of Ang-(1-9) did not inhibit neointimal formation 7 or 14 days after carotid ligation. Ang-(1-9) did not inhibit VSMC 7 days after carotid ligation as determined by incorporation of EdU. However, it was observed that Ang-(1-9) infusion inhibited carotid artery VSMC nuclear PCNA expression 14 days after cessation of blood flow. The data presented here is the first evidence the Ang-(1-9) diminishes circulating MCP-1 protein levels and *CCL2* gene expression in murine aorta in a model of neointimal formation. This study also revealed an intriguing effect of Ang-(1-9) on IL-9. Taken together, it is uncertain whether Ang-(1-9) is protective in the carotid artery ligation model as protective effects were not consistently observed and no detectable effect of neointimal area was determined.

4 Chapter 4 An investigation into the effects of Ang-(1-9) on HSVSMC migration and proliferation

4.1 Introduction

It is accepted that VSMCs in healthy adult blood vessels proliferate at an exceptionally low rate (Owens et al., 2004). However, VSMCs are plastic by nature and, in response to the acute vascular injury experienced in revascularisation surgeries, VSMCs proliferate, migrate and become more synthetic. This transition is characterised by the loss of 'healthy' contractile genes and is known as VSMC phenotype switching (Chappell et al., 2016). As a result of proliferation, VSMC accumulate within the intima leading to the narrowing of the grafted or stented blood vessel. Studies using human coronary arteries have reported that intimal VSMCs display increased DNA replication and expression of PCNA (Gordon et al., 1990b). This process, termed intimal hyperplasia, is part of an exacerbated healing mechanism that underlies the development and progression of vein graft disease (Hofstra et al., 1996, Doran et al., 2008). Furthermore, pre-existing neointimal legions can be present in coronary artery bypass grafts and 1 year follow up computed tomography (CT) coronary angiography has been used to determine that this is associated with increased narrowing of the vessel lumen (Kon et al., 2007). Inhibiting VSMC proliferation and migration is being actively investigated as a method to prevent the occlusion of vein grafts in order to improve the long-term outcomes of CABG surgeries. However, new candidate therapies are needed.

It is well established that excessive Ang II/AT_1R signalling promotes vascular remodelling and hypertension (Montezano et al., 2014). Ang II also has pro-thrombotic and pro-atherosclerotic effects (Ridker et al., 1993). Ang II/AT_1R signalling leads to the induction of proliferation and migration of VSMCs (Yaghini et al., 2010). Therefore, it is clear that Ang II signalling plays a role in the development and progression of vein graft disease. Classical inhibition of Ang II signalling through ACE inhibition and ARBs has been efficacious in the treatment of hypertension and atherosclerosis (Dagenais et al., 2006). However, in patients with coronary heart disease (72% of which had previously undergone revascularisation), ACE inhibition with trandolapril (Mavik) had no effect on the incidence of death, MI or the requirement for repeat revascularisation surgery (ACEi 21.9% vs 22.5% placebo) (Braunwald et al., 2004). Importantly, quinapril (Accupril) (another ACE inhibitor) had no effect on the outcomes of CABG

surgeries at a 3 year follow up examination(Rouleau et al., 2008). It is clear that new ways to inhibit Ang II signalling are needed.

A novel axis of the RAS exists that acts to counter-regulate the effects of Ang II/AT_1R. Effectors of the counter-regulatory RAS axis can signal via the AT_2R (Zhang et al., 2017a) or the Mas receptor (Villalobos et al., 2016) which are both well known for antagonising the effects of the AT_1R in the vasculature. Ang-(1-7) is a seven amino acid long angiotensin metabolite which is a ligand for the Mas receptor (Santos et al., 2003). Ang-(1-9) is a biologically active nonapeptide that is thought to act through the AT_2R to confer cardio-protection (Flores-Muñoz et al., 2011). With the use of receptor antagonists, several studies have demonstrated that the protective effects of Ang-(1-9) are AT_2R dependent and do not rely on the Mas receptor (Ocaranza et al., 2010, Flores-Munoz et al., 2012).

The majority of studies to date have investigated the effects of Ang-(1-9) in the heart (Zheng et al., 2015, Flores-Munoz et al., 2012, Mendoza-Torres et al., 2018). However, lesser is known about the effects of Ang-(1-9) in the vasculature. Recently, Ang-(1-9) infusion led to the inhibition of neointima formation in arterial and venous models of vascular injury demonstrating its status as an emerging candidate for use in revascularisation surgeries (McKinney et al, 2019). But it is pertinent to understand whether therapeutic interventions studied in pre-clinical mouse models are relevant to human disease. McKinney et al., reported that Ang-(1-9) inhibited serum and Ang II induced responses in HSVSMCs in culture (McKinney et al., in preparation). However, the pathophysiology of vascular remodelling is complex. In response to vascular injury, ECs and VSMCs produce *multiple* growth factors and cytokines which further promote hyperplasia as well as attract immune cells to the site of injury (Doran Amanda et al., 2008). This may explain why classical inhibition of the RAS have been unsuccessful in clinical trials for CABG and PCI. Therefore, it is important to assess the effect of Ang-(1-9) on VSMC proliferation induced by independent cytokines such as PDGF-BB that have an established role in VGF (other than Ang II) (MERCATOR, 1992, Kenagy et al., 2009).

PDGF-B is a potent mitogen that as a homodimer (PDGF-BB) stimulates VSMC proliferation and migration and has a well characterised role in the pathogenesis

of VGF and restenosis (Nurminskaya et al., 2014, George et al., 1996, Sterpetti et al., 1996). It is also partly responsible for phenotype switching of VSMCs (Yoshida et al., 2007, Deaton et al., 2009). PDGF-BB stimulation inhibits expression of contractile markers such as aSMA, SM22α and SM-MHC, an affect that is accompanied by upregulation of proto-oncogenes such as c-fos (Dandre and Owens, 2004).

The mechanisms by which PDGF-B exerts its effects converge substantially with RAS signalling. Firstly, Ang II and PDGF-B signalling lead to similar transcriptional changes. Both activate the transcription factor protein C-ets-1 (ETS1), in turn upregulating expression of PDGF-B and PDGF receptors, leading to proliferation of VSMCs (Naito et al., 1998, Hultgardh-Nilsson et al., 1996, Zhan et al., 2005).

In VSMCs, exposure to Ang II and PDGF-BB results in tyrosine phosphorylation of the PDGFR-B (Heeneman et al., 2000, Linseman et al., 1995). Interestingly, the mitogenic effects of Ang II and PDGF-BB both involve ERK1/2 signalling (Yaghini et al., 2010, Kingsley et al., 2002). ERK1/2 is the master kinase of the MAPK pathway and is widely known for its pro-proliferative actions (Bornfeldt et al., 1997, Lille et al., 1997, Pagès et al., 1993). It is noteworthy that some ERK1/2 inhibitors are now approved and currently are in clinical trials for cancer studies (Caunt et al., 2015). Although their efficacy in revascularisation surgeries have yet to be determined, it is clear that inhibiting this kinase signalling pathway is an excellent strategy to inhibit cell proliferation. Taken together both Ang II and PDGF-B are major players in vein graft disease and dual inhibition of these mitogens through targeting points where their pathways converge may be an effective treatment to inhibit VSMC proliferation.

With vein graft disease as a key focus, the study presented here assessed the effects of PDGF-BB on human VSMC isolated from the surplus segment of preimplantation HSV. Here, a mechanistic investigation into the effects of Ang-(1-9) on HSVSMC proliferation and migration as well as downstream signalling events was performed.

4.2 Aims

- To investigate the effect of Ang-(1-9) on PDGF-BB induced HSVSMC migration and proliferation.

- To investigate the mechanisms involved in Ang-(1-9) mediated inhibition of HSVSMC migration and proliferation.

4.3 Results

4.3.1 HSVSMC phenotyping

VSMC were isolated from surplus segments of HSV donated by patients undergoing SV CABG. It was first important to determine that the cells isolated were VSMC rather than and other vascular cell types such as ECs. To do so, immunofluorescence and confocal microscopy was performed to detect the presence or absence of the classical VSMC markers aSMA and smooth muscle protein 22 alpha (SM22a) in VSMC isolated from three independent patients (Li et al., 1996). HSVSMC from three independent patients either expressed SM22a or aSMA to varying degrees (Figure 4.1).

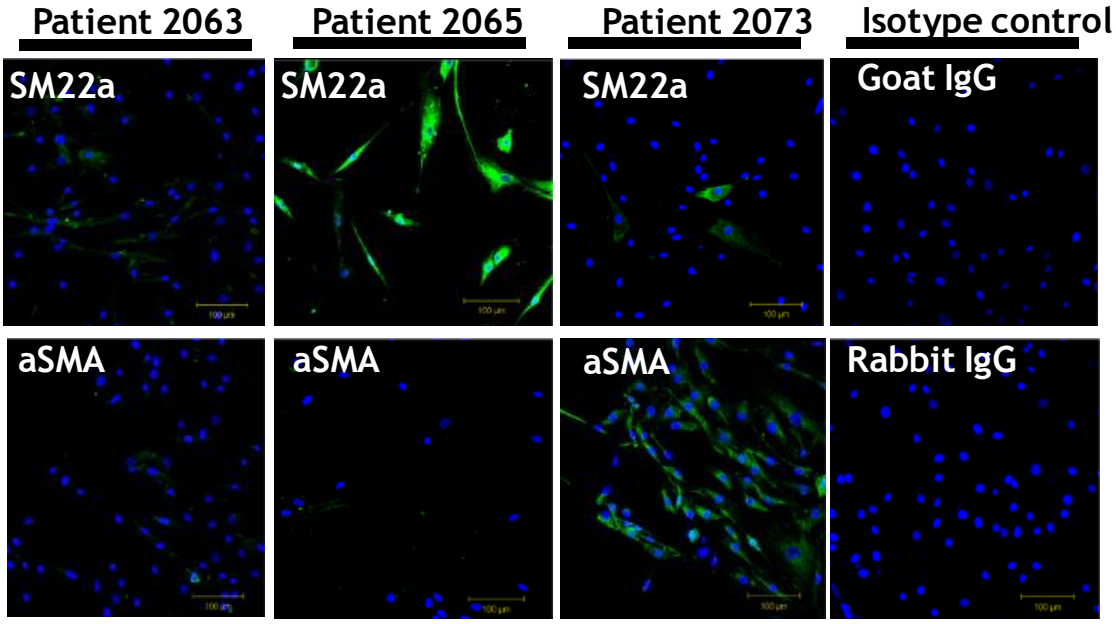

Figure 4.1 Human saphenous vein vascular smooth muscle cells (VSMC) phenotyping. Vascular smooth muscle cells (VSMC) were isolated from the surplus saphenous vein of CABG patients. Immunofluorescence detected SM22a/aSMA green, DNA was detected by DAPI staining (blue). 40X objective. Scale bar 100μm n=3 independent patients.

4.3.2 PDGFRB expression in HSVSMC

Before investigating the effects of PDGF-BB on HSVSMCs, expression of the PDGF-receptor beta (*PDGFRB*) in this cell type was determined. Quantitative real time PCR confirmed mRNA expression of the *PDGFRB* in HSVSMC isolated from three independent patients (Average *Ct 24.6*±0.08) (Figure 4.2A). Thus agreeing with previous studies that have reported that venous VSMCs strongly express *PDGFRB* (Li et al., 2011a). The expression of PDGFRB was further substantiated and validated at the protein level by immunoblotting which detected a single band at the correct estimated molecular weight of 190 kDa (Figure 4.2B Top panel). 18S was used as housekeeper during QRTPCR and GAPDH was utilised during immunoblotting (Figure 4.1.2B bottom panel)

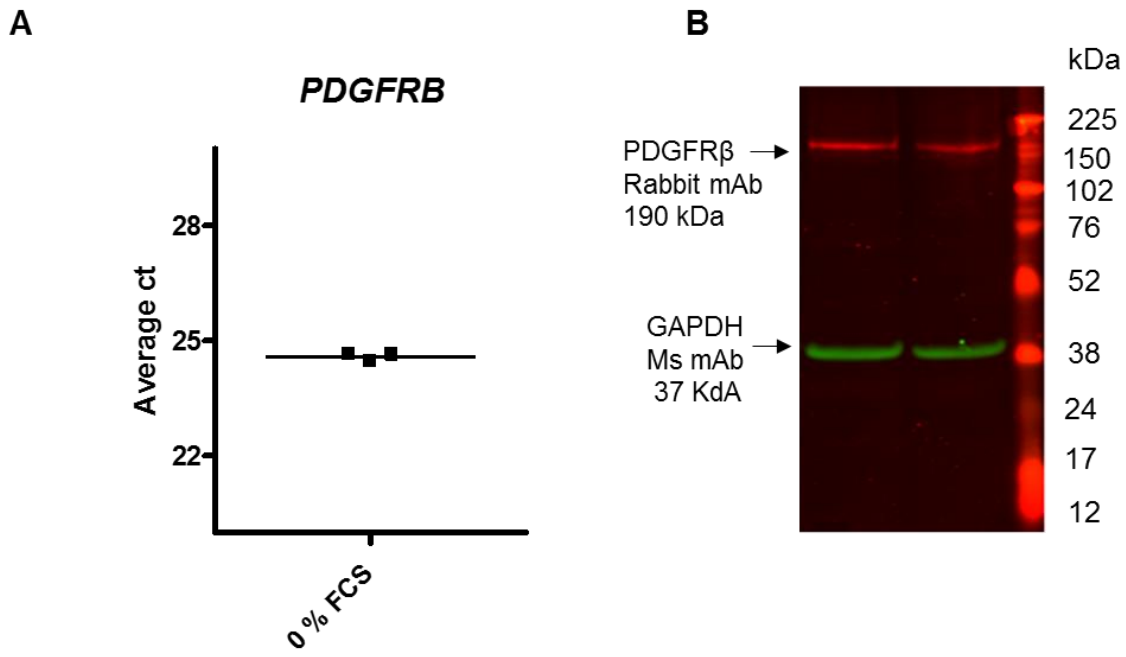

Figure 4.2 HSVSMC express the PDGFB Receptor

(A) Quantitative real time PCR detected *PDGFRB* at the gene level in cultured HSVSMC. Values represent the mean Ct ± SEM n=3 independent patients (B) immunoblotting for the PDGFRB detected protein expression in HSVSMC lysates n=1 independent patient.

4.3.3 The effect of Ang-(1-9) on PDGF-BB induced HSVSMC proliferation

VSMC proliferation is a hallmark of neointima formation. To dissect the mechanism of action of Ang-(1-9) on HSVSMC proliferation, the effect of Ang-(1-9) on PDGF-BB induced proliferation was assessed as it has an established role in VGF and is known to be present in serum. HSVSMC proliferation was quantified by assessing the incorporation of a synthetic thymidine analogue bromodeoxyuridine (BrdU) using an ELISA based method. HSVSMC exposed to PDGF-BB exhibited significantly elevated levels of BrdU incorporation [0% FCS 0.32±0.03 vs PDGF-BB 0.69±0.011 $p<0.01$). Pre-incubation with 1 μM Ang-(1-9) significantly inhibited PDGF-BB induced proliferation [Ang-(1-9) 0.46±0.05 $p<0.05$]. This inhibitory effect was not observed at the lower dose of 200 nM Ang-(1-9) therefore displaying a dose-dependent effect (Figure 34.3).

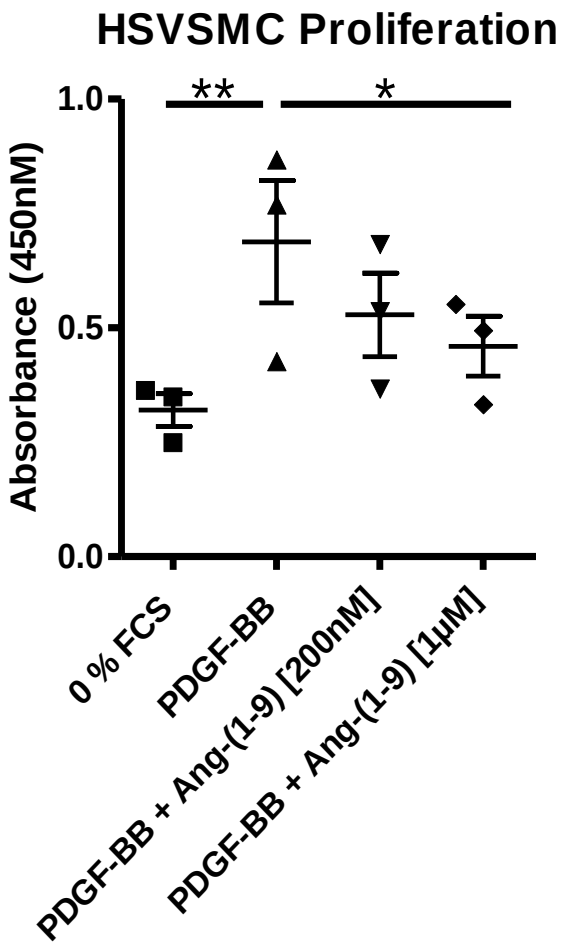

Figure 4.3 Ang-(1-9) inhibits PDGF-BB induced HSVSMC proliferation.

Quiesced HSVSMC were exposed to PDGF-BB in the presence or absence of Ang-(1-9) at a dose of 0.2 or 1μM. At 48 hours BrdU incorporation was determined by sandwich ELISA and colorimetry. Values represented as the mean absorbance values ± SEM n=3 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test *p<0.05 **p<0.01.

4.3.4 Ang-(1-9) supresses PDGF-BB induced HSVSMC directional migration

HSVSMC migration is another critical factor in neointima formation (Gerthoffer William, 2007). It was therefore decided to investigate the effect of pre-incubation with Ang-(1-9) peptide on PDGF-BB induced HSVSMC migration. One of the most commonly used methods to investigate VSMC migration, the scratch wound assay, was employed (Liang et al., 2007). The scratch wound assay assesses directional migration (chemokinesis) that is achieved by an elevation in cellular motility either induced by presence of a pro-migratory agent or by the scratch wound itself affecting basal levels of migration (Liang et al., 2007, Louis and Zahradka, 2010).Basal migration of serum starved HSVSMCs as determined by percentage wound closure was approximately 34% PDGF-BB induced directional migration [0% FCS 34.4±3.8% vs PDGF-BB: 78.1±2.7% $p<0.05$] (Figure 4.4). Again, pre-incubation with 200 nM Ang-(1-9) had no significant effect of PDGF-BB induced effects. However, HSVSMC directional migration was significantly reduced by the higher dose of Ang-(1-9) [1 μM] [PDGF-BB 78.1±2.7% vs Ang-(1-9) + PDGF-BB 49.6±0.7% $p<0.01$) (Figure 4.4).

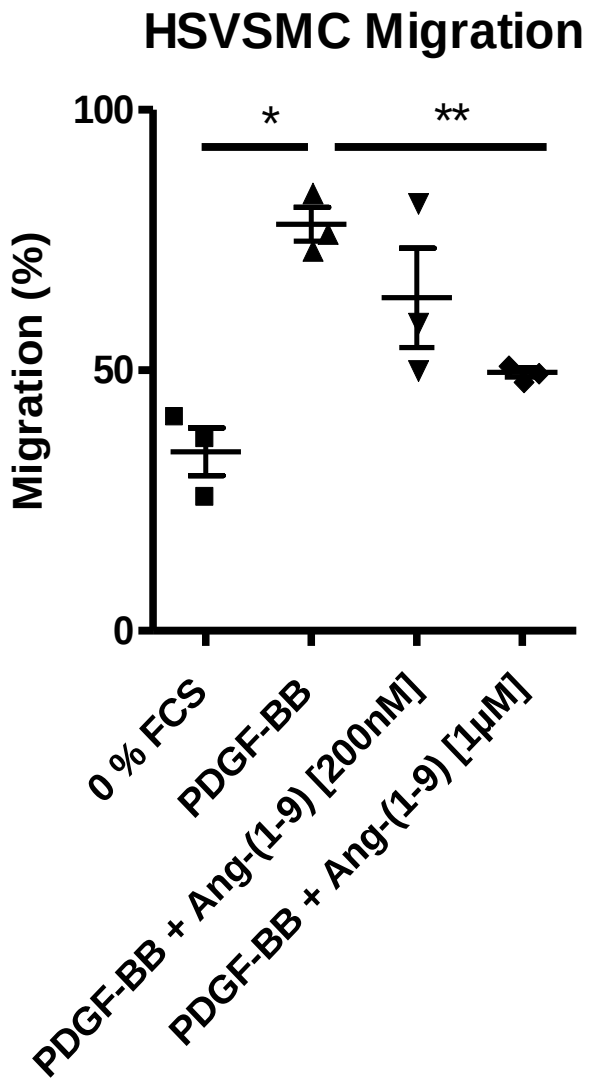

Figure 4.4 Ang-(1-9) inhibits PDGF-BB induced directional migration.

The scratch assay was used to assess HSVSMC directionally migration. A vertical scratch was performed in a monolayer of quiescent HSVSMC which were then exposed to PDGF-BB in the presence of absence of Ang-(1-9). The distance of the scratch was measured at 0hour and 24 hours. Values represented as the mean percentage (%) migration after 24 hours ± SEM n=3 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test *p<0.05 **p<0.01

4.3.5 Ang-(1-9) supresses PDGF-BB induced HSVSMC chemotaxis

Chemotaxis infers a chemical gradient through which VSMC or other cell types migrate toward the area which the agents are most concentrated. For example, increased shear stress promotes dysfunction of ECs which release PDGF-BB, this local release promotes VSMC chemotaxis toward the intima thus promoting intimal thickening (Dardik et al., 2005). To investigate chemotaxis, a modified version of the Boyden chamber transwell assay was used (Chen, 2005). Representative confocal microscopy images are presented for each condition. Under control conditions (0% FCS) the number of HSVSMC that migrated to the lower side of the chamber was 3.72±0.32 nuclei per field of view (Figure 4.5A). It was then investigated whether PDGF BB at 20 ng/mL in the lower chamber induced VSMC migration (Figure 4.5B). The presence of PDGF-BB in the lower chamber of the transwell plate resulted in a significant increase in the number of HSVSMC migrating through the 8 micron porous membrane to the lower side of the chamber [0% FCS 3.72±0.32 vs PDGF-BB 13.34±1.55 $p<0.05$] (Figure 4.5A) (see sections 2.4.2 for schematic). Co-incubating HSVSMC with 1 μM Ang-(1-9) significantly inhibited PDGF-BB induced chemotaxis [PDGF-BB 13.34±1.55 vs Ang-(1-9) 7.07±0.65 $p<0.01$] (Figure 4.5A).

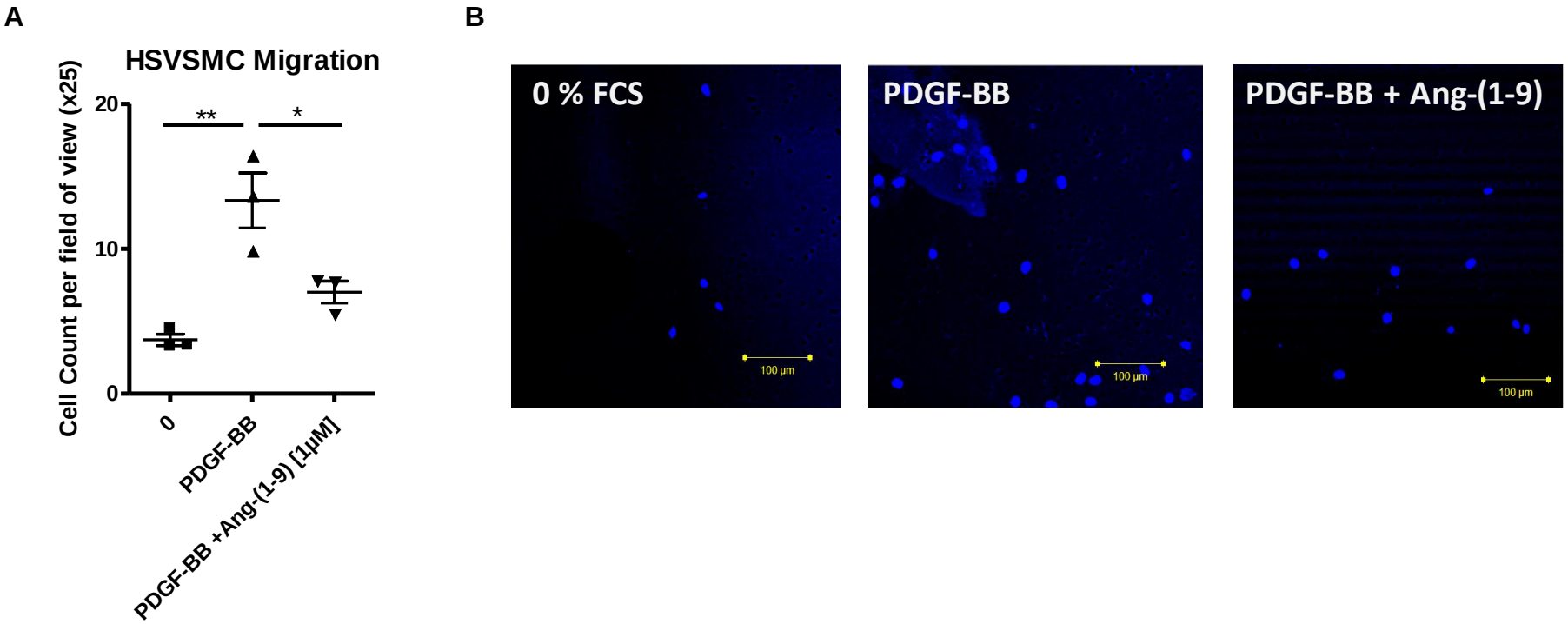

Figure 4.5 Ang-(1-9) significantly reduces chemotaxis-based migration.

The Boyden chamber assay was used to investigate the effect of Ang-(1-9) (1μM) and PDGF-BB (20 ng/mL) on chemotaxis-based migration. After 24 hours the number of nuclei that had migrated through the porous membrane was counted manually (B) DAPI staining and confocal microscopy was used to visualise the nuclei (blue) of HSVSMC that had migrated to the lower side of the chamber (X20) Scale bar 100μm. Data represented as the mean number of nuclei per field of view ± SEM. n=3 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test *$p<0.05$ **$p<0.01$

4.3.6 Effect of Ang-(1-9) on PDGF-BB induced intracellular signalling

Next, the effect of Ang-(1-9) on the activation of alternative intracellular signalling pathways by PDGF-BB was assessed (n=5 independent patients). A commercially available kit (see 2.5.13) that utilised an array of antibodies complementary to phosphorylated and cleaved members of signalling pathways was used to indicate activation by fluorescent read-out (Figure 4.6).

PDGF-BB had no effect on activation of signal transducer and activator of transcription (STATs) amongst other intracellular proteins at an acute time point (5 minutes) (Figure 4.7). However, the data clearly demonstrate that PDGF-BB stimulation led to a significant upregulation of phosphorylation of ERK1/2 (Thr202/Tyr204) [4.2 fold, $p<0.001$], Akt (Thr308) [2.7 fold, $p<0.01$], Akt (Ser473) [8.8 fold, $p<0.01$] and PRAS40 (Thr246) [3.5 fold, $p<0.001$] (Figure 4.8). At this acute time point, Pre-incubation with Ang-(1-9) for 30 minutes significantly inhibited PDGF-BB dependent phosphorylation of ERK1/2 (Thr202/Tyr204) [Total signal: PDGF-BB 66990±9378.74 vs PDGF-BB + Ang-(1-9) 34900±5938.57 $p<0.05$] (Figure 4.8).

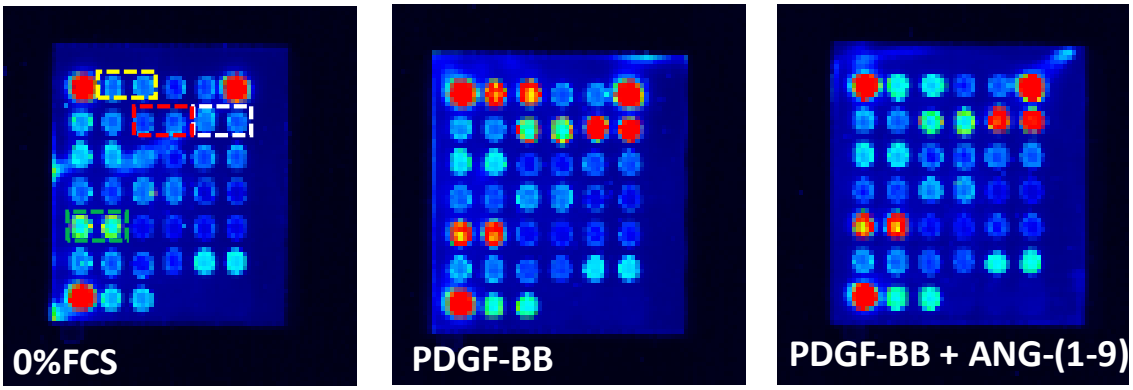

Figure 4.6 Ang-(1-9) inhibits PDGF-BB induced ERK1/2 phosphorylation.

Quiescent HSVSMC were exposed to PDGF-BB for 5 minutes in the presence or absence of Ang-(1-9). Cell lysates were then probed using the PathScan® Intracellular Signalling Array Kit (A) Representative images of the array under each condition. ERK1/2 (yellow), AKT (Ser473/Thr308) (red/white) and PRAS40 (green) Colour refers to the dashed boxes indicating target of array spot. HSVSMC lysates taken from 5 independent patients**.**

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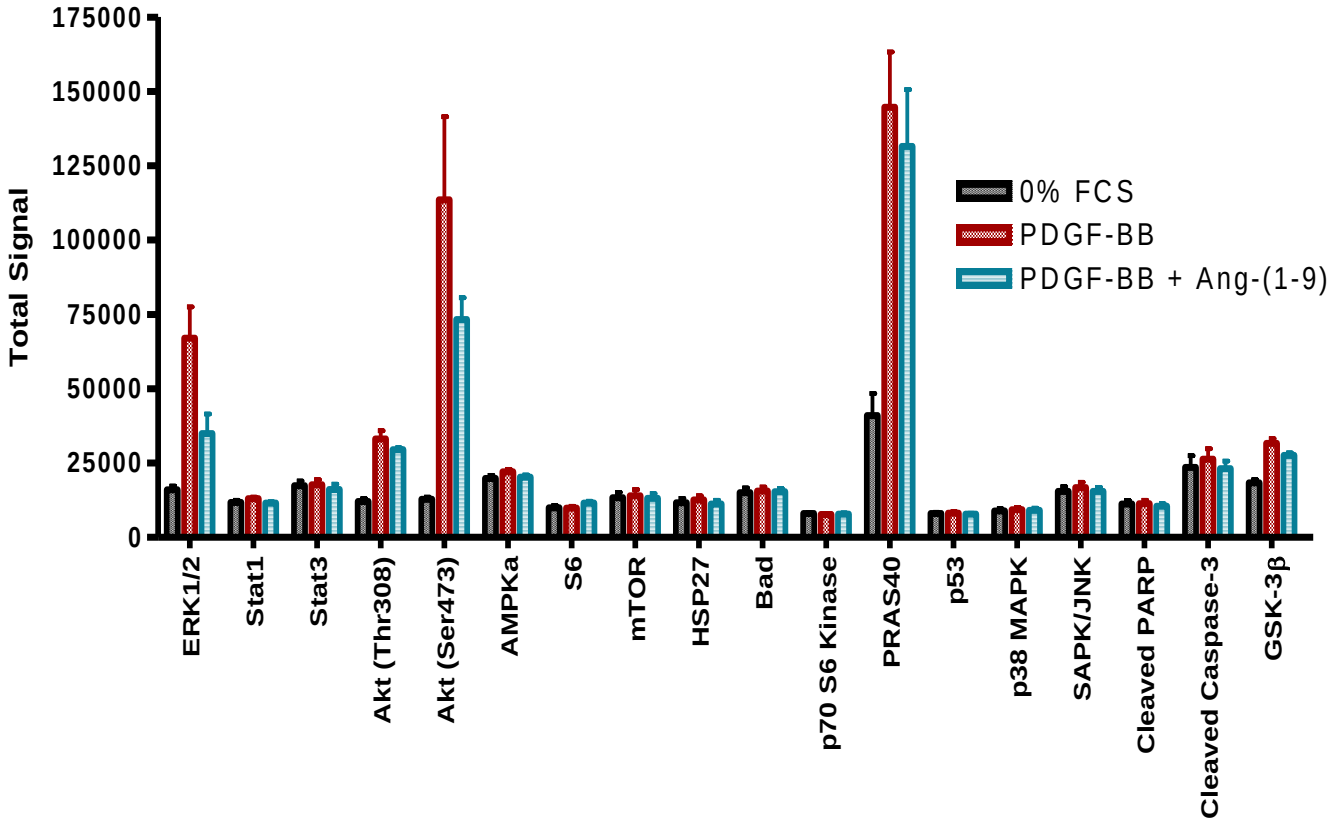

Figure 4.7 Ang-(1-9) inhibits PDGF-BB induced ERK1/2 phosphorylation.

Quiescent HSVSMC were exposed (black) to PDGF-BB for 5 minutes in the absence (red) or presence of Ang-(1-9) (blue). Cell lysates were then probed using the PathScan® Intracellular Signalling Array Kit. The levels of 18 different cleaved or phosphorylated intracellular signalling members were assessed by densitometry. Values represent mean total signal from five independent patients. Error bars represent S.E.M.

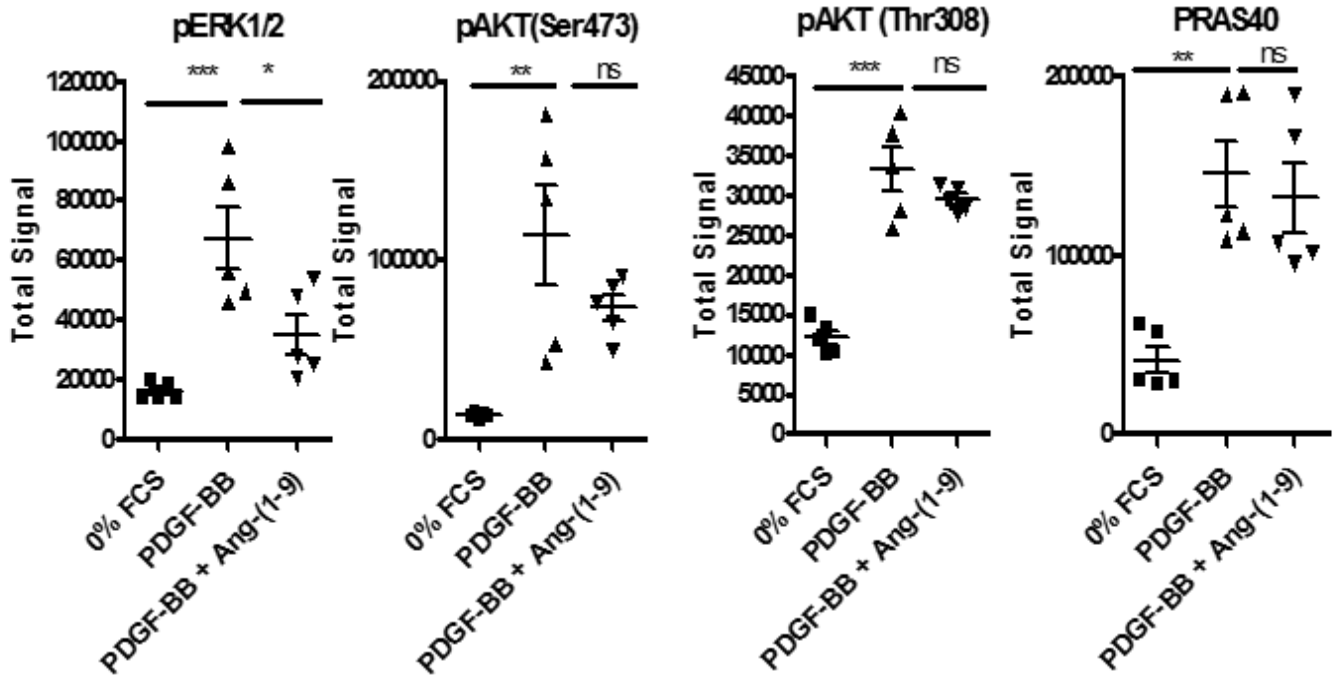

Figure 4.8 Ang-(1-9) inhibits PDGF-BB induced ERK1/2 phosphorylation.

Quiescent HSVSMC were exposed to PDGF-BB for 5 minutes in the presence or absence of Ang-(1-9). Cell lysates were then probed using the PathScan® Intracellular Signalling Array Kit. The total signal ± SEM was determined by standard densitometry and compared using a one-way ANOVA + Tukey's multiple comparison post hoc test *p<0.05 **p<0.01 ***p<0.001 n=5 independent patients.

4.3.7 The effect of MEK inhibition on PDGF-BB induced HSVSMC proliferation

In order to determine whether inhibition of the ERK1/2 pathway could inhibit PDGF-BB induced proliferation the BrdU incorporation assay was utilised in combination with a MEK1 inhibitor (U1026) (Figure 4.9). (Favata et al., 1998) Due to variation in absorbance values between patients the average fold change has been presented (Figure 4.9B). BrdU assays showed that PDGF-BB once again elicited a pro-proliferative response as compared to unstimulated cells [PDGF-BB: 2.76±0.09 compared to 0% FCS control $p<0.001$] (Figure 4.9B). Pre-incubation with U1026 inhibited PDGF-BB induced HSVSMC proliferation compared to cells treated with PDGF-BB and vehicle control [PDGF-BB: 2.76±0.09 vs PDGF-BB+DMSO: 1.23±0.07 $p<0.001$] (Figure 4.9B).

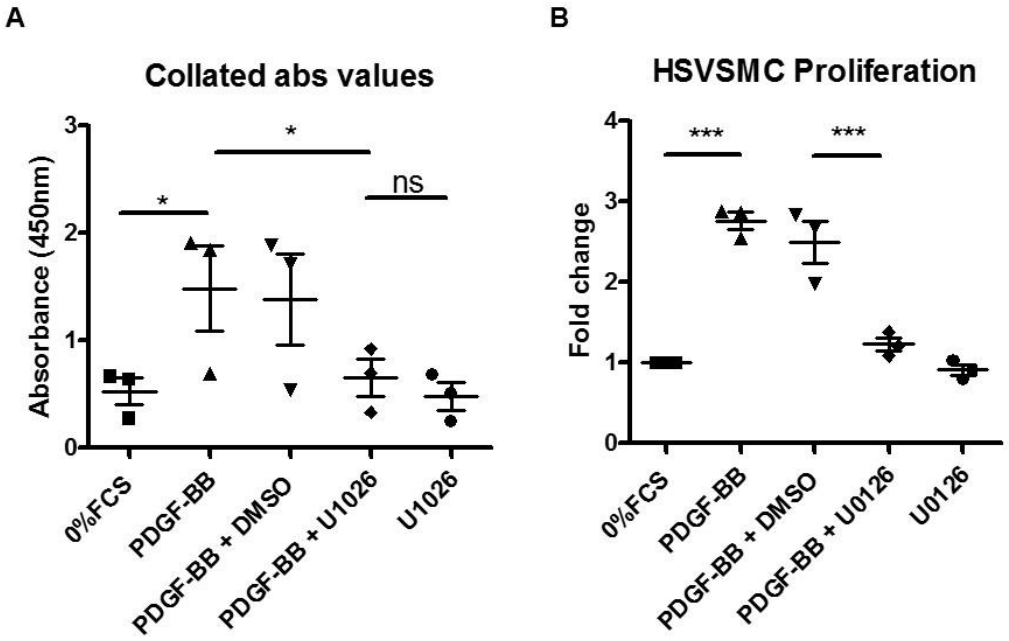

Figure 4.9 U1026 inhibits PDGF-BB induced HSVSMC proliferation.

Quiescent HSVSMC were exposed to PDGF-BB in the presence or absence of MEK1 inhibitor U1026. 48 hours later BrdU incorporation was determined by sandwich ELISA and colorimetry. Values represented as (A) the mean absorbance values and the (B) mean fold change ± SEM n=4 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test *p<0.05 ***p<0.001.

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4.4 Discussion

Vein graft failure (VGF) following CABG occurs due to proliferation and migration of VSMCs forming a neointima that blocks the graft lumen (Wan et al., 2012). Currently there are no pharmacological interventions which prevent late VGF. Dysregulation of the RAS promotes vascular remodelling via Ang II stimulation of the AT_1R (Montezano et al., 2014). Previous studies have identified counter-regulatory RAS peptides, including Ang-(1-7) and Ang-(1-9), which inhibit VSMC proliferation and migration (Zhang et al., 2016, McKinney et al., 2015). Here, evidence is presented showing that Ang-(1-9) has a therapeutic effect in the vasculature. This is the first study to identify that Ang-(1-9) can counteract the mitogenic effects of PDGF-BB in HSVSMC. Here Ang-(1-9) was found to inhibit PDGF-BB induced HSVSMC directional and chemotactic migration as well as proliferation via inhibition of ERK1/2 phosphorylation.

Recently, McKinney et al., investigated the effect of exogenous Ang-(1-9) peptide on serum-induced HSVSMC proliferation (McKinney et al., 2015). In an attempt to further dissect the mechanism of inhibitory action, we chose to evaluate the effect of pre-exposure to Ang-(1-9) on PDGF-BB, a growth factor known to be in FCS and established proliferative effects on VSMCs (Kingsley et al., 2002, Sterpetti et al., 1996). PDGF-BB has been demonstrated to elicit an enhanced proliferative response in VSMC isolated from SVs as compared to mammary artery (Li et al., 2011a). This is due to PDGF-BB induced upregulation MAPKs and downregulation of cell cycle inhibitors, an effect seen only in venous VSMC as compared to arterial cells (Yang et al., 1998).

The BrdU incorporation assay determined that Ang-(1-9) inhibited PDGF-BB induced proliferation. This was seen as a dose-dependent effect in which a higher concentration of Ang-(1-9) was required to significantly suppress proliferation. It is not known why this was the case however it could be speculated that the differences in the half-life of Ang-(1-9) and PDGF-BB play a role. Pre-treatment with Ang-(1-9) also inhibited HSVSMC directional migration in a scratch assay and as well as reducing chemotaxis in the Boyden chamber assay. The variability observed between patient samples in these assays is reflective of the heterogeneity of the patient samples. This heterogeneity was

clearly identified by immunofluorescence analysis of smooth muscle cell markers.

Early studies identified that Ang-(1-7) inhibited PDGF-BB induced VSMC proliferation (Freeman et al., 1996). More recently, it was reported that Ang-(1-7) inhibited PDGF-BB induced migration of VSMC and that this is associated with a reduction in phosphorylation of tyrosine 1009 on the PDGFRB (Tallant et al., 2012). Future work could investigate the effect of Ang-(1-9) on PDGF-BB mediated PDGFRB phosphorylation at Y1009 and other autophosphorylation sites.

PDGF-BB, importantly, is known to activate MAP kinases such as ERK1/2 to elicit pro-proliferative responses in VSMC through activation of the transcription factors c-Jun, Ras and Src (Barone and Courtneidge, 1995, Zhan et al., 2002b, Irani et al., 1994). ERK1/2 signalling also contributes to VSMC migration (Kingsley et al., 2002). A wealth of studies support that the AT_2R negatively regulates AT_1R signalling in part by inhibition of ERK1/2 phosphorylation (Horiuchi et al., 1997, Yamada et al., 1996, Inuzuka et al., 2016). McKinney et al., have demonstrated that Ang-(1-9) can inhibit Ang II induced ERK1/2 phosphorylation and that this effect is reversed by AT_2R antagonist PD123,319 (McKinney et al., in preparation).

In this study, an unbiased screening approach was used to simultaneously measure 18 different phosphorylated or cleaved members of intracellular signalling cascades. In agreement with the previous literature PDGF-BB stimulated phosphorylation of Akt and ERK1/2 (Zhan et al., 2003a, Kaplan-Albuquerque et al., 2003).

A trend toward a decline in Akt phosphorylation at serine residue 473 was observed but not at threonine residue 308 after pre-incubation with Ang-(1-9). No difference was reported in the level of PRAS40, a residue downstream of Akt. In VSMC, PDGF-BB induced Akt signalling results in phenotype switching via down regulation of classic SMC genes such as aSMA, this is concomitant with a change in serum response factor (SRF) subcellular location (Kaplan-Albuquerque et al., 2003). Further studies could investigate the effect of Ang-(1-9) or the AT_2R on PDGF-BB induced VSMC phenotype switching. This could be achieved through Ang-(1-9) treatment or adenoviral mediated constitutive overexpression of the

AT_2R in VSMC followed by gene expression analysis of classical SMC markers after treatment with PDGF-BB.

The cell-based assays employed in this study clearly demonstrate that Ang-(1-9) inhibited HSVSMC proliferation and migration. ERK1/2 is key in these processes (Zhan et al., 2003a). Importantly, in the unbiased screening approach presented here, pre-incubation with Ang-(1-9) significantly decreased PDGF-BB induced ERK1/2 phosphorylation. This is in good agreement with the findings of McKinney et al., who described Ang-(1-9) inhibited Ang II-induced migration via blocking Ang II-mediated ERK1/2 phosphorylation (McKinney et al., in preparation). Subsequently, the BrdU incorporation assay was used in combination with a MEK1 inhibitor (U1026) in order to inhibit ERK1/2 and not Akt (Favata et al., 1998). Inhibition of the ERK1/2 pathway markedly inhibited PDGF-BB induced HSVSMC growth. This provides evidence that selective ERK1/2 inhibition is sufficient to inhibit HSVSMC proliferation. The effect of U1026 on PDGF-BB induced HSVSMC migration was not assessed. In studies from other laboratories this effect has also been reported. Vinpocetine, a chemical synthetically derived from a plant based extract (Szobor and Klein, 1976) inhibited neointima formation in the carotid ligation model and importantly inhibited VSMC migration and proliferation via attenuation of ERK1/2 phosphorylation but did not inhibit Akt phosphorylation levels (Cai et al., 2012). Further studies could assess the effect of Ang-(1-9) on other ERK1/2 agonists. Moreover, further investigations into the precise mechanism of action of Ang-(1-9) are required especially with regard to HSVSMC migration and intracellular signalling. For example, it is still unclear how Ang-(1-9) mediated inhibition of ERK1/2 phosphorylation.

In the present study, it was not investigated whether the effects of Ang-(1-9) were AT_2R dependent. However, it has been previously reported that PDGF-BB induced an exacerbated proliferative response in AT_2R KO embryonic aortic VSMC (Akishita et al., 1999). This supports the hypothesis that, in general, AT_2R negatively regulates proliferation induced by growth factors other than Ang II. This could be a novel finding supporting the notion that Ang-(1-9) acts through the AT_2R or it is possible that Ang-(1-9) is metabolised to Ang-(1-7) via ACE mediated enzymatic cleavage (Donoghue et al., 2000) (and other peptides through subsequent hydrolysis) to elicit an effect through other receptors such

as the Mas receptor. This is important to consider as *in vitro* studies show that other angiotensin peptides such as Ang II are rapidly broken down into other peptides (Ang-(1-7)/Ang IV) in culture (Basu et al., 2015).

Future work could include *ex vivo* models of saphenous VGF using whole sections of HSV. These models can include *ex vivo* vein perfusion systems where pulsatile laminar flow is utilised to recapitulate the increase in haemodynamic pressure experience in the arterial environment of the heart (Prandi et al., 2015, Longchamp et al., 2014). The effect of Ang-(1-9) treatment could be assessed by monitoring well known proliferation markers such as PCNA (Moldovan et al., 2007). The same could be achieved in a more simplistic model where the SV is cultured in a petri dish containing growth medium with and without PDGF-BB or Ang II. Furthermore, the effect of signalling downstream of the AT_2R could be assessed by fluorescence *in situ* hybridisation (FISH). Kukida et al reported that ATIP and PPAR gamma form a complex to bind to PPAR response element (PPR) (Kukida et al., 2016). This leads to inhibition of pro-proliferative and inflammatory responses in VSMC (Kukida et al., 2016). Investigating whether the same effect occurs in human SVs *ex vivo* after Ang-(1-9) treatment would be highly relevant to CABG surgeries and in turn the prevention of VGF.

In summary, Ang-(1-9) inhibited PDGF-BB induced proliferation and migration of HSVSMCs *in vitro,* two hallmarks of neointima formation. Furthermore, pre-incubation with Ang-(1-9) led to decreased levels of PDGF-BB induced ERK1/2 phosphorylation. ERK1/2 inhibition via pharmacological inhibition of MEK1 potently inhibited PDGF-BB induced proliferation *in vitro*. But further investigations into Ang-(1-9) mediated intracellular signalling are required. Overall, the data presented here are promising because classical inhibition of the RAS through ACE inhibition have not been successful in clinical trials (Rouleau et al., 2008). PDGF-BB is of utmost importance in the progression of vein graft disease and dual inhibition of PDGF-BB effects and the effects of Ang II could be a more optimal dual treatment strategy. These data lend further support to the view that Ang-(1-9) is protective in the vasculature

5 Chapter 5 An investigation into the effect of adenoviral vector-mediated overexpression of Ang-(1-9) on human vascular cells *in vitro* and neointima formation *in vivo*

5.1 Introduction

In the previous chapter, delivery of Ang-(1-9) as an exogenous peptide inhibited HSVSMC proliferation *in vitro*. However, the therapeutic application of angiotensin peptides is hampered by their rapid half-life. In Wistar albino rats the half-lives of Ang II and Ang III are 16.1 and 14.1 seconds respectively (following intravenous injection) (Al-Merani et al., 1978). In Sprague Dawley rats, the half-life of Ang-(1-7) after intravenous administration was found to be between 9 and 11 seconds (Yamada et al., 1998). Following subcutaneous delivery, the half-life of Ang-(1-7) in humans is around 29 minutes (Rodgers et al., 2006). The half-life of Ang-(1-9) *in vivo* is not yet known however, Chen et al., analysed the metabolism of angiotensin peptides by ACE *in vitro*. The authors reported that Ang-(1-9) was hydrolysed 18% slower than Ang I and 30% slower than Ang-(1-7) (Chen et al., 2005). Therefore, it is possible that the half-life of Ang-(1-9) is longer than that of Ang-(1-7), however, broadly speaking angiotensin peptides have rapid half lives *in vivo*. For this reason, viral gene therapy vectors have been previously engineered to constitutively over-express these peptides. These vectors may therefore be useful novel tools to counter-regulate the RAS in the setting of vascular injury (van Kats et al., 2001a, Methot et al., 1997).

The PREVENT trial series is the only clinical trial to date that has evaluated gene therapy in the setting of VGF following CABG surgery. Targeting VSMC proliferation via *ex vivo* treatment of the human SV with edifoligide (an E2F antisense oligonucleotide) was deemed safe, however despite promising results from early-phase trials, long-term outcomes reported from Phase III trials by the PREVENT investigators were disappointing (as discussed in section 1.7.3). Edifoligide failed to improve the outcomes of CABG surgeries between one and five years (Mann and Conte, 2003, Alexander et al., 2005, Lopes et al., 2012).

CABG offers a unique opportunity to utilise viral vectors by treating the venous graft *ex vivo* in the operating theatre before implantation. This provides local transgene expression and prevents the risks of systemic exposure to the viral vector. George et al., targeted VSMC migration and reported that *ex vivo* transduction of a venous graft using Ad-TIMP3 led to a sustained inhibition of neointima formation 3 months after engraftment in a pig model of interposition

SV grafting (George et al., 2011). This research is now progressing as the first-in-human phase I/II adenoviral gene therapy clinical trial in the CABG setting, with a view to assess its impact on preventing neointima formation following CABG (A randomised double blind Placebo-contROlled Trial of Ad-TIMP3 to prEvent Coronary artery bypass grafT failure [PROTECT]) (Ylä-Herttuala and Baker, 2017).

Replication deficient adenoviral, lentiviral and adeno-associated viral vectors have previously been used as gene therapy vectors to successfully counter-regulate the RAS in diverse pathologies. The engineering of expression cassettes encoding a fusion protein containing a furin-cleavable angiotensin peptide has made it possible to overproduce angiotensin peptides *in vitro* and *in vivo* (Methot et al., 1997). A human prorenin signal peptide ensures secretion and is linked to a mouse crystallizable region fragment (Fc region) of IgG2B which provides molecular mass in order to prevent hydrolysis and breakdown by nonspecific proteases. This Fc region fragment is linked by a human prorenin prosegment that contains a cleavage site for furin protease which facilitates release of the adjacent angiotensin peptide (Methot et al., 1997). This approach was originally used to investigate tissue specific overexpression of angiotensin peptides and upregulated Ang II formation in the heart by up to forty fold leading to pathophysiological cardiac remodelling (van Kats et al., 2001a). Van Kats et al., generated transgenic mice which over-expressed a fusion protein that contained a furin-cleavable Ang II peptide specifically in cardiomyocytes. Cardiac specific overexpression of the Ang II fusion protein led to an increase of 20-40-fold Ang II content in the heart leading to pathological cardiac remodelling. It is notable that leakage of Ang II into the circulation was not sufficient to induce hypertension in this study (van Kats et al., 2001a). Later, transgenic rats with testicular overexpression of an Ang-(1-7) fusion protein were used to increase circulating levels by 2.5 fold in order to enhance lipid and glucose metabolism, improve kidney function and confer cardio-protection (Botelho-Santos et al., 2007, Santos et al., 2004, Ferreira et al., 2006, Santiago et al., 2010).

Unsurprisingly, this approach was soon utilised in viral gene therapy (Flores-Munoz et al., 2012, van Kats et al., 2001a, Takayanagi et al., 2012). Lentiviral-

mediated delivery of Ang-(1–7) was therapeutic in bleomycin-induced pulmonary fibrosis and MCT induced pulmonary hypertension models (Shenoy et al., 2010). In the pulmonary fibrosis model, overexpression of Ang-(1-7) decreased collagen deposition and gene expression of the AT_1R and TGF-B_1. In the pulmonary hypertension model, intratracheal delivery of Lenti-Ang-(1-7) attenuated remodelling in the lungs and heart as well as decreasing right ventricular systolic pressure (Shenoy et al., 2010). Lentiviral mediated gene transfer of Ang-(1-7) has also been utilised in mouse models of MI where it increased fractional shortening, decreased left ventricular end-diastolic pressure, and decreased cardiac hypertrophy (Qi et al., 2011).

Flores-Munoz and colleagues utilised a replication deficient human adenovirus serotype 5 (Ad5) vector to overexpress Ang-(1-7) or Ang-(1-9) to inhibit H9c2 and adult rabbit primary cardiomyocyte hypertrophy *in vitro* (Flores-Munoz et al., 2012). Both direct transduction and the conditioned media taken from RAdAng-(1-7) and RAdAng-(1-9) transduced HeLa cells inhibited Ang-II induced hypertrophy in a fashion blocked by the Mas receptor antagonist A779 or AT_2R antagonist PD123,319 respectively. In this study, RAdAng-(1-7) and RAdAng-(1-9) also inhibited cardiomyocyte hypertrophy induced by isoproterenol and vasopressin thus suggesting that these vectors can be used to inhibit pathological processes independent of Ang II (Flores-Muñoz et al., 2012). Recently, a single injection of AAV serotype 9 encoding a furin-cleavable Ang-(1-9) peptide improved cardiac function over a period of 8 weeks in a mouse model of MI (Fattah et al., 2016) (as discussed in detail in section 1.7.4).

The counter-regulatory axis of the RAS has also drawn a great deal of interest in the field of cancer research due to the tumour promoting effects of Ang II/AT_1R signalling (Pinter and Jain, 2017). Pei et al., utilised a recombinant AAV serotype 8 vector to introduce an Ang-(1-7) fusion protein expression cassette under a liver specific promoter in order to utilise the liver as a factory and successfully raised circulating levels of Ang-(1-7) by approximately 6 fold (Pei et al., 2016). This had a functional and therapeutic effect in murine xenograft models of nasopharyngeal, liver and lung carcinoma (Pei et al., 2016, Chen et al., 2017, Mao et al., 2018). The authors also further improved this model by mutating tyrosine residues in the AAV capsid in order to improve liver transduction. A

single injections of AAV8-Y703F-Ang-(1-7) led to a dramatic increase (>50 fold) in sera Ang-(1-7) levels (Mao et al., 2018).

Therefore, it clear that viral vectors can be utilised to overexpress components of the counter-regulate RAS. Overexpressing angiotensin peptides *in vivo* through the use of viral vectors has not been investigated as a strategy to inhibit neointima formation following injury. Here, the effect of adenoviral mediated overexpression of Ang-(1-9) on HSVEC and HSVSMC proliferation was investigated. Furthermore, a proof-of-concept study employed intravascular delivery of an adenoviral vector that encoded a Ang-(1-9) Fc fusion-protein [RAdAng-(1-9] to assess the effect of adenoviral-derived Ang-(1-9) on neointima formation following carotid artery wire injury (WI) (Lindner et al., 1993).

5.2 Aims

- Investigate the effect of adenoviral-mediated delivery of Ang-(1-9) on HSVEC and HSVSMC proliferation and migration by conditioned media assays and direct transduction of target cells.

- Investigate the effect of adenoviral-mediated delivery of Ang-(1-9) in the murine carotid wire injury model of neointima formation following intravenous administration.

5.3 Results

5.3.1 Production and titration of recombinant adenoviral vectors

Stocks of adenoviral vectors were generated as described in section 2.6. The micro BCA assay was used to determine the titer in number of viral particles per mL. The titer in plaque forming units and the ratios between the two titers are presented in Table 5.1.

Date of Isolation	Ad vector	VP	PFU	Ratio
13/02/2016	AdGFP	$1.35x10^{12}$	$1.79x10^{10}$	1 in 75.4
20/05/2017	RAdAng (1-9)	$6.6x10^{12}$	$1.75x10^{11}$	1 in 37.7
20/05/2017	Ad*LacZ*	$1.87x10^{12}$	$7x10^{10}$	1 in 26.4

Table 5.1 Recombinant adenoviral vector titers Table of adenoviral vectors generated and their titer in viral particles per mL (VP/mL) and plaque forming units (PFU).

5.3.2 VSMC transduction with recombinant human adenovirus serotype 5 leads to transgene expression after 48 hours

Ex vivo transduction of the human SV prior to engraftment into the arterial circulation of the heart is currently being investigated in the clinic. Similarly, coronary artery stent-based delivery of viral vectors for use in PCI is also being investigated but is still at in preclinical development (Fishbein et al., 2017, Appleby et al., 2014, Fishbein et al., 2008). Here, HSVSMC and human coronary artery SMCs (HCASMC) were transduced with adenoviral vectors encoding either green fluorescence protein (GFP) or *Lac*Z in order to determine whether VSMC from these different vascular beds could be effectively transduced. Fluorescence microscopy detected GFP expression two days after HSVSMC and HCASMC had been transduced with AdGFP (Figure 5.1 Top and bottom panels). No fluorescence was observed in control cells. GFP expression in HSVSMC appeared to increase as the concentration of adenoviral vector increased (100-600 pfu/cell) (Figure 5.1: top panels). The same trend was observed when HSVSMC were transduced with Ad*Lac*Z and transgene expression assessed by detection of ß-galactosidase activity (Figure 5.1: bottom panels). These data represent a single biological repeat.

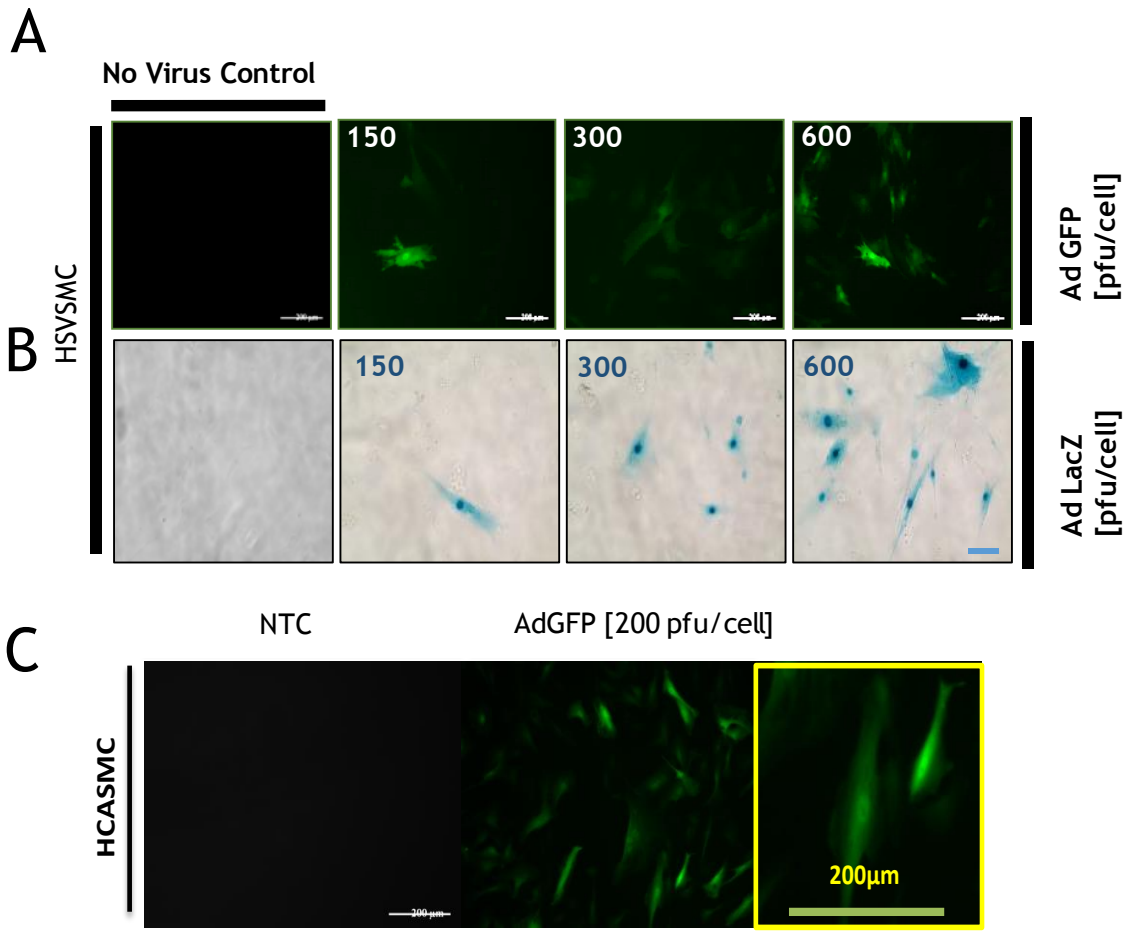

Figure 5.1 Adenoviral transduction of VSMC.

Transduction efficacy was assessed by the ability of AdGFP/Ad*LacZ* to transduce VSMC at a multiplicity of infection of 150, 300 and 600 pfu/cell. Cells were visualised 48 hours after transduction using fluorescence or light microscopy. A) Fluorescence microscopy of AdGFP transduced HSVSMC (10X objective, scale bar: 200µm).((B) X-Gal staining (20X objective scale bar: 100µm) (C) HCASMC were transduced with AdGFP at 200 pfu/cell and transgene expression assessed by fluorescence microscopy 48 hours later (10X objective, scale bar: 200µm) n=1 independent patient.

5.3.3 VSMC transduced with RAdAng-(1-9) express the Ang-(1-9) fusion protein

The short half-life of angiotensin peptides limits their utility as therapeutics. An adenoviral vector originally engineered by Flores-Munoz et al., to express a fusion protein which releases Ang-(1-9) [RAdAng-(1-9)] was propagated on a large scale, purified and titer determined as $1.75x10^{11}$ pfu/cell (Flores-Muñoz et al., 2012). In order to detect expression of the Ang-(1-9) fusion-protein, HSVSMC were transduced with RAdAng-(1-9) at 30, 300 and 1000 pfu/cell. After 48 hours of transduction, western immuno-blotting of cell lysates with a fluorophore-conjugated Goat anti-Mouse IgG (488nM) detected the murine IgG2B fragment of the fusion protein at the correct molecular weight of 32 kDa (300 and 1000 pfu/cell) (Figure 5.2A). The same approach was employed to assess whether HCASMC transduced with RAdAng-(1-9) express the fusion protein. HCASMC transduced with RAdAng-(1-9) [200 pfu/cell] expressed the fusion protein (Figure 5.2B). Additionally, immunofluorescence experiments detected the fusion protein within HSVSMC by probing for Mouse IgG (red). Positive staining for Ms IgG was observed in a punctate pattern around the nucleus suggesting trafficking of the fusion protein through the endoplasmic reticulum (Figure 5.3A). The fusion protein was not detected in HSVSMC transduced with a control vector (AdLacZ) (Figure 5.3B).

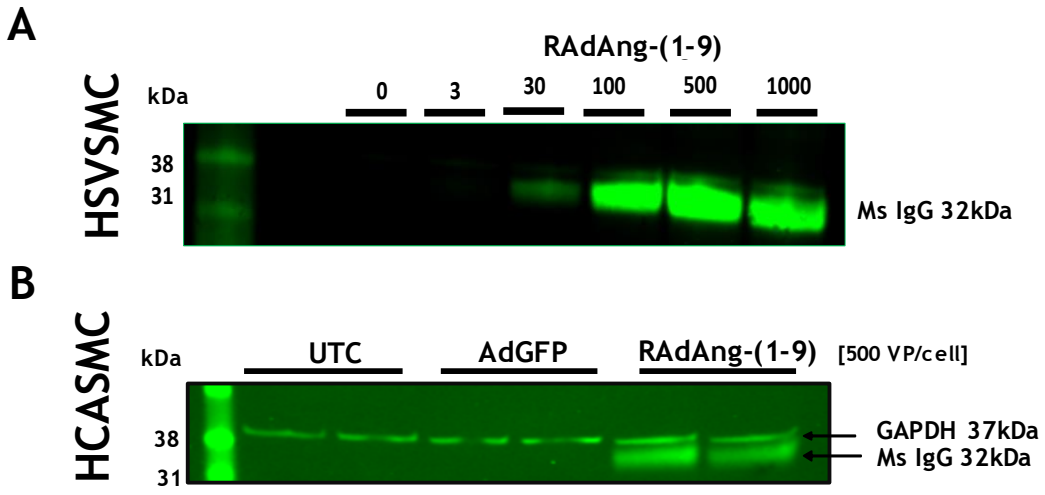

Figure 5.2 VSMC transduced with RAdAng-(1-9) produce the IgG fusion protein

Immunoblotting of cell lysates demonstrated (A) HSVSMC (n=3 independent patients) and (B) HCASMC (n=1) transduced with RAdAng-(1-9) produce the IgG fusion protein (Ms IgG 32kDa, GAPDH 37 kDa). UTC (un-transduced control).

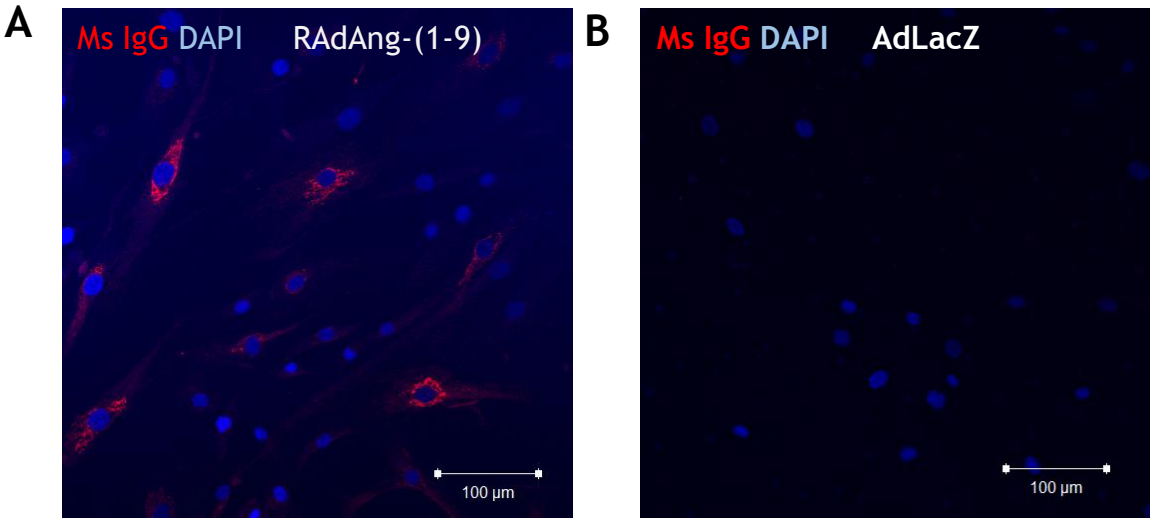

Figure 5.3 HSVSMC transduced with RAdAng-(1-9) harbour the Ang-(1-9 fusion protein as determined by Ms IgG detection.

Immunofluorescence and confocal microscopy detected the fusion protein (red) in HSVSMC transduced with (A) RAdAng-(1-9) (B) the fusion protein was not detected in AdLacZ transduced HSVSMC (Goat anti-mouse IgG AlexaFluor 533) (25X objective, scale bar: 100µm). Representative Images n=3 independent patients.

5.3.4 Direct transduction with RAdAng-(1-9) inhibits Ang II induced HSVSMC migration

Having previously provided evidence that VSMC can be readily transduced with RAdAng-(1-9) the next aim was to assess the functional effect of the adenoviral vector on HSVSMC. Therefore, the effect of direct transduction with RAdAng-(1-9) on HSVSMC migration was assessed. HSVSMC were serum starved for 72 hours in the presence or absence of RAdAng-(1-9)/RAdControl (RAd60) transduction. A scratch wound was then performed and HSVSMC re-stimulated with Ang II (200nM) +/- exogenous Ang-(1-9) (200 nM) peptide as controls. Ang II increased migration as compared to serum starved HSVSMC [0.5% FCS 30.15±1.6% wound closure vs 54.4±5% wound closure Ang II $p<0.05$]. Pre-incubation with 200 nM soluble Ang-(1-9) peptide significantly decreased Ang II induced migration by approximately 30% [Ang II 54.4±5% wound closure vs 37.73±3.15% wound closure + Ang-(1-9) $p<0.05$]. A more robust inhibitory effect (~78%) was observed in cells transduced with RAdAng-(1-9) as compared to RAdControl cells treated with Ang II [RAdAng-(1-9) 12.8±4.4 vs 59.2±9.2 RAdControl $p<0.001$] (Figure 5.4A/B). In order to confirm IgG fusion protein expression in RAdAng-(1-9)-transduced cells, protein lysates were prepared from transduced, scratched cells and immunoblotting was performed. The Fc portion of the fusion protein was consistently detected in the cell lysates of HSVSMC transduced with RAdAng-(1-9) providing evidence that transgene expression occurred during the experiment (Figure 5.4C).

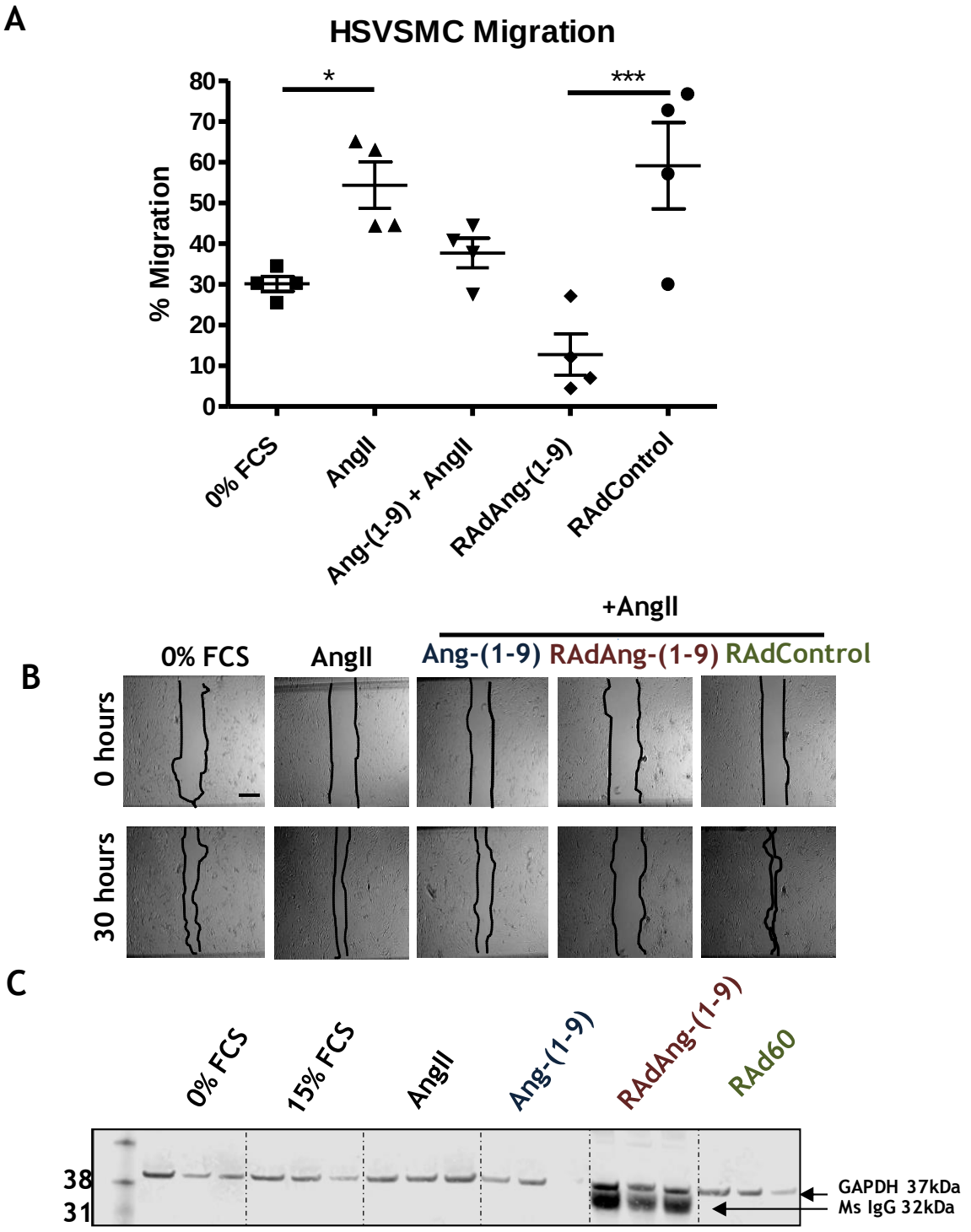

Figure 5.4 Direct transduction with RAdAng-(1-9) inhibits Ang II induced HSVSMC migration. Transduced HSVSMC (300-600 pfu/cell) were serum starved for 72 hours. A vertical scratch was made down each monolayer of cells. HSVSMC were then left untreated or exposed to Ang II [200nM]. (A) Migration was measured after 30 hours. Values represented as percentage migration ± SEM n=4 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test *p<0.05 ***p<0.001. (B) phase contrast microscopy was utilised to image each well at 3 different points down the scratch (C) Immunoblotting of cell lysates demonstrated HSVSMC transduced with RAdAng-(1-9) produced the IgG fusion protein (Ms IgG 32kDa, GAPDH 37 kDa)

5.3.5 Direct transduction with RAdAng-(1-9) promotes HSVSMC proliferation

Next, HSVSMC were directly transduced with RAdAng-(1-9) and the subsequent effect on HSVSMC proliferation was assessed. Following 72 hours transduction with RAdAng-(1-9) or RAdControl (RAd60), HSVSMC were stimulated with 5% FCS and the effect on proliferation determined by MTS assay (Figure 5.5). The addition of 5% FCS stimulated proliferation of HSVSMC as compared to serum starved cells [0.5% FCS 0.94±0.06 vs 1.51±0.01 5% FCS $p<0.001$]. However, direct transduction with RAdAng-(1-9) enhanced the pro-proliferative effect induced by serum [5% FCS 1.51±0.01 vs 2.11±0.014 RAdAng-(1-9) $p<0.001$]. Likewise, transduction with RAdAng-(1-9) at 600 pfu/cell also enhanced the effect of serum as compared to HSVSMC transduced with an equal concentration of control vector [RAdAng-(1-9) 2.11±0.014 vs 1.48±0.03 RAdControl $p<0.001$] (Figure 5.5).

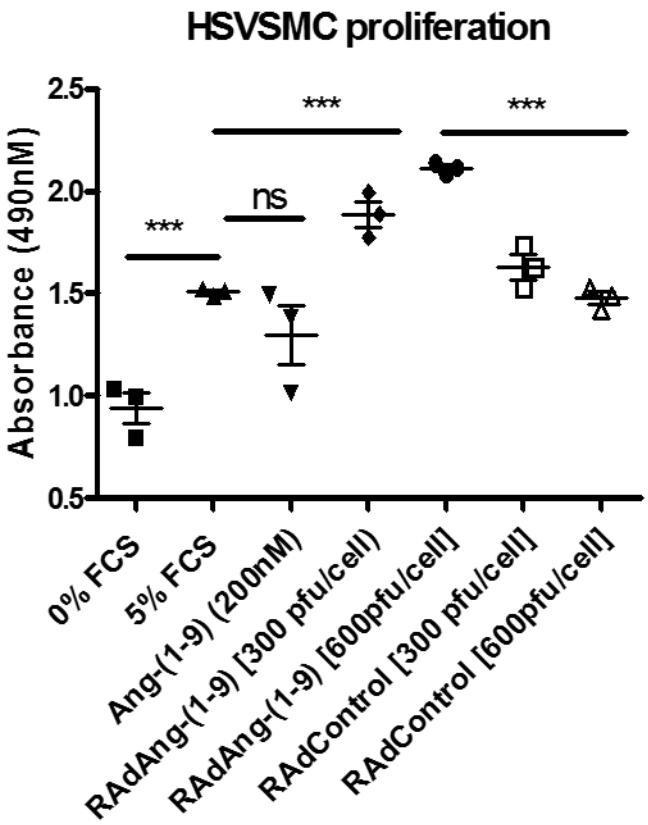

Figure 5.5 Direct transduction with RAdAng-(1-9) promotes HSVSMC proliferation. Quiesced HSVSMC were transduced with RAdAng-(1-9) or RAdControl (300-600 pfu/cell). HSVSMC were re-stimulated with 5%FCS. Proliferation was measured by MTS assays 48 hours later. Values represented as the mean absorbance values ± SEM n=3 independent patients. One-way ANOVA + Tukey's multiple comparison post hoc test ***p<0.001

5.3.6 Phenotyping of isolated HSVEC

Ex vivo adenoviral transduction of human SV leads to effective transgene expression in VSMC but also ECs and resident cell types (George et al., 1998). It was therefore important to also assess the effect of RAdAng-(1-9) on ECs. ECs previously isolated from human SVs of CABG patients (HSVEC) were first subject to immunofluorescence analysis to assess the expression of classical smooth muscle cell and EC markers. Cells isolated from two independent patients were assessed. HSVEC from both patients expressed the endothelial glycoprotein von Willebrand factor (VWF) (Figure 5.6A: red channel). Presence of VSMC contamination was tested by probing for aSMA. Neither sample expressed aSMA (Figure 5.6A: green channel). Next, HSVECs were transduced with RAdAng-(1-9) [600 pfu/cell] and transgene expression detected by immunofluorescence as previously described. A large proportion of HSVEC were effectively transduced at this MOI (Figure 5.6B).

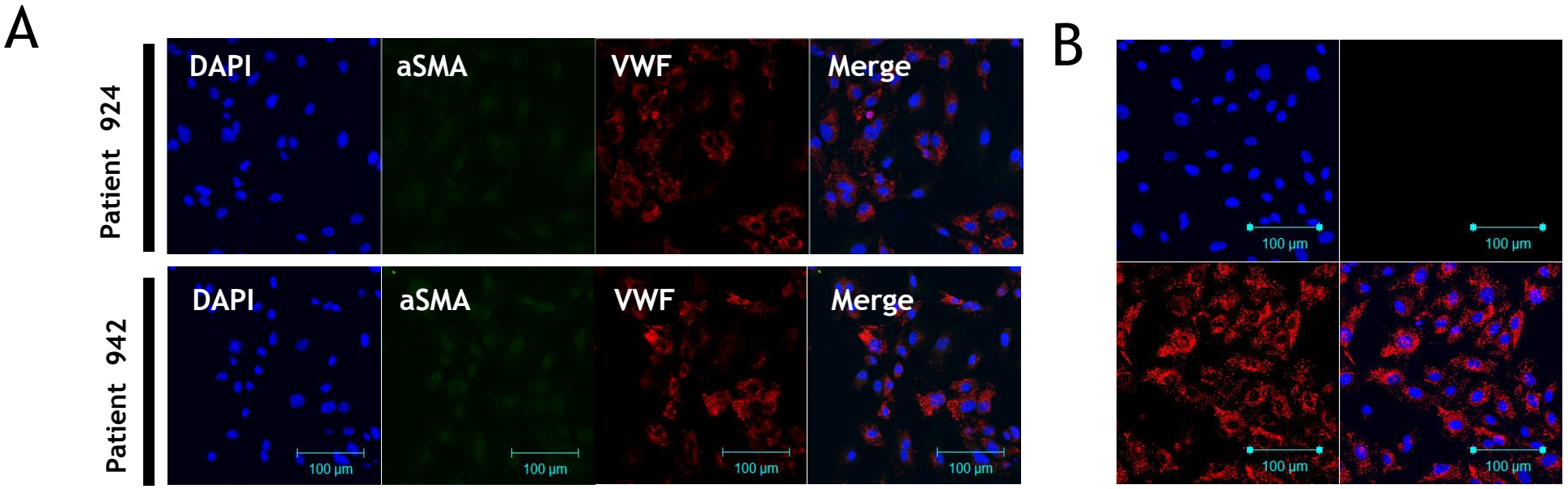

Figure 5.6 Phenotyping and transgene expression in HSVECs isolated from the unused section of saphenous vein donated by CABG patients. (A) HSVECs were fixed and probed by immune-fluorescence for VWF (red) and aSMA (green), DNA was detected by DAPI staining (blue) Representative images n=2 independent patients (B) HSVEC transduced with RAdAng-(1-9) express the fusion protein. Immunofluorescence and confocal microscopy detected the fusion protein (red) in HSVEC transduced with RAdAng-(1-9) (Goat anti-mouse IgG AlexaFluor 533) (40X objective, scale bar: 100µm) Representative images n=1 independent patient

5.3.7 Effects of RAdAng-(1-9) transduction on HSV endothelial cell proliferation

To gain more information on the effects of RAdAng-(1-9) transduction on relevant cell types, ECs isolated from the human SV (HSVEC) of CABG patients were transduced with RAdAng-(1-9) and the effect on proliferation assessed by MTS assay (Figure 5.7). In this experiment HSVECs isolated from different patients displayed varied responses to serum. The presence of 5% serum increased the average levels of proliferation however this was not statistically significant [0% FCS 0.53±0.092 vs 5% FCS 0.78±0.16]. However, quiesced ECs in 0% FCS transduced with RAdAng-(1-9) at 600 pfu/cell displayed significantly increased proliferation in comparison to RAdControl transduced cells at the equivalent MOI [RAdAng-(1-9) 0.95±0.11 vs RAdControl 0.55±0.057 $p<0.01$]. Similarly, in the absence of serum, RAdAng-(1-9) transduced HSVECs at 200 pfu/cell [0.86±0.04 $p<0.05$] and 600 pfu/cell [0.945±0.11 $p<0.01$] increased proliferation as compared to the quiesced HSVECs in 0% FCS (Figure 5.7). RAdControl had no effect on HSVEC proliferation in this model.

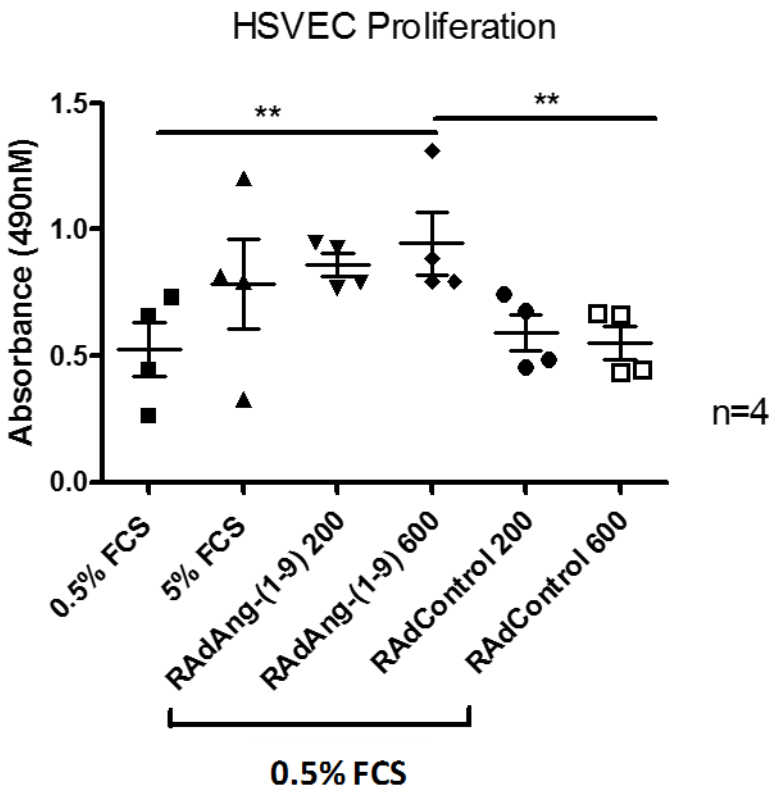

Figure 5.7 Direct transduction with RAdAng-(1-9) promotes HSVEC proliferation.

Quiesced HSVEC were transduced with RAdAng-(1-9) or RAdControl (200-600 pfu/cell). HSVEC were placed in 0.5% FCS for the duration of the experiment with the exception of the positive control. Values represented as the mean absorbance values ± SEM n=4 independent patients. Repeated measures ANOVA + Tukey's multiple comparison post hoc test *p<0.05 **p<0.01.

5.3.8 Confirmation of transgene expression and fusion protein secretion

The therapeutic rationale for our *in vivo* experiments was to deliver RAdAng-(1-9) via tail vein injection in order to utilise the liver as a factory to secrete the Ang-(1-9) transgene and examine the distant effects on neointima formation following wire injury of the carotid artery. To model this scenario *in vitro* transgene expression and fusion protein secretion was confirmed in a hepatocyte model using the human HepG2 hepatoma cell line (Figure 5.8). This cell model has been widely utilised as a hepatocyte cell model for adenoviral vector studies (Alba et al., 2009, Zeilinger et al., 2016). First, HepG2 cells were transduced with AdGFP at increasing MOIs (500 and 1000 VP/cell). At both MOIs, efficient GFP transgene expression was detected using fluorescence microscopy (Figure 5.8A) To determine whether the fusion protein was produced and secreted, the culture media was collected from HepG2 hepatocytes 48 hours after transduction with RAdAng-(1-9) and concentrated using 20 kDA centrifugal filter units. Immunoblotting of concentrated conditioned media with a fluorophore conjugated goat anti-mouse IgG (488nM) detected the murine IgG2B fragment of the fusion protein at the correct molecular weight of 32kDa (100, 500,1000 VP/cell) (Figure 5.8B).

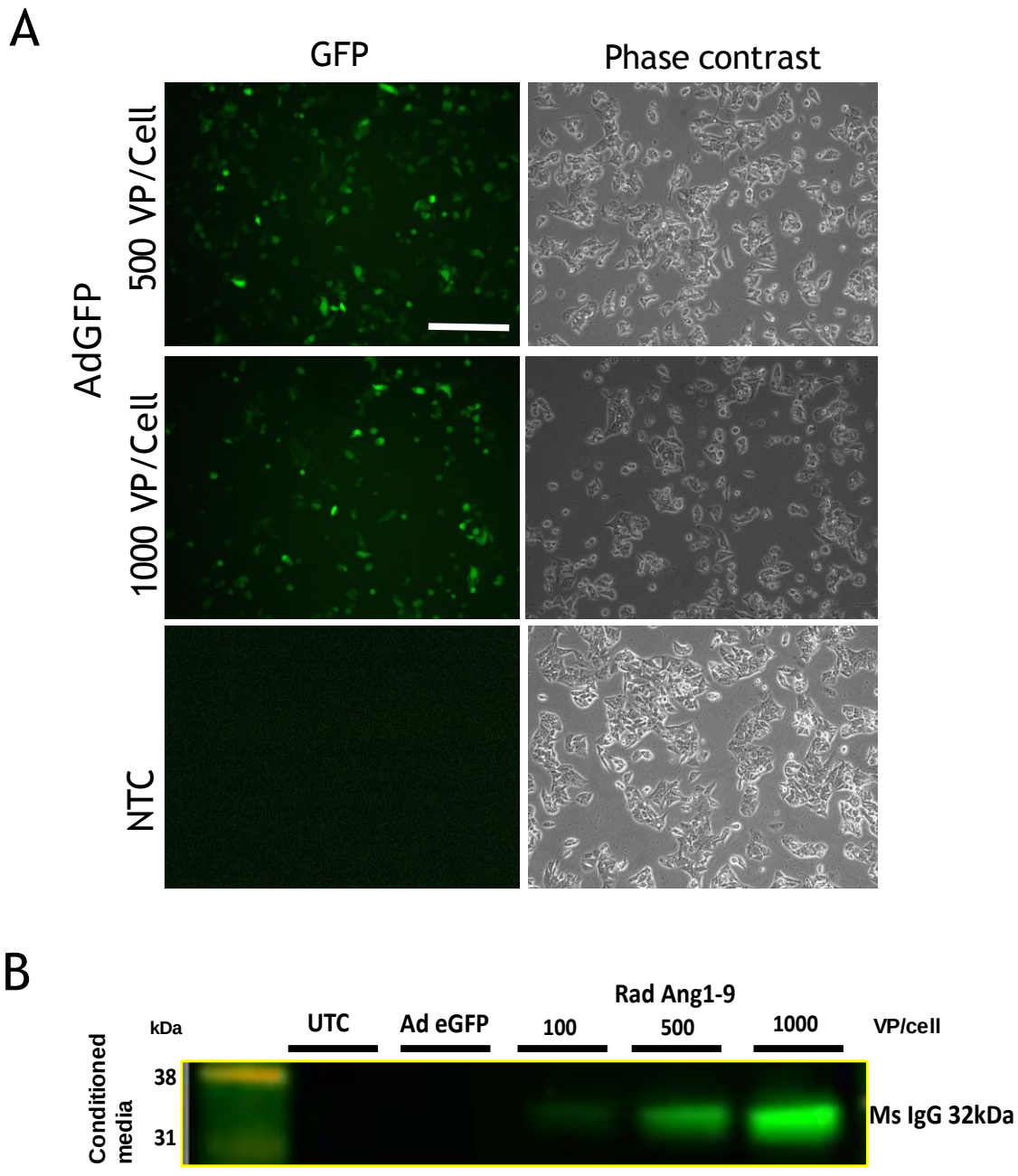

Figure 5.8 Assessment of adenoviral mediated gene transfer to HepG2 cells *in vitro.*

(A) HepG2 cells transduced with AdGFP at 500 and 1000 VP/cell were assessed for GFP expression two days later by fluorescence microscopy. Representative images n=1 (B) HepG2 cells express and secrete the IgG fusion protein. microscopy (10X objective, scale bar: 200µm**).** HepG2 cells were transduced with 100, 500, 1000 VP/cell RAdAng-(1-9) or RAdControl. Immunoblotting of the concentrated conditioned media taken from HepG2 cells that had been transduced with 100, 500 and 1000 VP/cell. A rabbit anti mouse secondary antibody detected the Fc portion of the Ms IgG section of the fusion protein (32 kDa) n=3 biological repeats.

5.3.9 Effect of conditioned media from RAdAng-(1-9) transduced HepG2 cells on HSVSMC proliferation

To evaluate the effect of hepatocyte-secreted Ang-(1-9) on VSMC proliferation, conditioned media transfer experiments were performed using HSVSMC. HepG2 cells were transduced with RAdAng-(1-9) (200 pfu/cell) and incubated in serum free media for 72 hours. An empty control vector (RAd60) was used to control for adenoviral transduction. Next, the conditioned media (CM) was transferred to recipient HSVSMC in the presence or absence of 5% FCS. After a further 48 hours incubation, proliferation was measured using the MTS assay (Figure 5.9A) (Mosman 1983). Conditioned media from non-transduced cells were used as a control. The addition of 5% FCS to this conditioned media stimulated HSVSMC proliferation [CM 0% FCS 0.58±0.04 vs CM+ 5% FCS 0.92±0.08; $p<0.01$] (Figure 5.9B). Conditioned media from RAdAng-(1-9) transduced HepG2 cells significantly inhibited 5% FCS induced proliferation in comparison with the RAd60 control at a MOI of 200 pfu/cell [RAdControl CM+ 5% FCS 0.95.92± 0.073 vs RAdAng-(1-9) CM + 5% FCS 0.54± 0.023 $p<0.01$] (Figure 5.9B).

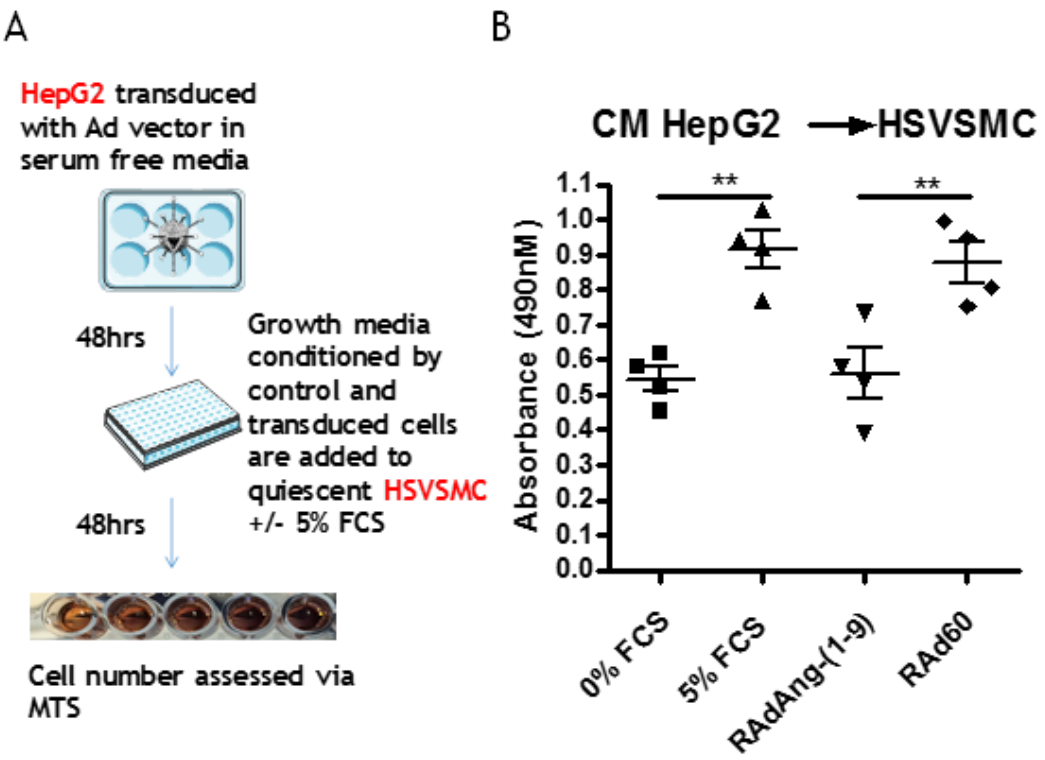

Figure 5.9 Ang-(1-9) secreted from RAdAng-(1-9) transduced HepG2 cells inhibits HSVSMC proliferation

(A) Schematic of experimental design (B) Conditioned media from untreated and adenoviral vector transduced HepG2 cells [200VP/cell] was transferred to quiescent HSVSMC. The effect of 5%FCS on HSVSMC proliferation was determined by MTS assay 48 hours later. **p<0.01 One-way ANOVA with Tukey's multiple comparison post hoc test. Values represent the mean absorbance values. n=4 independent patients.

5.3.10 HeLa cells express ß-galactosidase three days after adenoviral transduction with AdLacZ

Next, assessment of successful delivery of adenoviral vectors to the liver via tail vein injection was assessed utilising a control adenoviral vector expressing *Lac*Z (Ad*Lac*Z). It is important to include a reporter control adenoviral vector when investigating the effect of a potential therapeutic vector in order to control for the effects of viral transduction. First, an adenoviral vector overexpressing *Lac*Z was propagated by standard methods (see section 2.6). HeLa cells were transduced with Ad*Lac*Z and ß-galactosidase activity determined by X-gal staining. Adenoviral transduction at 50 and 500 VP/cell resulted in efficient transgene expression in a dose-dependent manner with strong X-Gal staining detected at an MOI of 500 (VP/cell) (Figure 5.10–blue). ß-galactosidase activity was not detected in PBS treated control HeLa cells. This confirmed this batch of Ad*Lac*Z was producing β-galactosidase and was suitable for *in vivo* gene delivery assessment.

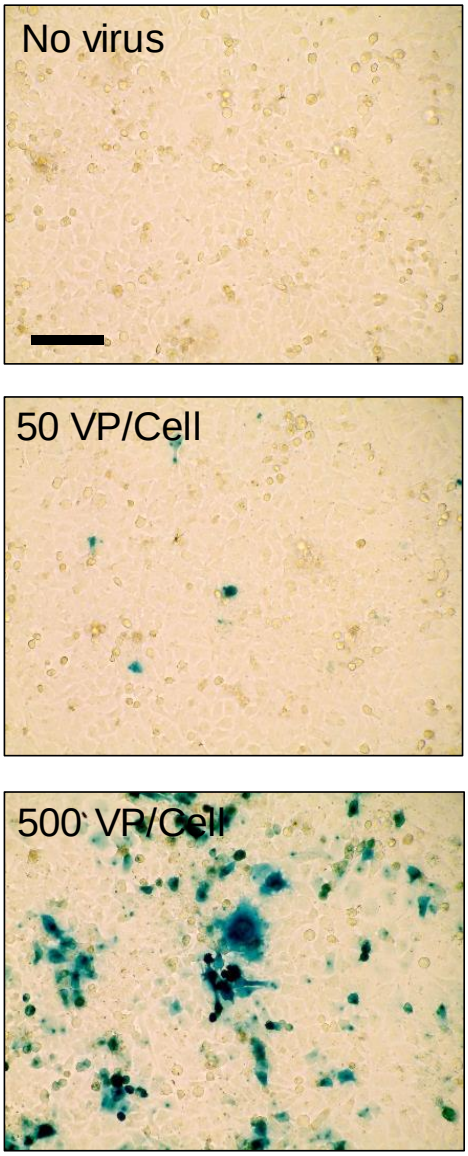

Figure 5.10 HeLa cells transduced with Ad*LacZ* express ß-galactosidase.

HeLa cells were transduced with 50 or 500 VP/cell or left untreated. 72 hours later ß-galactosidase activity was determined by .X-gal staining. X10. Scale bar 100 µm n=1

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5.3.11 Liver ß-galactosidase expression after intravascular delivery of AdLacZ

It is well established that intravascular delivery of adenoviral vectors in mice leads to strong liver transgene expression (Huard et al., 1995, Waddington et al., 2008). In order to evaluate the chosen dose and delivery method either saline or Ad*Lac*Z ($1x10^{11}$ viral particles) was administered via tail vein injection. Three days later, ß-galactosidase activity was detected by X-Gal staining. At this dose, liver transgene expression was observed in all mice administered Ad*Lac*Z (Figure 5.11). ß-galactosidase activity was not detected in the livers of mice that were administered saline.

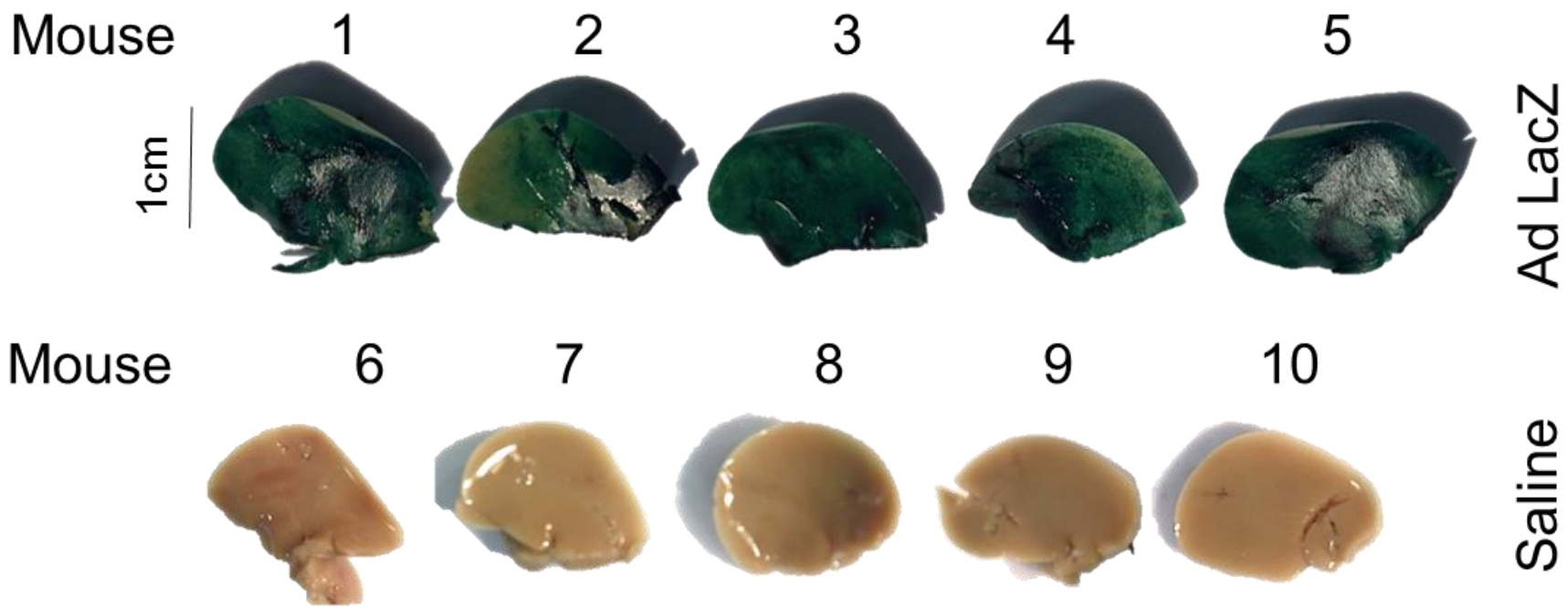

Figure 5.11 *In vivo* liver gene transfer following intravascular delivery of recombinant adenoviral vectors.

Ad*Lac*Z ($1x10^{11}$ VP) (1-5) or PBS (100 µL) was administered to C57 BL6/J mice via tail vein injection. 48 hours later transgene expression in the liver was detected by ß-galactosidase activity using X-Gal staining. (n=5).

5.3.12 Intravascular delivery of RAdAng-(1-9) prevents neointima formation 28 days after wire injury

Viral vector-derived Ang-(1-9) has previously been shown to improve cardiac function after MI in mice (Fattah et al., 2016). To investigate whether gene therapy with Ang-(1-9) can prevent neointima formation after injury C57 BL/6 mice were injected intravenously with 1×10^{11}VP of recombinant adenoviral vectors 48 hours before the carotid artery endothelium was denuded using a modified wire injury surgery (Lindner et al., 1993, Greig et al., 2017). Vessel morphology and neointima formation were then quantified using standard histological methods 28 days after injury (Figure 5.12).

After 28 days, carotid artery wire injury elicited a significant increase in media area in the wire-injured mice compared to sham-operated animals (Figure 5.13 middle panel) [Sham 25036.2±1484.4 μm^2 vs Saline WI 38442.7±1543.6 μm^2 $p<0.001$]. Furthermore, development of an occlusive neointima region was apparent in the wire injured group as compared to sham-operated animals [Sham 196±68.7μm^2 vs Saline WI 21555.9±2955.3 μm^2 $p<0.001$] (Figure 5.13 left panel). Moreover, a significant increase in mean medial and intimal area 28 days after surgery was also observed in control animals receiving I.V delivery of $1x10^{11}$ VP of Ad*Lac*Z (Figure 5.13 right panel). In contrast intravenous tail vein injection of $1x10^{11}$ VP of RAdAng-(1-9) 48 hours before carotid wire injury resulted in a sustained attenuation of neointima formation as compared to mice injected with Ad*Lac*Z [Ad*Lac*Z 22704± 4531.1 μm^2 vs RAdAng-(1-9) 3501.76±1540.4μm^2 $p<0.001$] (Figure 5.13: left panel). This effect was also observed when the neointima area and medial area was expressed as a ratio (NI:M) and compared [Ad*Lac*Z 0.5±0.09 vs RAdAng-(1-9) 0.09±0.03 $p<0.01$] Moreover, medial hypertrophy was also reduced 28 days after wire injury following RAdAng-(1-9) delivery as compared to Ad*Lac*Z treated mice [Ad*Lac*Z 45443.6±2171 μm^2 vs RAdAng-(1-9) 31773.4±2860.5 μm^2 $p<0.01$] (Figure 5.13 central panel).

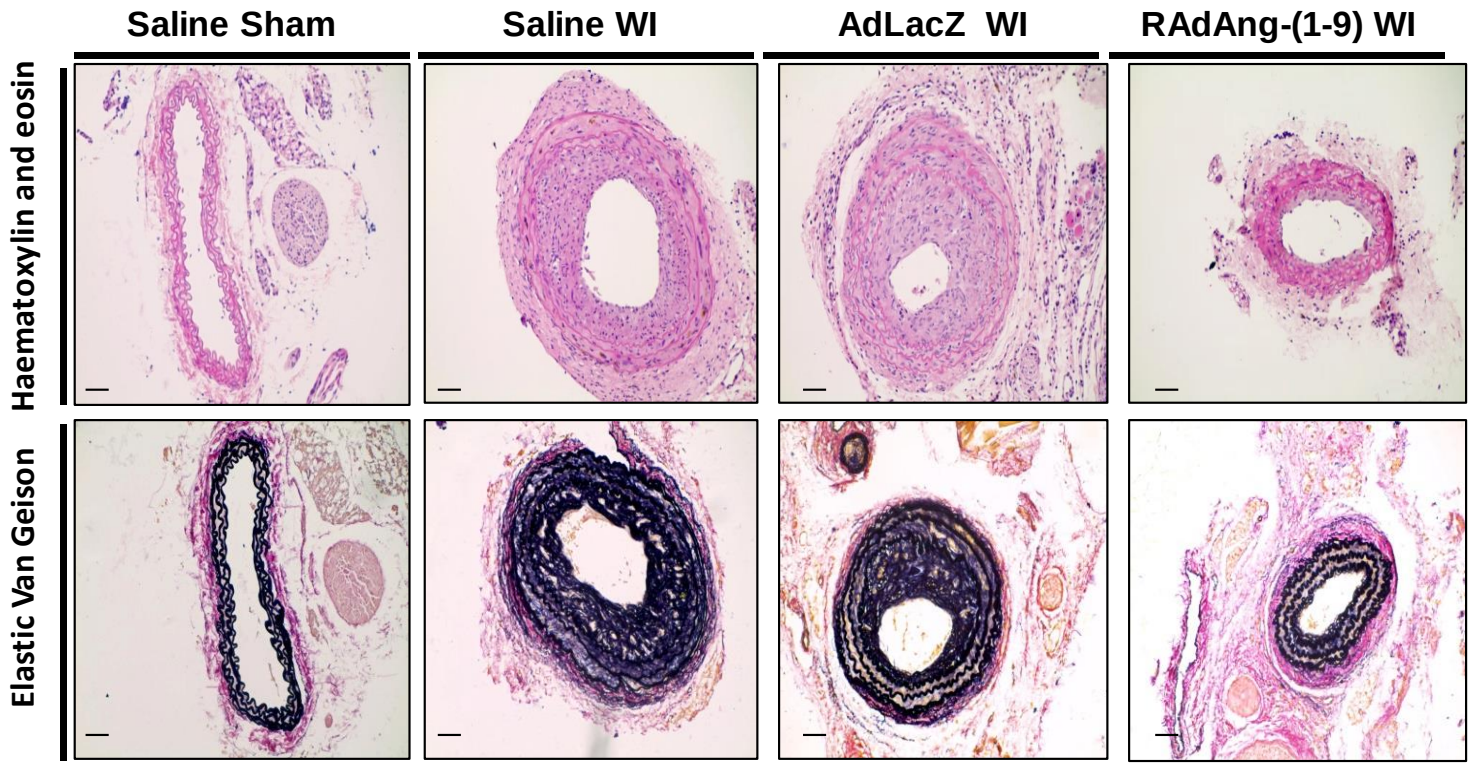

Figure 5.12 *In vivo* gene transfer of Ang-(1-9) reduces intima and media area 28 days after carotid wire injury.

8-10-week-old C57 BL/6 mice were injected via the tail vein with Ad*Lac*Z, RAdAng-(1-9) or Saline 2 days before wire injury surgery. Four weeks later the left carotid arteries were isolated and fixed in paraformaldehyde for histology and quantification of morphology. Bottom panels are representative sections stained with (A) haematoxylin and eosin (B) Elastic van geisen stain n=6-9 mice Objective ×20 Scale bar 100μm**.**

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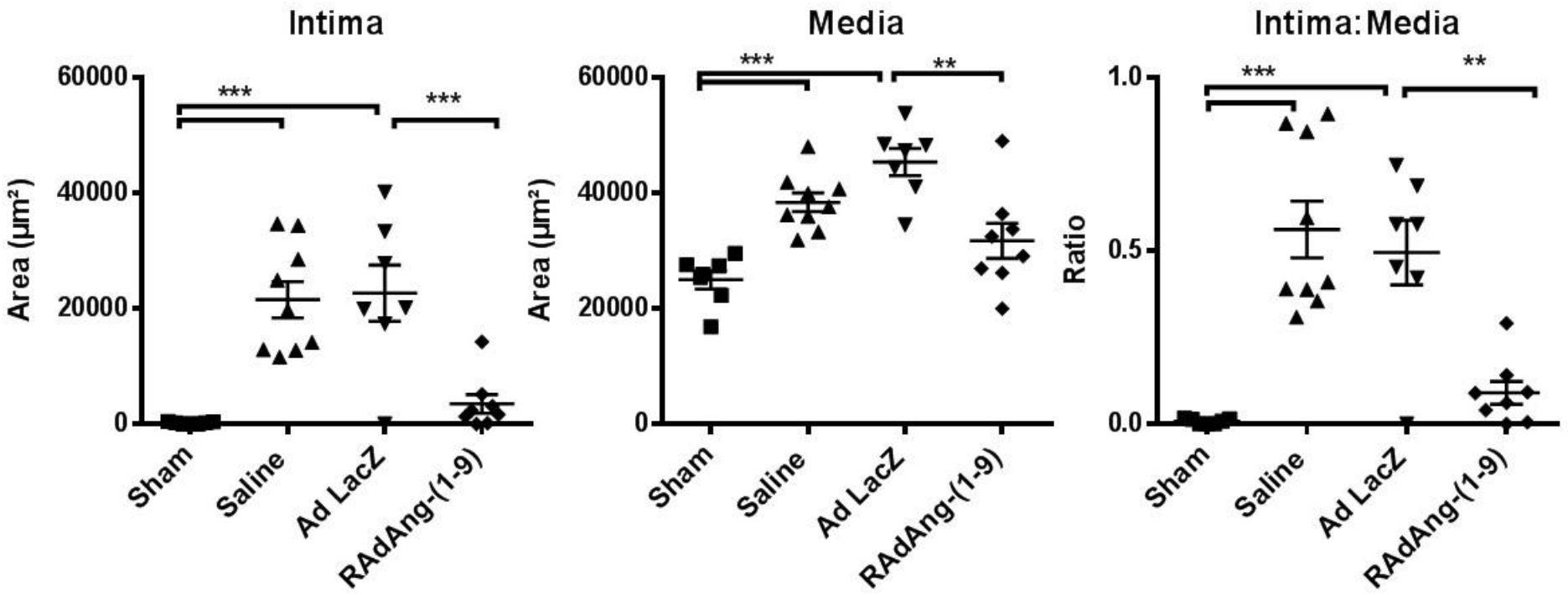

Figure 5.13 *In vivo* gene transfer of Ang-(1-9) reduces intima and media area 28 days after wire injury.

8-10-week-old C57 BL/6 mice were injected via the tail vein with Ad*Lac*Z, RAdAng-(1-9) or Saline 2 days before wire injury surgery. Morphometric analyses of carotid arteries 28 days after surgery. One way ANOVA and Tukey's multiple comparison post hoc test, **<0.01 ***p<0.001; n=6-9 mice**.**

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5.3.13 The effect of RAdAng-(1-9) on serum cytokine and chemokine profile 28 days after wire-injury

Next, mice were either injected with saline, RAdAng-(1-9) or AdLacZ. Two days later the same mice were subject to sham or wire injury surgery. At the study end point (28 days post-injury) sera was taken from each mouse and stored at -80ºC. It was decided to screen these sera samples for inflammatory markers in order to give an indication whether gene delivery of Ang-(1-9) had any sustained effect of cytokine profile (as compared to RAdControl). The inflammatory profile of the sera from AdLacZ and RAdAng-(1-9) treated mice were assessed using immuno-multiplexing technology. IL-1α, IL-2, IL-3, IL-5, IL-6, MIP-1α, MIP-1ß, IFNg and GM-CSF were present at concentrations close to the lower detection limit. IL-9, IL-10, IL-12-p70, IL13, Eotaxin, RANTES, G-CSF and MCP-1 were detected at mid-range concentrations. Pronounced concentrations of IL-1B, IL12-α and TNF-α were detected at concentrations above 100 pg/mL. Of the 23 cytokines measured, none were significantly altered by RAdAng-(1-9) delivery at this time point (Figure 5.14).

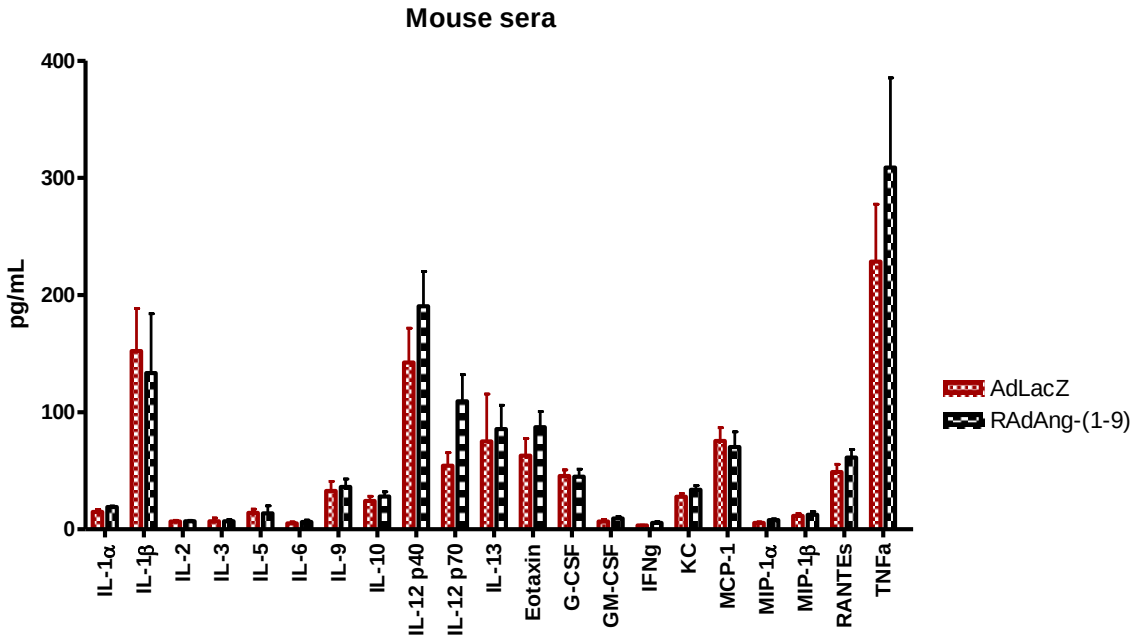

Figure 5.14 Gene therapy with Ang-(1-9) does not alter the inflammatory prolife in serum 28 days after wire injury.

Serum concentrations of 23 circulating cytokine and chemokines as determined by the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay Sera from n=7-8 animals per group. Values represent the means ± SEM. A student's *t* test was performed to compare the effects of RAdAng-(1-9) and AdLacZ.

5.4 Discussion

This chapter describes an investigation into the use of gene therapy with Ang-(1-9) in the setting of vascular injury. First, the use of adenoviral vectors to induce transgene expression in human vascular smooth muscle cells was characterised. HCASMC and HSVSMC transduced with the reporter gene vectors AdGFP and Ad*Lac*Z expressed the transgene product after 48 hours. Cells from only one independent patient were utilised in this initial reporter gene transfer experiment and the data is presented to reflect the correct working of the expression cassette rather than the efficiency of gene transfer. Similarly, HSVSMC transduced with RAdAng-(1-9) expressed the fusion protein as determined by immunoblotting. Detection of the Ms IgG Fc portion of the fusion protein was used as a surrogate for Ang-(1-9). In HSVSMC, the Ang-(1-9)/IgG fusion protein was visualised being trafficked by the endoplasmic reticulum using immunofluorescence and confocal microscopy. Next, *in vitro* biochemical and cell-based approaches were adopted to investigate the effect of RAdAng-(1-9) on HSVSMC migration and proliferation. Direct transduction of HSVSMC with RAdAng-(1-9) inhibited Ang II induced migration. HSVSMC transduced with RAdControl migrated as expected when stimulated with Ang II. Unexpectedly, direct transduction with RAdAng-(1-9) enhanced serum induced proliferation of HSVSMCs. Transduction with a control vector did not affect HSVSMC proliferation. Similarly, direct transduction of HSVECs with RAdAng-(1-9) induced a pro-proliferative phenotype as compared to their non-transduced and RAdControl transduced counterparts. This anti-migratory, pro-proliferative effect in vascular cells was unexpected and remains to be elucidated therefore further investigations are required.

A new therapeutic strategy was devised which would deliver the vector systemically rather than locally, in order to more closely resemble previous studies where soluble Ang-(1-9) peptide was delivered systemically. In order to mimic this scenario *in vitro,* a hepatocyte cell line (HepG2) was transduced with RAdAng-(1-9) and the conditioned media added to recipient HSVSMC. Transduction of a HepG2 cells with RAdAng-(1-9) promoted expression and secretion of the Ang-(1-9) fusion protein as determined by immunoblotting of the conditioned culture media. Conditioned media taken from HepG2 harbouring the Ang-(1-9) fusion protein gene inhibited HSVSMC proliferation induced by 5%

FCS. Conditioned media collected from non-transduced and RAdControl transduced HepG2 cells did not alter serum induced proliferation.

In vivo gene transfer studies demonstrated that systemic delivery of Ad*Lac*Z by tail vein injection led to strong liver transgene expression. Administration of Ad*Lac*Z prior to wire injury did not affect carotid artery neointima formation four weeks after endothelial denudation. RAdAng-(1-9) was administered IV two days before wire injury surgery. A single administration of RAdAng-(1-9) led to sustained inhibition of neointima formation four weeks after injury. Therefore, gene therapy with Ang-(1-9) can be used to inhibit VSMC proliferation but the manner in which this novel tool is used requires careful consideration.

5.4.1 Gene transfer of Ang-(1-9) to HSVSMC

Ex vivo transduction of venous bypass grafts with an adenoviral vector is currently being pursued at the clinical stage (see section 1.7.3). However, the effect of constitutive overexpression of Ang-(1-9) on vascular cells has not yet been reported. For this reason, HSVECs and HSVSMC were directly transduced with RAdAng-(1-9) and the effect on migration and proliferation assessed.

RAdAng-(1-9) conferred an antimigratory effect as determined by scratch wound assay. It is clear that overproduction of Ang-(1-9) in this *in vitro* setting prevented pro-migratory actions of Ang II. It is possible that this antagonism is mediated by the AT_2R in a fashion similar to that reported in previous studies performed by our laboratory (Flores-Muñoz et al., 2011, Flores-Munoz et al., 2012) and others (Ocaranza et al., 2010, Ocaranza et al., 2014). It is also possible that continuous production of Ang-(1-9) is leading to an accumulation of anti-migratory angiotensin metabolites such as Ang-(1-7). Ang-(1-7) has previously been shown to inhibit Ang II induced migration of human VSMCs (Bihl et al., 2015) and rat VSMCs (Zhang et al., 2016). Western blotting studies have identified that Ang-(1-7) inhibits Ang II induced phosphorylation of Akt and ERK1/2 which have known roles in promoting VSMC migration (Zhang et al., 2016). In *Chapter 4*, an antibody-based protein array demonstrated that Ang-(1-9) could inhibit ERK1/2 phosphorylation. This may be important in mediating the anti-migratory effect reported in this chapter. However, the subsequent findings that describe the pro-proliferative phenotype of RAdAng-(1-9) treated HSVSMC

may suggest that this is not the case. It is equally as possible that in response to RAdAng-(1-9) transduction, HSVSMC develop a more synthetic phenotype, producing extracellular matrix components and inhibitors of MMPs leading to a failure to migrate in response to Ang II. This highlights a clear limitation of the scratch wound assay for assessing directional migration. Gene expression analysis of collagens, TIMPs and MMPs may be insightful. Furthermore, the pro-proliferative phenotype induced by RAdAng-(1-9) may suggest that the experimental design was not be optimal for assessing migration. Here HSVSMC were transduced overnight before being subject to 72 hours of serum starvation before the scratch was made. This means VSMC at the time of scratch may have displayed different densities and phenotype across the treatment conditions.

Unexpectedly, direct transduction of VSMC with RAdAng-(1-9) induced a pro-proliferative response as determined by MTS assay. It has been reported in some studies that overexpression of counter-regulatory RAS components leads to unfavourable effects in the heart. Adenoviral delivery of the AT_2R in cardiomyocytes induced hypertrophy in an AT_1R dependent manner (D'Amore et al., 2005). Moreover, cardiac specific overexpression of the AT_2R had differential dose dependent effects (Xu et al., 2014). Low levels of AT_2R over-expression was beneficial in a model of MI, however increased AT_2R over-expression can be deleterious (Xu et al., 2014). In addition, AAV mediated overexpression of ACE2 in the heart led to onset of cardiac fibrosis (Masson et al., 2009). Together, these studies highlight that members of the counter-regulatory RAS axis may have differential effects depending on their locality and expression levels.

It is also possible that elevated levels of intracellular Ang-(1-9) may be acting on intracellular angiotensin receptors that have been previously detected on the mitochondrial (Abadir et al., 2011) and nuclear membrane (Eggena et al., 1993). Mitochondrial AT_2R are localised on the inner mitochondrial membrane and upon stimulation modulated NO production and respiration (Abadir et al., 2011). Further studies would need to demonstrate that in this *in vitro* setting Ang-(1-9) accumulates within the cells as well as the extracellular space. This would be a possible scenario if Ang-(1-9) is secreted in extracellular vesicles. Moreover, this is possible because it has previously been reported that Ang II is internalised with the AT_1R and accumulates intracellularly (Ingert et al., 2002). Furthermore

, plasma derived radiolabelled Ang II was identified in the mitochondrial protein fraction isolated from the kidney and adrenal glands (van Kats et al., 2001b). Liposome based delivery of Ang II has been shown to promote proliferation of rat aortic VSMC in a fashion that was independent from plasma membrane bound AT_1R (Filipeanu et al., 2001). Moreover, intracellular Ang II promoted proliferation of cardiac fibroblast through nuclear membrane bound AT_1R (Tadevosyan et al., 2017). Therefore, the intracellular RAS provides an added element of complexity that should be considered when approaching gene transfer-based delivery of Ang-(1-9).

Alternatively, in this *in vitro* constitutive overexpression setting, excessive degradation of Ang-(1-9) to other products such as Ang IV could potentially mediate different effects on VSMCs via inhibition of the insulin-regulated aminopeptidase (IRAP) receptor, previously known as the AT_4R (Esteban et al., 2005, Albiston et al., 2001). To evaluate this hypothesis, aminopeptidase inhibitors could be used to selectively block the degradation of Ang-(1-9) to Ang IV thereby assessing its effect on HSVSMC *in vitro*. It is important also to realise that local delivery of RAdAng-(1-9) may have different effects following whole tissue (SV, other conduit or vessel) transduction due to changes in phenotypes such as metabolism, haemodynamic factor levels, or cell to cell cross talk.

Inhibiting VSMC proliferation whilst maintaining the regenerative capacity of the endothelium is important in targeting vein graft intimal hyperplasia. Here RAdAng-(1-9) did not inhibit HSVEC proliferation, in fact, a pro-proliferative effect was reported. RAdAng-(1-9) transduced HSVECs were significantly more proliferative than non-transduced HSVECs or HSVECs transduced with RAdControl. It is of note that 5% FCS did not promote a proliferative response that was deemed statistically significant. Therefore, future studies should ensure a more robust positive control, this could be a higher percentage of serum or an independent growth factor to enable further investigation of the effects on HSVEC.

5.4.2 Conditioned media from RAdAng-(1-9) transduced HepG2 cells inhibits VSMC proliferation *in vitro*

The aforementioned *in vitro* assays investigated the effects of local viral mediated overexpression of Ang-(1-9). Previous studies (McKinney et al., in preparation), have reported the therapeutic effects on vascular remodelling following systemic infusion of Ang-(1-9). It was therefore hypothesised that systemic delivery, rather than local delivery, may be important in mediating the therapeutic effects of Ang-(1-9). For this reason, a proof of concept study was designed to test the effects of systemic delivery of RAdAng-(1-9) on neointima formation *in vivo*. Adenoviral vectors are sequestered in the liver and previous studies have reported utility of the liver as a biological pump to deliver therapeutic peptides (Chen et al., 2017). Furthermore, intravenous administration of first generation adenoviral vectors have previously been utilised to deliver secreted transgenes to the liver in order to inhibit vascular remodelling processes (Johnson et al., 2006). In $ApoE^{-/-}$ mice, systemic delivery of RAdTIMP2 inhibited atherosclerotic plaque development (Johnson et al., 2006). Similarly, in an atherosclerotic vein graft model, systemic delivery of an adenoviral vector encoding a secreted chemokine inhibitor [CC-CK binding protein (35K)] inhibited vein graft atherosclerosis (Ali Ziad et al., 2005)

In this chapter, conditioned media taken from RAdAng-(1-9) transduced HepG2 cells inhibited serum induced proliferation of HSVSMC. HepG2 are commonly used in lieu of primary hepatocytes and were utilised here in attempt to best mimic the scenario *in vivo* (Zeilinger et al., 2016, Alba et al., 2009). The data here suggests that Ang-(1-9) accumulates in the extracellular space/culture media following transduction. To strengthen the hypothesis that this growth inhibitory effect is mediated by adenoviral-derived Ang-(1-9), co-administration of an AT_2R antagonist could be utilised in further studies. Nevertheless, the anti-proliferative effect here is in good agreement with the effect of acute exposure to synthetic Ang-(1-9) peptide reported in *Chapter 4* and by McKinney et al., and therefore suggests that viral delivery of Ang-(1-9) can mimic the effects of exogenous peptide (McKinney et al., 2015). Future studies could provide more insight into the effect of adenoviral derived Ang-(1-9) on HSVSMC migration, apoptosis and intracellular signalling pathways.

5.4.3 Systemic administration of RAdAng-(1-9) inhibits neointima formation *in vivo*

The modified wire injury model was utilised here because this is the model best characterised in other studies from our laboratory. McKinney et al., performed extensive investigations into the effect of Ang-(1-9) on wire injury induced neointima formation using the receptor antagonists PD123,319 and A779 in wild-type and AT_2R KO mice (McKinney et al., in preparation). In this chapter, systemic delivery of RAdAng-(1-9) suppressed neointima formation when compared to the control adenovirus (Ad*Lac*Z) and saline treated mice thus copying the phenotype of subcutaneous minipump infusion of the peptide. Similarly, these data agree with the effect of the AT_2R ligand C21 in other models of vascular injury. Kukida et al., reported that daily intraperitoneal injection of the AT_2R agonist C21 (10 μg/kg per day) resulted in suppression of neointima formation induced by polyethylene-cuff placement in mice (Kukida et al., 2016).

Following systemic delivery, replication deficient adenoviral vectors are sequestered in the liver leading to strong liver transgene expression as demonstrated in this chapter by detection of ß-galactosidase activity after systemic delivery of Ad*Lac*Z. Previous literature has reported that this transgene expression is transient only lasting 7-14 days and is not detectable at 21 days (Yang et al., 1994). It is therefore clear that delivery of RAdAng-(1-9) led to a sustained inhibition of neointima formation suggesting an early therapeutic window. This notion is supported for by evidence in the literature as discussed below (George et al., 2011, Bennett et al., 1997, Morishita et al., 1993).

Lindner et al., characterised the first carotid wire injury model and reported that proliferation of *medial* VSMCs peaked at five days after surgery and *intimal* VSMC peaked eight days after endothelial denudation before dropping dramatically by 14 days (Lindner et al., 1993). In other vascular injury models, the neointimal lesion does not increase in size between 14-28 days (Kumar and Lindner, 1997, Clowes et al., 1983). This suggests that the events that occur in these first two weeks are key to the establishment of an occlusive neointimal lesion. As such it is postulated that suppression of VSMC proliferation and migration in this period may be sufficient to markedly decrease vascular

remodelling over a longer period. In the carotid wire injury model the endothelium has regenerated by approximately 75% after 2 weeks (Lindner et al., 1993). It is possible that agents need only to suppress VSMC proliferation until the re-establishment of a healthy endothelium. Indeed, it has been reported that ECs can act to quiesce VSMC through producing vasodilatory and anti-proliferative agents such as NO and prostacyclin (Garg and Hassid, 1989b, Castellot et al., 1981, Shirotani et al., 1991). *In vitro* studies presented here reported that direct transduction with RAdAng-(1-9) increased HSVEC proliferation as compared to a control vector. It may be possible for this to be exploited in order to accelerate regeneration of a healthy endothelium however a caveat of this strategy is that uncontrolled proliferation of the endothelium would not be warranted.

Sustained effects on neointima formation after a single dose of anti-proliferative agent was first reported over 20 years ago (Morishita et al., 1993). In this study antisense oligonucleotides against CDK2 and PCNA led to a sustained inhibition of neointima formation after 8 weeks (Morishita et al., 1993). This sustained effect was observed despite antisense oligonucleotides becoming inactive just four days after delivery (Morishita et al., 1993, Bennett et al., 1997). However, other research groups have suggested that chronic inhibition of neointima formation is due to inhibition of VSMC migration in the four days following injury (Bennett et al., 1997). Data presented in *Chapter 3* and also by McKinney et al., reported that Ang-(1-9) inhibited VSMC PCNA nuclear translocation after injury (McKinney et al., in preparation). Therefore, this marker of proliferation may in fact be key to the mechanism of action of Ang-(1-9).

Sustained effects have also been reported after delivery of either anti-proliferative or anti-migratory genes in preclinical models. Adenoviral mediated overexpression of PTEN in a canine SV graft model led to sustained inhibition of neointima formation 90 days after CABG (Hata et al., 2005). *Ex vivo* transduction of a porcine SV graft with AdTIMP3 led to prolonged inhibition of neointima formation three months after being engrafted within the carotid artery (George et al., 2011) .The same trend has also been demonstrated in rat models where systemic delivery of TIMP1 adenovirus showed a decrease in neointima formation 16 days after injury despite no increase in TIMP1 in the plasma at this time point

(Furman et al., 2002). Similarly, systemic delivery of an adenovirus has been used to overexpress a broad spectrum chemokine inhibitor attenuated neointima formation in mouse atherosclerotic vein graft model (Ali Ziad et al., 2005).

It has also been reported that potent ACE inhibition at the time of balloon injury reduced arterial expression of proto-oncogenes c-fos and c-jun by up to 50% in rabbits (Van Belle et al., 1995). Similarly, inhibition of the RAS via AT_1R antagonism with the ARB candesartan (Atacand) [116-10 mg $kg^{-1}d^{-1}$] attenuated mRNA expression of c-fos and c-jun in a rat model of neointima formation (Kim et al., 1995). These previous studies are noteworthy as Ang-(1-9) has previously been reported to inhibit actions of Ang II/AT_1R signalling but also has been reported to be a competitive inhibitor of ACE (Kokkonen et al., 1997, Flores-Muñoz et al., 2011). Future work could analyse the expression of these protooncogenes in the carotid artery in the hours and days following wire injury and gene transfer.

In *Chapter 3*, continuous infusion with soluble Ang-(1-9) peptide for 14 days after carotid ligation surgery significantly inhibited systemic concentrations of MCP-1. In the study presented in this chapter the systemic cytokine profile in the study mice was also quantified. The high concentrations of pro-inflammatory cytokines measured following systemic administration of Ad5 in these experiments indicate that systemic delivery of a recombinant human Ad5 vector may not be appropriate in the clinical setting. This is likely due to liver toxicity caused by exposure to adenovirus, in particular TNFα is associated with adenoviral vector infection (Muruve et al., 1999, Shayakhmetov et al., 2004). Local delivery of Ang-(1-9) to the carotid artery or vein graft could avoid this and may therefore be of value. Alternatively, rare adenoviral vector serotypes such as Ad35 are less immunogenic, and could be engineered to express the RAdAng-(1-9) expression cassette (Vogels et al., 2003, Abbink et al., 2007, Seshidhar Reddy et al., 2003).

The data presented in this chapter indicate that direct treatment of SV grafts with RAdAng-(1-9) may not be appropriate and some modifications to the expression cassette may be needed. However, systemic delivery of RAdAng-(1-9) resulted in reduction of neointima formation in a murine model of vascular injury. Therefore, there is still the potential to utilise gene delivery with Ang-(1-9) using a different strategy. Prompting regeneration of the endothelium by

endothelial targeted gene delivery of Ang-(1-9) may be a promising line of investigation.

These studies suggest overexpression of an angiotensin peptide via viral vector delivery can be used to inhibit intimal hyperplasia after acute vascular injury. Here, adenoviral-derived Ang-(1-9) can also be used to mimic the effects of synthetic Ang-(1-9) peptide that inhibit human VSMC proliferation and migration. In summary, RAdAng-(1-9) may be a novel therapeutic tool in vascular injury.

6 Chapter 6 General Discussion

6.1 General summary

Revascularisation procedures such as PCI or CABG are vital treatments for patients with CHD. Globally, CABG surgeries are being performed at an average annual rate of 44 per 100 000 (Head et al., 2017) with over 200,000 surgeries performed in the US alone each year (Weiss and Elixhauser, 2014). The long-term patency of SV grafts following CABG is poor, with almost half of vein grafts failing after a decade (Goldman et al., 2004). The research described in this thesis aimed to characterise the role and therapeutic application of Ang-(1-9) in pathological processes that contribute and lead to VGF.

The carotid artery ligation model of neointima formation (Kumar and Lindner, 1997) was employed to investigate the effect of Ang-(1-9) on vascular remodelling processes following cessation of blood flow. In the present study, Ang-(1-9) did not inhibit neointima formation 7 or 14 days following carotid ligation. Ang-(1-9) also failed to inhibit VSMC proliferation 7 days after carotid ligation. Ang-(1-9) infusion did however inhibit VSMC proliferation 14 days after carotid ligation as determined by nuclear PCNA expression in aSMA positive cells. Initial investigations into the effects of Ang-(1-9) on inflammation were conducted at this timepoint and revealed that Ang-(1-9) significantly decreased circulating levels of MCP-1. However, the same effects were not seen at day 7. Fibrotic processes (e.g. deposition of collagen) were assessed by picrosirius red staining and qRTPCR, however, Ang-(1-9) had no detectable effect on fibrosis in this model.

It is pertinent that therapeutic effects observed in small animal models are relevant to human disease. Hence, mechanistic studies on Ang-(1-9) were performed in HSVSMC. Here, Ang-(1-9) inhibited the pro-migratory and pro-proliferative actions of PDGF-BB in HSVSMC. Furthermore, Ang-(1-9) selectively inhibited PDGF-BB mediated ERK1/2 phosphorylation.

These *in vitro* findings highlighted that soluble Ang-(1-9) peptide may have therapeutic effect of VSMC proliferation and migration. However, angiotensin peptides belong to a complex metabolic pathway and thus they have short half-lives. This pharmacokinetic profile hinders the therapeutic application of angiotensin peptides. Therefore, an adenoviral vector [RAdAng-(1-9)] encoding

an intracellular cleaved and secreted Ang-(1-9) peptide was characterised (Flores-Muñoz et al., 2012). HSVSMC transduced with RAdAng-(1-9) expressed the fusion protein as determined by immunofluorescence and immunoblotting in cell lysates.

Subsequent studies aimed to determine the suitability of Ang-(1-9) as a candidate for a gene transfer approach in CABG. It was noted that direct transduction of HSVSMC with RAdAng-(1-9) inhibited Ang II induced migration. However, direct transduction of HVSMCs and HSVECs resulted in a pro-proliferative phenotype either by enhancing serum induced proliferation or promoting proliferation in the absence of supplementary growth factors. This indicated that local delivery of Ang-(1-9) with the current adenoviral vector may not be suitable for application in CABG and some modifications to the expression cassette may be needed.

Genetically modified mice (Santos et al., 2004) as well as viral vectors (Chen et al., 2017) utilising an expression cassette encoding Ang-(1-7) have been used to drive tissue-specific transgene expression in order to increase systemic levels of Ang-(1-7). Therefore, it was hypothesised that intravascular delivery of RAdAng-(1-9) would lead to increased circulating concentrations of Ang-(1-9) and the distant effect on neointima formation could be investigated. Gene transfer via this route leads to effective liver transduction (Huard et al., 1995, Waddington et al., 2008) and this strategy has been previously employed to deliver a chemokine inhibitor to prevent vein graft occlusion in atherosclerotic mice (Ali Ziad et al., 2005). Systemic delivery of RAdAng-(1-9) therefore aimed to more closely mimic the previously described murine studies that utilised systemic delivery of Ang-(1-9) via subcutaneous implantation of an osmotic minipump. This would utilise the liver as a biological pump to delivery Ang-(1-9), an analogous approach that is seen naturally within the RAS for the production of angiotensinogen (Hackenthal et al., 1990). First, this approach was modelled *in vitro* using HSVSMC and HepG2 human hepatoma cells (Parker et al., 2006, Alba et al., 2009). The culture media taken from HepG2 cells transduced with RAdAng-(1-9) significantly inhibited HSVSMC proliferation, whereas conditioned media from RAdControl transduced HepG2 cells did not. Next, it was confirmed that intravascular delivery of a reporter adenoviral vector (Ad*Lac*Z) promoted

strong murine liver transgene expression without any effect on neointima formation in a modified endothelial denudation by wire injury model (Lindner et al., 1993). Adenoviral-mediated delivery of Ang-(1-9) *in vivo* significantly attenuated neointima formation 28 days after carotid artery wire injury.

Therefore the work presented here demonstrates that soluble Ang-(1-9) peptide can inhibit VSMC proliferation and migration *in vitro*. Furthermore, gene therapy with Ang-(1-9) can confer protective effects in the vasculature following injury .

6.2 Limitations of findings

When interpreting the data presented in this thesis a number of caveats and limitations must be considered when conceptualising the results in a broader context.

The limitations of the *in vitro* research presented here include the heterogeneity of biological samples isolated from independent human saphenous veins. This was most obviously observed when HSVSMC from independent patients were subject to phenotyping by immunofluorescence. All three patients' samples appeared to have a different expression pattern of the classical VSMC markers: aSMA and SM22a. Further analysis of HSVSMC phenotyping by flow cytometry would provide an accurate view of VSMC phenotypic subsets and heterogeneity. This could include the addition of fibroblast markers such as fibroblast specific protein -1 (Strutz et al., 1995).

Functional heterogeneity between patient samples was also observed in cellular based assays and when intracellular signalling was assessed by phospho-protein immuno-array. In some cell-based assays, only three independent biological repeats were analysed. Although this is acceptable, it is now recommended by top journals that a minimum of five biological repeats are performed. Given the obvious heterogeneity of patient samples, further biological repeats may be warranted to provide a more representative result.

Five independent patient samples were utilised when performing the phospho-protein immuno array (*Chapter 4*). This identified that PDGF-BB mediated ERK1/2 phosphorylation was significantly inhibited by Ang-(1-9). However, it was

apparent that this only occurred in three out of the five patient samples (see Figure S1D). Therefore, it is speculative whether Ang-(1-9) would have the same effect if a larger study was performed.

It is possible that this heterogeneity may be explained by passage number. HSVSMC were only cultured until passage 6, however due to differences in rate of proliferation, some cells will have been in culture longer than other. Furthermore, HSVSMC used were not always the same passage number. This is important as many believe VSMCs become more plastic as time in culture increases. Rat aortic VSMCs express higher levels of *PDGFRB* and lower levels of contractile genes such as aSMA (*ACTA2*) and transgelin (*TAGLN*) as passage number increases (Chang et al., 2014). Similarly, low passage porcine VSMCs are more contractile than high passage porcine VSMCs (Sugita et al., 2019).

Here the scratch assay (Liang et al., 2007) was used to investigate HSVSMC migration in *Chapter 4* and *5*. In this cost-effective assay, a vertical scratch is made to a confluent monolayer of VSMCs and the distance of gap (wound) closure is measured over a time course. It is important to note that there are some limitations to this method. Firstly, no chemical gradient is made and therefore the extent of migration can be affected by cells undergoing mitosis (infilling). In order to limit this, cells were first serum started for 72 hours, although some basal proliferation may still occur at this time. Next, a final time point of either 24 or 36 hours was set to assess migration. This time point is appropriate because the doubling time HSVSMC in complete culture media is approximately 48 hours (Newman et al., 1995). However, the analysis of migration could be more reliably measured using live cell microscopy coupled with motion tracking. Similarly, this could be combined with a fluorescence ubiquitination cell cycle indicator (FUCCI), that would allow for a more accurate representation of how HSVSMC are affected by Ang-(1-9) or RAdAng-(1-9) (Piltti et al., 2018). In *Chapter 5*, RAdAng-(1-9) inhibited Ang II induced migration in the scratch assay but promoted proliferation in separate assays. Therefore, it appears that the inhibitory effect of RAdAng-(1-9) on migration is distinct from infilling by proliferation. It is possible that overexpression of Ang-(1-9) is causing HSVSMC to over-produce ECM or downregulate MMPs thus encasing HSVSMC within a matrix rendering them unable to migrate. This is speculative however

and it is clear that further investigations are required to fully understand this result.

In *Chapter 5*, the MTS assay was used to assay HSVSMC proliferation. Tetrazolium based assays such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) indirectly measure cell growth by virtue of the metabolic reduction of tetrazolium to a coloured formazan product (Mosmann, 1983). These assays are cheap and amenable to high throughput 96 well plate assays and as such have been used widely to study proliferation with the rational that the amount of product correlates with the number of viable cells (Riss et al., 2016). Tetrazolium based assays can reproduce data obtained using more direct methods for measuring proliferation such as tritiated thymidine incorporation (a measurement of DNA replication) (Riss et al., 2016). However, a number of limitations and confounding factors are present with this assay (Wang et al., 2010). Firstly, the reduction of tetrazolium salts is dependent on NADPH and the mitochondrial electron transport chain and therefore primarily a measurement of mitochondrial activity (Berridge and Tan, 1993). Moreover, as cells proliferate and become more confluent *in vitro*, changes in metabolism can occur independently (Gal et al., 1981) or as a result of contact inhibition (Vlodavsky et al., 1978). Therefore, future work should assess the reproducibility of this data using gold standard methods such as flow cytometric analysis of 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Salic and Mitchison, 2008). This assay replaces the ethyl group at position 5 of deoxyuridine with an alkyne group when cells are progressing through S-phase. The alkyne group present in the newly synthesised DNA can then be labelled with fluorescent azides in a Cu(I)-catalysed [3+2] cycloaddition reaction (“click” chemistry) (Salic and Mitchison, 2008).

The limitations of the *in vivo* work presented here are primarily related to how closely the *in vivo* experiments actually model human disease. Both models presented here are arterial models of neointimal formation. These models do not mimic the setting of a vein graft procedure but similar biological and molecular events following endothelial denudation or ligation of the carotid occur during or following vein grafting. The two arterial models used here were

used principally to study the effect of Ang-(1-9) on a prominent event underlying neointimal formation *in vivo*: VSMC migration and proliferation. However, both models are not entirely relevant to VGF and CABG procedures because they involved the permanent cessation of blood flow and therefore does not resemble the physiological setting of a revascularisation surgery and therefore the mechanism of neointimal formation may be distinct. Moreover, both models used are arterial models rather than venous models.

In *Chapter 3,* the ligated carotid arteries of mice infused with water (control) or Ang-(1-9) were assessed by histological staining and morphometric image analysis. Seven days after permanent ligation of the carotid artery, there was not a quantifiable change in neointimal area as determined by morphometric and statistical analyses. This is commonly seen at this time point and markers of proliferation are normally assessed rather than quantitative analysis of neointimal area (Allagnat et al., 2017, Kumar and Lindner, 1997). However, the later 14-day time point is well characterised, and at this time point the area of the neointima should be significantly greater than that of sham-operated animals (Kumar and Lindner, 1997). Despite this, in *Chapter 3,* the average area of the neointima in the carotid artery of control ligated mice was not significantly greater than that seen in sham-operated mice. Because of the simplistic nature of this procedure, it is unlikely that the surgeries themselves failed technically. It is more likely that this is a result of how the carotid arteries were processed and sectioned. Each carotid artery was embedded with the ligation site distal to the point of sectioning by microtome. Therefore, it is possible that some carotid arteries may not have been fully analysed at the point of ligation, either because of the microtome being sterically blocked from the site of ligation or due to curvature at the site of ligation. This curvature was sometimes observed after the carotid arteries were processed overnight (section 2.8.1). Future work could involve and independent researcher melting down the remaining paraffin wax embedded tissue for subsequent re-embedding and processing. However, this might be technically challenging. It is important to note that this histological read out of failed to validate the model and therefore conclusions cannot be reliably drawn from these data. For this reason, a more sensitive read out was performed to analyse VSMC proliferation, immuno-histo-fluorescence.

In 1993, Lindner and colleagues developed the first murine arterial injury model caused by endothelial denudation with a flexible wire (Lindner et al., 1993). This model involved the insertion of a flexible wire through the external left carotid artery where it was passed down to the aortic arch in a twisting motion to mechanical remove the EC layer. The external left carotid is then ligated, and blood flow restored through the internal left carotid. Platelet adherence to the denuded surface occurs immediately and VSMC rich neointimal lesions are present by day 8 (Lindner et al., 1993). Since then, Sata and colleagues have developed a femoral artery wire injury model (Sata et al., 2001). Both models allow assessment of VSMC proliferation and neointima formation in response to endothelial denudation with little effect of blood flow, thereby mimicking some aspects observed in VGF but more closely resemble PCI and ISR.

In *Chapter 5,* a modified version (Tennant et al., 2008, Greig et al., 2017) of Lindner's carotid artery wire injury model was utilised (Lindner et al., 1993). In this modified surgical procedure, an incision is made in the common carotid artery, the whole stretch of the vessel endothelium is denuded, and the common carotid artery remains ligated. This model allows for the reproducible creation of expansive neointimal lesions. Therefore, this model is suitable for assessing the effects on VSMC migration and proliferation but is likely to be less relevant to vein graft failure and ISR because of the permanent ligation that remains distal of the bifurcation. Another limitation is that neointima formation may be more linked to thrombus formation caused by exposure of underlying thrombogenic ECM surface due to the added effect of cessation of blood flow. However, blood flow is still pulsatile at stretches of carotid artery that are situated farther (>2 mm) from the ligation site (Kumar and Lindner, 1997). Furthermore, the dynamics and time course of regeneration of the endothelium is uncharacterised. Therefore, this model is a cruder model of neointima formation. Despite these limitations, effects observed using this modified wire injury model appear potent and robust. Future work should utilise the vena cava to carotid interposition vein graft model. This would allow for a more representative investigation into the effect of RAdAng-(1-9) *in vivo* due to the opportunity to treat the donor vena cava with RAdAng-(1-9) before grafting.

Lastly the statistical tests used in this thesis assume gaussian distribution. However, small samples sizes can produce unreliable results when testing for gaussian distribution due to a tendency to pass normality tests (Ghasemi and Zahediasl, 2012).

6.3 Future perspectives

In *Chapter 3* the carotid artery ligation model was used to investigate the effect of Ang-(1-9) on neointima formation following cessation of blood flow. Ang-(1-9) infusion had no effect on neointima formation in this model, while a pronounced inhibitor effect was observed following gene transfer of an Ang-(1-9) fusion protein in a wire injury model. A key difference between the two models is the presence of an intact endothelium. It is therefore possible that the beneficial effects of Ang-(1-9) are dependent on the endothelium. Future studies could assess whether Ang-(1-9) infusion or gene transfer promotes regeneration of the endothelium following injury.

With vein graft failure as a focus, future studies should aim to employ venous rather than arterial models and human rather than murine tissue where possible. Future work could employ the use of human SV segments in an *ex vivo* culture system that utilises controlled changes in haemodynamic factors to assess intimal hyperplasia (Longchamp et al., 2014). Perfusion settings such as pulsatile flow and pressure can be controlled to mimic conditions experienced by the SV in the arterial environment of the heart (Miyakawa et al., 2008). This is an appropriate next step to the research presented here as it removes the effect of the local and systemic immune response and also of circulating mitogens thus allowing a more focused analysis of the effect of Ang-(1-9) on vascular remodelling induced by changes in blood flow. Following the period of perfusion segments of SV can be taken for gene/protein analysis. Similarly, sections can be fixed in PFA for histological and immunofluorescence probing of intracellular pathways. These techniques could elucidate novel insights into human vein graft disease and the translational therapeutic potential of Ang-(1-9) in this setting. This could also be a valuable system for testing the effect of viral vectors that encode Ang-(1-9) or other targets. Moreover, this would be cost effective and in alignment with the “three R’s” of animal research as our laboratory routinely receive human SV from bypass patients, thus removing the immediate need for

animal models (Russell and Burch, 1959). Therefore, the establishment of an *ex vivo* perfusion system or collaboration with an institute that specialises in this technique would be beneficial in driving this and other projects forward.

If these studies were successful, larger animal models of vein grafting may be warranted to assess efficacies *in vivo*. Progression to larger animal models that better resemble human anatomy is an important step in progressing any preclinical research. Rabbit, canine, pig and non-human primate models are all available if ethical permission is granted. Porcine vein graft models more closely mimic human pathophysiology and involve the exposure of the carotid artery by cervical incision followed by interposition of section of autologous SV (Southgate et al., 1999). Other porcine models of bypass graft stenosis include engraftment of the IMA within the LAD (Bonatti et al., 2002). The disadvantage of these models is that they are financially costly and require specialised skills in pre-and post-operative care of pigs. (Mehta et al., 1996). Local adenoviral delivery of Ad-TIMP3 has sequentially progression from *ex vivo* models through to *in vivo* porcine models and has now been granted permission and funding for clinical trial. Thus, investigating the right gene in the right models can pave the wave for new clinical trials as the medical need to improve outcomes of CABG remains.

It is unclear whether neointima formation would be inhibited or sustained after treatment with RAdAng-(1-9) in larger animal models. For example, more sustained or higher levels of transgene expression may be needed. To resolve this other viral vectors such as AAVs could be used as they are known to result in far longer, sometimes almost lifelong transgene expression (Nathwani et al., 2011). These vectors could be first tested in the mouse model described here. Ultimately however, this would present other issues as to whether systemically raised levels of Ang-(1-9) is safe for extended periods of time. Of note, patients medicated with ACE inhibitors and ARBs experience symptomatic hypotension after prolonged treatment (Bagger, 1997). Likewise, ACE expression in rats can be upregulated by prolonged treatment with the ACE inhibitor captopril (Fyhrquist et al., 1980). Hence, it may be important to be able to stop transgene expression. For this reason, the transient nature of adenoviral mediated gene transfer may be preferred.

In *Chapter 4*, Ang-(1-9) inhibited HSVSMC proliferation and migration mediated by PDGF-BB signalling, specifically by attenuating ERK1/2 phosphorylation. This effect could be linked to activation of phosphatases by Ang-(1-9) via AT_2R (Bedecs et al., 1997). Previous *in vitro* and *in vivo* studies have demonstrated that cardiovascular protective effects of Ang-(1-9) can be blocked by coadministration of the AT_2R antagonist PD123,319 (Flores-Muñoz et al., 2011, Flores-Munoz et al., 2012, Ocaranza et al., 2014). Future research could therefore assess whether the effects of Ang-(1-9) on PDGF-BB mediated proliferation and migration can be attenuated by coadministration of either PD123,319 (Widdop et al., 1992) or the tyrosine phosphatase inhibitor sodium orthovanadate (Zhang et al., 1997).

In *Chapter 5*, RAdAng-(1-9) inhibited Ang-II induced HSVSMC migration, and conditioned media from transduced HepG2 cells inhibited serum induced HSVSMC proliferation. However, HSVSMCs and HSVECs directly transduced with RAdAng-(1-9) unexpectedly displayed a pro-proliferative phenotype. It is possible that constitutive overexpression of Ang-(1-9) *in vitro* leads to over-activation of the AT_2R. It has previously been reported that cardioprotective effects of the AT_2R are dependent on expression levels. In a mouse model of heart failure, low levels (1 and 4 copies) of AT_2R over-expression in the heart reduced mortality after experimental MI surgery and increased cardiac eNOS expression (Xu et al., 2014). However, cardiac specific high level (9 copies) overexpression of the AT_2R transgene upregulated expression of TGF-β_1 and the AT_1R resulting in no therapeutic effect *in vivo* (Xu et al., 2014). It is unknown whether overexpression of AT_2R agonists elicits similar effects to overexpression of AT_2R itself, however this may partly explain the *in vitro* observations presented here. Equally, continuous overproduction of Ang-(1-9) may saturate the AT_2R if the AT_2R is expressed at low levels. This could result in off target effects. For example, at low doses Ang-(1-7) inhibited Ang II induced vasoconstriction of human resistance vessels however, Ang-(1-7) had the opposite effect at higher doses (Ueda et al., 2000). This may have been an off-target effect via the AT_1R (Teixeira et al., 2017) and similar events may explain the pro-proliferative effect reported in *Chapter 5*. Future studies will inevitably have to address these unexpected effects on HSVSMC proliferation in further detail.

Here, RAdAng-(1-9) promoted proliferation of serum starved HSVEC in the absence of any additional mitogenic supplements. This was a particularly interesting effect because promoting EC proliferation facilitates regeneration of the endothelium and has been shown to be protective in vascular injury (Kipshidze et al., 2004a). Previous studies have reported that regeneration of the endothelium prevents neointima formation in pre-clinical models of vein grafting (Qu et al., 2017) and re-engineering the RAdAng-(1-9) expression cassette to be under transcriptional control of an EC specific promoter such as VWF or flt-1 (Fms Related Tyrosine Kinase 1) could be used to promote regeneration of the endothelium of the SV after CABG (Reynolds et al., 2001, Jahroudi and Lynch, 1994).

Promisingly, intravascular delivery of RAdAng-(1-9) attenuated neointima formation in the murine wire injury model of vascular injury. An important step was to measure concentrations of Ang-(1-9) in the serum following gene transfer. To the author's knowledge, presently there is no Ang-(1-9) ELISA that has been cited in a peer reviewed manuscript that has be demonstrated to effectively measure concentrations of the peptide. During the present study, two unreviewed commercially available ELISA kits from independent companies failed to detect Ang-(1-9) in culture media spiked with exogenous Ang-(1-9) peptide. Both ELISAs detected a signal in murine serum samples (see section 2.5.14), however no increase was observed in serum samples from mice implanted with mini pumps infusing Ang-(1-9) at a dose of 48 µg/kg/hour. Taken together, these data show that it is challenging to measure Ang-(1-9) levels. Since angiotensin peptides differ by the presence or absence of only one or two amino acids, it is important that future research is undertaken to quantify systemic Ang-(1-9) concentrations following gene transfer. This could be explored using high performance liquid chromatography (HPLC) (Brosnihan and Chappell, 2017). Liquid chromatography mass spectrometry services for measuring angiotensin peptides are commercially available from Attoquant, a private company that have been successfully quantifying levels of angiotensin peptides and displaying them as "RAS-Fingerprints™" in a number of recent high impact studies (Basu et al., 2017, Binder et al., 2019, Uijl et al., 2019).

The pathway of angiotensin peptide formation and metabolism is complex, with ACE, ACE2 , neutral endopeptidase (NEP), prolylendopeptidase (POP), prolylcarboxypeptidase (PCP) and thimet oligopeptidase (TOP) all contributing to the formation and degradation of angiotensin peptides (Mendoza-Torres et al., 2015). Therefore, it is unclear whether the data presented here is due to Ang-(1-9) alone, or includes contributions from Ang-(1-7), Ang-(1-5) or other metabolites generated under the experimental conditions utilised. It is noteworthy that angiotensin peptides as short as two amino acids in length (Val-Tyr) are vasoactive (Axelband et al., 2012, Dias et al., 2017). LCMS could therefore potentially elucidate the relative quantities of circulating specific peptides following osmotic mini pump delivery of Ang-(1-9) or *in vivo* gene transfer of Ang-(1-9). Furthermore, as a substrate of ACE it is possible that overexpressing or delivering Ang-(1-9) may reduce circulating Ang II levels to an extent (Kokkonen et al., 1997).

Through the use of LCMS, ACE inhibitors have been shown to prevent the experimental degradation of Ang-(1-7) and Ang-(1-9) (Poglitsch et al., 2012, Trask and Ferrario, 2007). Considering that many patients are already being medicated with ACE inhibitors, this could potentially contribute to a substantial advancement in improving the pharmacokinetics of Ang-(1-9) following therapeutic delivery, making it a more realistic option.

Other immediate future work could also involve a further characterisation of the gene transfer model at an earlier time point to assess proliferation markers and inflammation. While other delivery strategies could be investigated such as local intra-arterial or adventitial delivery which may be more clinically relevant with respect to *ex vivo* treatment of SVs in CABG. However, based on the data presented here, Ang-(1-9) may need to be delivered systemically in order to have therapeutic effects. Additionally, because *ex vivo* gene therapy in CABG is safe, it may be the case that other therapeutic genes are better suited to the direct human SV delivery approach.

6.3.1 Other novel tools to deliver Ang-(1-9)

Here, an adenoviral vector [RAdAng-(1-9)] was used to deliver Ang-(1-9) in a proof of concept pre-clinical study. Despite the effectiveness of intravascular

delivery of RAdAng-(1-9) in the mouse model presented here, it is important to consider the most optimal way to utilise Ang-(1-9) going forward. Gene therapy in CABG favours a local delivery approach whereby the conduit would be transduced *ex vivo* thus minimising systemic exposure to the vector. Initial studies presented in *Chapter 5* demonstrated that direct transduction with RAdAng-(1-9) was pro-proliferative. Although this observation requires further validation in *ex vivo* and *in vivo* models of neointima formation, it suggests that the current Ang-(1-9) vector may not be suitable for a gene transfer approach in CABG. Modifying the vector (e.g. to selectively target ECs, which are denuded following vein graft implantation) may be a useful strategy to harnessing the pro-proliferative effects of RAdAng-(1-9) whilst avoiding deleterious effects on the underlying VSMCs. Moreover, other strategies to deliver Ang-(1-9) should be considered. Liposomes and extracellular vesicles are other strategies being actively investigated as a method to delivery angiotensin peptides themselves, or the gene transfer peptide expression cassette (Shil et al., 2014, Frezard et al., 2015, Downie et al., 2018). Alternatively, a more conventional approach could be to administer daily subcutaneous injections of Ang-(1-9) before and in the months after surgery. In the past, mini pumps have been used in clinical trials. Subcutaneous infusion of angiopeptin for 4 days following balloon angioplasty inhibited stenosis rates (Placebo: 12% vs 40% Angiopeptin) after 6 months (Eriksen et al., 1995). One year after angioplasty, death, MI and need for further surgery was decreased by 9% as compared to the placebo group (Eriksen et al., 1995).

6.3.2 Implications for related diseases

Both models of neointima formation utilised here are arterial injury models and therefore the data presented may also suggest that Ang-(1-9) could have therapeutic potential in the context of arterial restenosis. It is possible that Ang-(1-9) may therefore be a candidate for use in a drug eluting stent. An Ang-(1-7) eluting stent improved vasodilatory function of the rat aorta following implantation (Langeveld et al., 2008). Therefore, this may be a delivery strategy well suited to Ang-(1-9) in the context of ISR. While Ang-(1-9) may also be an exciting candidate to use in other models linked to VSMC proliferation and migration such as abdominal aortic aneurysms (AAA). Habashi and colleagues reported that AT_1R antagonism by losartan (Cozaar) prevented AAA in a

preclinical Marfan syndrome mouse model (Habashi et al., 2006). The group bolstered their evidence by providing a direct link with AT_2R agonism and the attenuation of AAA. Blocking the AT_1R pharmacologically (enalapril) and redirecting signalling through the AT_2R decreased ERK1/2 phosphorylation, a major mediator in TGF-B induced AAA (Habashi et al., 2011). This elegant set of experiments have traversed the gap between basic science and disease and as a result losartan is now used effectively against AAA in the clinic. In an initial study of 18 paediatric patients with Marfan syndrome the rate of aortic root enlargement was suppressed after treatment with losartan (Brooke et al., 2008).

Members of the counter-regulatory RAS pathway have attracted investment from the biotechnology and pharmaceutical industries. For example, GlaxoSmithKline have exclusive rights to a form of recombinant human ACE2 (GSK2586881 formally APN01) which has been shown to be well tolerated in healthy humans and patients with ARDs (Haschke et al., 2013, Khan et al., 2017). Furthermore, clinical formulations of Ang-(1-7) have also been developed, e.g. TXA127 (Tarix Pharmaceuticals). There are 19 clinical trials currently registered on *ClinicalTrials.gov* that are testing drugs based on Ang-(1-7). Therefore, there may be interest in the commercial exploitation of Ang-(1-9), depending on the current intellectual property status. Ang-(1-9) is thought to mediate its effects through the AT_2R (Flores-Muñoz et al., 2011), but it has also been reported to be a competitive inhibitor of ACE, where ACE directed hydrolysis of Ang-(1-9) produces Ang-(1-7) (Jackman et al., 2002). Together this strengthens the notion that Ang-(1-9) may be an effective therapeutic. Clinical limitations such as the rapid half-life could be resolved by encapsulation of the peptide, constitutive overproduction by gene therapy vectors and formulation of a cyclic analogue similar to that which has been described for Ang-(1-7) (Durik et al., 2012).

7 Conclusion

The data presented in this thesis suggest that Ang-(1-9) has therapeutic benefits as demonstrated by inhibition of VSMC proliferation and migration in human saphenous vein VSMC *in vitro* models. Accompanying *in vitro* studies indicated that these effects may be linked to inhibition of ERK1/2 signalling. Furthermore, this is the first evidence that gene therapy with Ang-(1-9) can be utilised to inhibit human VSMC migration (by direct transduction of target cells) and

proliferation *in vitro* (by conditioned media) as well promote HSVEC proliferation. Moreover, gene therapy with Ang-(1-9) can be used to inhibit neointima formation in a murine model of mechanical vascular injury. However, the mechanism by which Ang-(1-9) is delivered will be of importance to future research. In summary, this thesis highlights the promise for Ang-(1-9) to be utilised therapeutically in the setting of neointimal formation.

8 Supplementary

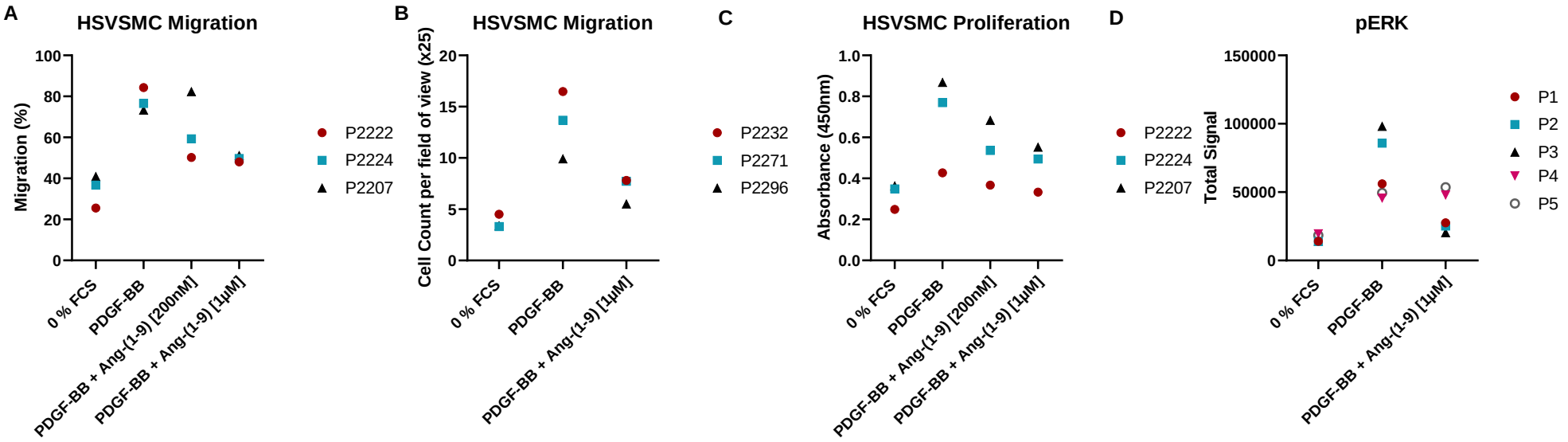

Figure S1: Key data from Chapter 5 represented as dot plots with the addition of colour coding clarification of data points from independent biological samples. The effect of Ang-(1-9) soluble peptide on HSVSMC migration as determined by (A) scratch (n=3) and (B) Boyden chamber assay (n=3). (C) The effect of Ang-(1-9) on HSVSMC proliferation as determined by BrdU incorporation assay (n=3). (D) Intracellular signalling events following PDGF-BB exposure in the presence or absence of Ang-(1-9) was assessed by a phospho-protein immunoblotting array. ERK1/2 phosphorylation is presented here. (n=5).

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