The Role of Angiotensin-(1-7) and Angiotensin-(1-9) in Vascular Remodelling

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Authors Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by me with the exception of analysis of microRNA and target expression, and microRNA inhibitor studies which were performed in collaboration with Miss Lisa McArthur. The plasmid used to express the angiotensin type 2 receptor in cell lines was generated by Mr Daniel Villela, and experiments using this plasmid were performed in collaboration with Mr Daniel Villela. This thesis has not been submitted previously for a higher degree. This research was carried out principally in the Institute of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Dr. Stuart A. Nicklin and Prof. Graeme Milligan. Investigation of nitric oxide release from cell lines and small vessel wire myography experiments were carried out in Prof. Robson A. Santos' lab within the Department of Physiology at the Federal University of Minas Gerais, Brazil.

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Table of Contents

List of Figures

List of Tables

List of Publications, Presentations and Awards

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Presentations

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Awards

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List of Abbreviations and Definitions

-/-	knockout
α-SMA	alpha smooth muscle actin
AAA	abdominal aortic aneurysm
ABC	avidin biotinylated enzyme complex
ACE	angiotensin converting enzyme
ACE2	angiotensin converting enzyme 2
ADAM	a disintigrin and metalloproteinase
Ago2	argonaute protein 2
AmpA	aminopeptidase A
AmpN	aminopeptidase N
Ang A	angiotensin A
Ang I	angiotensin I
Ang II	angiotensin II
Ang III	angiotensin III
Ang IV	angiotensin IV
Ang-(1-12)	angiotensin-(1-12)
Ang-(1-5)	angiotensin-(1-5)
Ang-(1-7)	angiotensin-(1-7)
Ang-(1-9)	angiotensin-(1-9)
Ang-(2-9)	angiotensin-(2-9)
Ang-(2-10)	angiotensin-(2-10)
Ang-(3-7)	angiotensin-(3-7)
ANOVA	analysis of variance
Apo $E^{-/-}$	apolipoprotein E knockout
ARAP1	type 1 Ang II receptor-associated protein
AT_1R	angiotensin type 1 receptor
AT_2R	angiotensin type 2 receptor
AT_4R	angiotensin type 4 receptor
ATBP50	AT_2R -binding protein of 50 kDa
ATIP1	AT_2R -interacting protein 1
ATRAP	AT_1R -associated protein
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor

BH_4	tetrahydrobiopterin
BK_2R	bradykinin type 2 receptor
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CABG	coronary artery bypass graft
CAD	coronary artery disease
cAMP	cyclic adenosine monophospate
CCR-2	C-C chemokine receptor 2
CDK	cyclin dependent kinase
CDKN1a	cyclin-dependent kinase inhibitor 1
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster ovary cell
CI	cell index
CKDN2C	cyclin-dependent kinase 4 inhibitor C
CNS	central nervous system
CO_2	carbon dioxide
COX	cyclo-oxygenase
CpA	cathepsin A
CPA2	carboxypeptidase A2
CREB	cAMP response element-binding protein
Ct	cycle threshold
CVD	cardiovascular diseae
CyD	cyclodextrin
DAB	3, 3'-diaminobenzidine
DAF-FM	4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate
DAG	diacylglycerol
dCt	delta cycle threshold
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOCA	deoxycorticosterone acetate
ECL	enhanced chemiluminescence

ECM	extracellular matrix
EDHF	endothelium derived hyperpolarizing factor
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ERK1/2	extracellular-signal-related-kinase 1/2
EVG	elastin van gieson
FAK	focal adhesion kinase
FCS	foetal calf serum
GLP	guanine nucleotide exchange factor-like protein
GnRH	gonadotrophin-releasing hormone
GPCR	G-protein coupled receptor
Grb2	growth factor receptor-bound protein 2
GRK	G-protein related kinase
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HB-EGF	herparin binding-epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HRP	horse radish peroxidise
HSVEC	human saphenous vein endothelial cell
HSVSMC	human saphenous vein smooth muscle cell
I.U	international units
ICAM-1	intercellular adhesion molecule 1
IGF-1	insulin-like growth factor-1
IHC	immunohistochemistry
IL	interleukin
IP_3	inositol-1,4,5-triphosphate
IκB	inhibitor of kappa b
JAK	Janus kinase
JNK	c-JUN n terminal kinase
KCl	potassium chloride
kDa	kilodalton
LDL	low density lipoprotein

L-NAME	NG-nitro-L-arginine methyl ester
MAC-2	macrophage antigen 2
MAPK	mitogen activated protein kinase
MCP1	monocyte chemoattractant protein 1
MEK	mitogen-activated protein kinase-kinase
µg	microgram
µM	micromolar (micromoles per litre)
mg	milligram
miRNA	microRNA
MKP-1	mitogen activated protein kinase phosphatase 1
mM	millimolar (millimoles per litre)
mmHg	millimetre of mercury
MMP	matrix metalloprotease
MrgD	mas related gene D receptor
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTUS1	mitochondrial tumour suppressor-1
NaCl	sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NEP	neutral endopeptidase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nM	nanomolar (nanomoles per litre
nm	nanometres
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NAD(P)H oxidase subunit
NTC	untransfected control
O_2^-	superoxide
^oC	degrees celcius
$ONOO^-$	peroxynitrite
PAI-1	plasminogen-activator inhibitor 1
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention

PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PGE_2	prostaglandin E2
PGI_2	prostacyclin 2
Phe	phenylephrine
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PLZF	promyelocytic zinc finger protein
PMS	phenazine sulphate
polyacrylamide	N,N'-methylene-bis-acrylamide
POP	prolyl endopeptidase
PP2A	protein phosphatase 2A
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PTEN	phosphatase and tensin homologue
Pyk2	proline-rich tyrosine kinase 2
qRT-PCR	quantitative real time polymerase chain reaction
RAS	renin angiotensin system
RASA1	RAS p21 protein activator 1
Rb	retinoblastoma gene product
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	RhoA/Rho-associated, coiled-coil containing protein kinase
ROS	reactive oxygen species
RQ	relative quantification
RVLM	rostral ventrolateral medulla
S.E.M	standard error of the mean

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase-2
SHR	spontaneously hypertensive rat
SHRSP	stroke prone spontaneously hypertensive rat
siRNA	short interfering RNA
SM-MHC	smooth muscle myosin heavy chain
STAT	signal transducer and activator of transcription
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-alpha
TOP	thimet endopeptidase
Tris	tris(hydroxymethyl)aminomethane)
UTR	untranslated region
v/v	volume/volume
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
w/v	weight/volume

Summary

Vascular remodelling is an adaptive process that allows vessels to respond to changes in haemodynamic conditions, however this process also underlies the pathogenesis of atherosclerosis, vein graft failure following coronary artery bypass graft (CABG) surgery and restenosis following stent deployment to an atherosclerotic vessel. Injury to the vessel wall causes denudation of the endothelial cell (EC) layer and the resultant pathological vascular remodelling involves growth and migration of vascular smooth muscle cells (VSMC) and degradation and reorganisation of the extracellular matrix (ECM). Regeneration of the endothelial layer, known as re endothelialisation, is essential for healing of injured vessels and therefore therapies that specifically target VSMC growth and migration, without preventing re-endothelialisation are optimal in these pathologies. Dysregulation of the renin angiotensin system (RAS) is one of the key contributing factors to remodelling of the vasculature, with the majority of the pathological processes involved, being mediated by angiotensin II (Ang II) signalling at the angiotensin type I receptor (AT_1R). A counter regulatory axis of the RAS has been identified, centred around the enzymatic actions of angiotensin converting enzyme 2 (ACE2), and the resultant production of Angiotensin-(1-9) [Ang-(1-9)] and Angiotensin-(1-7) [Ang-(1-7)] from Ang I and Ang II respectively. This axis counter-regulates the actions of Ang II via the AT_1R, thereby providing a vasculoprotective role. Ang-(1-7) acts via the receptor Mas and inhibits Ang II induced VSMC cell migration, proliferation, and vascular remodelling *in vivo*. Comparatively, less is known about the actions of Ang-(1-9), however it has been identified as a functional ligand for the angiotensin type 2 receptor (AT_2R), inhibiting cardiac hypertrophy *in vitro* and cardiac fibrosis *in vivo*. However, the role of Ang-(1-9) in the vasculature is unexplored. Therefore, the main aim of this thesis was to investigate the interaction of Ang II and the counter-regulatory peptides Ang-(1-7) and Ang-(1-9) in the vasculature using primary human VSMC and EC, and to provide a direct comparison of Ang-(1-7) and Ang-(1-9) in order to further understand their signal transduction pathways.

First, a model of VSMC proliferation was established in VSMC isolated from human saphenous vein tissue (HSVSMC). Here it was demonstrated that while Ang II had no effect on HSVSMC proliferation, foetal calf serum (FCS) induced HSVSMC proliferation in a concentration dependent manner. Ang-(1-7) and Ang-

(1-9) blocked FCS induced proliferation of HSVSMC via Mas or the AT_2R, respectively. Ang II-induced HSVSMC migration via the AT_1R, and was inhibited by both Ang-(1-7) and Ang-(1-9) via Mas and the AT_2R, respectively. Further investigation into the functional interplay of Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC migration identified alterations in extracellular signal-related kinase 1/2 (ERK1/2) activity and matrix metalloproteinase 2 (MMP2) and MMP9 expression as potential mechanisms contributing to the observed results. Additionally, Ang II has recently been demonstrated to regulate expression of the microRNA-132/-212 (miR-132/-212) cluster in rat aortic VSMC, thereby regulating a number of target genes involved in VSMC migration. This pathway was assessed in HSVSMC and it was found that Ang II-mediated HSVSMC migration was associated with an increase in miR-132 but not miR-212 expression, and a decrease in phosphatase and tensin homologue (PTEN) expression, a miR-132 target, at the messenger RNA level. These changes were found to be via the AT_1R and were inhibited by Ang-(1-7) and Ang-(1-9). However, PTEN protein levels were unchanged and no changes were observed in key proteins involved in the downstream signalling pathways of PTEN, such as Akt and monocyte chemoattractant protein 1 (MCP-1). The role of miRNA-132 in Ang II induced HSVSMC migration was further investigated through the use of a miR-132 inhibitor and downregulation of DICER, a key enzyme involved in miRNA biogenesis. Here it was found that miR-132 or regulation of an alternative miRNA via DICER is not essential for Ang II induced HSVSMC migration. However, inhibition of miR-132 or DICER enhanced basal migration of unstimulated HSVSMC.

Next, the effect of the RAS peptides, particularly Ang-(1-9), on EC growth, migration and function was assessed. Ang II, Ang-(1-7) or Ang-(1-9) have no effect on growth or migration of EC isolated from human saphenous veins (HSVEC). A direct effect of Ang-(1-9) on nitric oxide (NO) release from HSVEC and Chinese hamster ovary (CHO) cells expressing the AT_2R was demonstrated. Although in cell culture Ang-(1-9) induced NO release in an AT_2R sensitive manner, it was found that in vessels from AT_2R knockout ($AT_2R^{-/-}$) mice the biological effect of Ang-(1-9) was maintained and promoted vasodilation of both aortic and mesenteric artery rings. Furthermore, Ang-(1-9)-induced relaxation of $AT_2R^{-/-}$ aortic rings, but not mesenteric artery rings, was blocked by A779, suggesting that in large vessels Ang-(1-9) may mediate is vasodilatory effects via

conversion to Ang-(1-7) and signalling via Mas, while in resistance vessels Ang-(1-9) promotes vasodilation through an alternative mechanism.

The observation that Ang-(1-7) and Ang-(1-9) block HSVSMC, but not HSVEC, proliferation and migration, identified these peptides as potential therapeutic agents in vascular injury. A carotid artery wire injury model in mice was established, where injury to the carotid artery using a synthetic nylon fibre induced significant injury to the vessel, manifesting in the production of a large neointimal area at 28 days post injury. To assess the effects of Ang-(1-7) and Ang-(1-9), the peptides were delivered via subcutaneously via osmotic minipump. It was found that Ang-(1-7) infusion reduced neointimal formation and neointimal/media (NI/MA) ratio in comparison to control vessels via Mas. Similarly, Ang-(1-9) reduced neointimal formation and NI/MA ratio via the AT_2R, as the AT_2R antagonist PD123,319, but not the Mas antagonist A779,blocked the effects of Ang-(1-9), indicating that this was via a direct effect of Ang-(1-9), as opposed to conversion to Ang-(1-7) and signalling via Mas.

An interesting finding from the *in vivo* study was that a large proportion of vessels from animals co-infused with Ang-(1-7) and A779, or Ang-(1-9) and PD123,319 developed more complex lesions with increased vessel remodelling and neovascularisation, largely within the media, in comparison to all other groups. Analysis of the composition of these complex lesions revealed that they were composed of disorganised ECM and were highly cellular, containing a large number of VSMC, macrophages and proliferating cells. Re-endothelialisation had occurred on the lumenal lining of these vessels and neovascularisation of the complex lesion was observed.

In summary the data from this thesis demonstrates for the first time a direct biological role for Ang-(1-9) in the vasculature through inhibition of HSVSMC migration and proliferation, and increase NO bioavailability from HSVEC *in vitro* and reduced neointimal formation in an *in vivo* mouse model of vascular injury. Furthermore, this study provides a direct comparison of Ang-(1-9) and Ang-(1-7) in the vasculature and while the end biological effects are similar, they act via different receptors, the AT_2R or Mas, respectively, and differences exist in their signal transduction mechanisms. These findings highlight the potential of Ang-(1-9) and Ang-(1-7) as therapeutic agents in the setting of vascular remodelling.

Chapter 1

General Introduction

1.1 Cardiovascular disease

The term cardiovascular disease (CVD) encompasses all diseases involving the heart and circulatory system, including coronary artery disease (CAD), hypertension and stroke. CVD is the main cause of death in the UK, claiming the lives of approximately 180,000 people in 2010, around one third of all deaths that year (British Heart Foundation, 2012). Furthermore, CVD is one of the main causes of premature death in the UK (deaths before the age of 75), accounting for 28 % of premature death in men and 19 % in women in 2010 (British Heart Foundation, 2012). CVD also places a heavy burden on the UK economy; overall CVD is estimated to cost the UK £19 billion per year, with approximately 46% of this due to direct health care costs, 34% to productivity losses associated with death and illness in those of working age, and 20% of the informal care of people with CVD (British Heart Foundation, 2012). Although the numbers of people with CVD has been falling in recent years, there is still a vital need for continued research within this area leading to new prevention strategies and treatments.

1.1.1 Cardiovascular remodelling

CVD development and progression is associated with remodelling of the vasculature, the heart and other organs including the kidney. Remodelling is usually an adaptive process that occurs in response to long term-changes in haemodynamic conditions or following injury, which subsequently contributes to the pathogenesis of various CVD (Glagov *et al.*, 1987, Pasterkamp *et al.*, 2000).

Changes in haemodynamic conditions result in structural alterations of the vessel wall, indicated by changes in wall diameter and thickness (Gibbons and Dzau, 1994, Pasterkamp *et al.*, 2004, Ward *et al.*, 2000). While this is initially an adaptive process, eventually this leads to enhanced vessel reactivity or impaired relaxation, limiting blood pressure control and leading to the development of hypertension (Ward *et al.*, 2000, Gibbons and Dzau, 1994). Additionally, chronic changes in haemodynamic conditions leads to the production of reactive oxygen species (ROS), which creates an inflammatory environment resulting in endothelial damage and the development of atherosclerosis (Cai and Harrison, 2000). In fact, remodelling of the vasculature is one of the major causes of all CVD and ultimately promotes remodelling of the heart. For example,

hypertension causes an increase in haemodynamic load within the heart, resulting in an increase in left ventricular mass due to cardiomyocyte hypertrophy and ECM remodelling (Selvetella *et al.*, 2004). While this is initially an adaptive process to compensate for the increased haemodynamic load, it eventually becomes maladaptive leading to a reduction in left ventricular performance, cardiac impairment and an increased risk of cardiac events, which eventually culminates in heart failure (Selvetella *et al.*, 2004). Furthermore, the presence of atherosclerosis within the coronary arteries restricts blood flow to the heart, causing the myocardium to become ischaemic and resulting in chest pain and shortness of breath under resting conditions, symptoms characteristic of angina (Ross, 1999a). Rupture of the atherosclerotic plaque can lead to complete occlusion of the coronary artery, resulting in myocardial infarction (Ross, 1999a).

1.1.2 Vascular remodelling

The vascular wall is continuously exposed to haemodynamic forces such as luminal pressure and sheer stress. Alterations in these forces, either physiological or pathological, lead to both functional and structural alterations in the vascular wall (Glagov *et al.*, 1987, Ward *et al.*, 2000) (Figure 1.1). Acute changes in haemodynamic force, as in vasoconstriction or vasodilation, can modify vessel diameter. Chronic changes in haemodynamic forces result in structural alterations in the vessel wall, known as vascular remodelling, indicated by changes in wall diameter or thickness (Gibbons and Dzau, 1994). Vascular remodelling is therefore an adaptive process of structural remodelling that occurs in response to long-term changes in haemodynamic forces (Gibbons and Dzau, 1994). Vascular remodelling involves changes in at least four different cellular processes; cell growth, cell migration, cell death, and production or degradation of extracellular matrix (ECM) (Gibbons and Dzau, 1994). While vascular remodelling is initially an adaptive process within the vessel to cope with the altered haemodynamic force, it contributes to the pathology of various CVDs including hypertension and atherosclerosis. However, vascular remodelling is not solely determined by haemodynamic force, and a role for inflammatory responses and changes in ECM components has been identified (Hacking *et al.*, 1996, Pasterkamp *et al.*, 2000, Pasterkamp *et al.*, 2004). Furthermore, the vascular wall is also drastically changed following injury, when a neointima

forms as part of the reparative response, and its formation involves thrombosis, migration and vascular smooth muscle cell (VSMC) proliferation, matrix production, and the infiltration of inflammatory cells (Gibbons and Dzau, 1994).

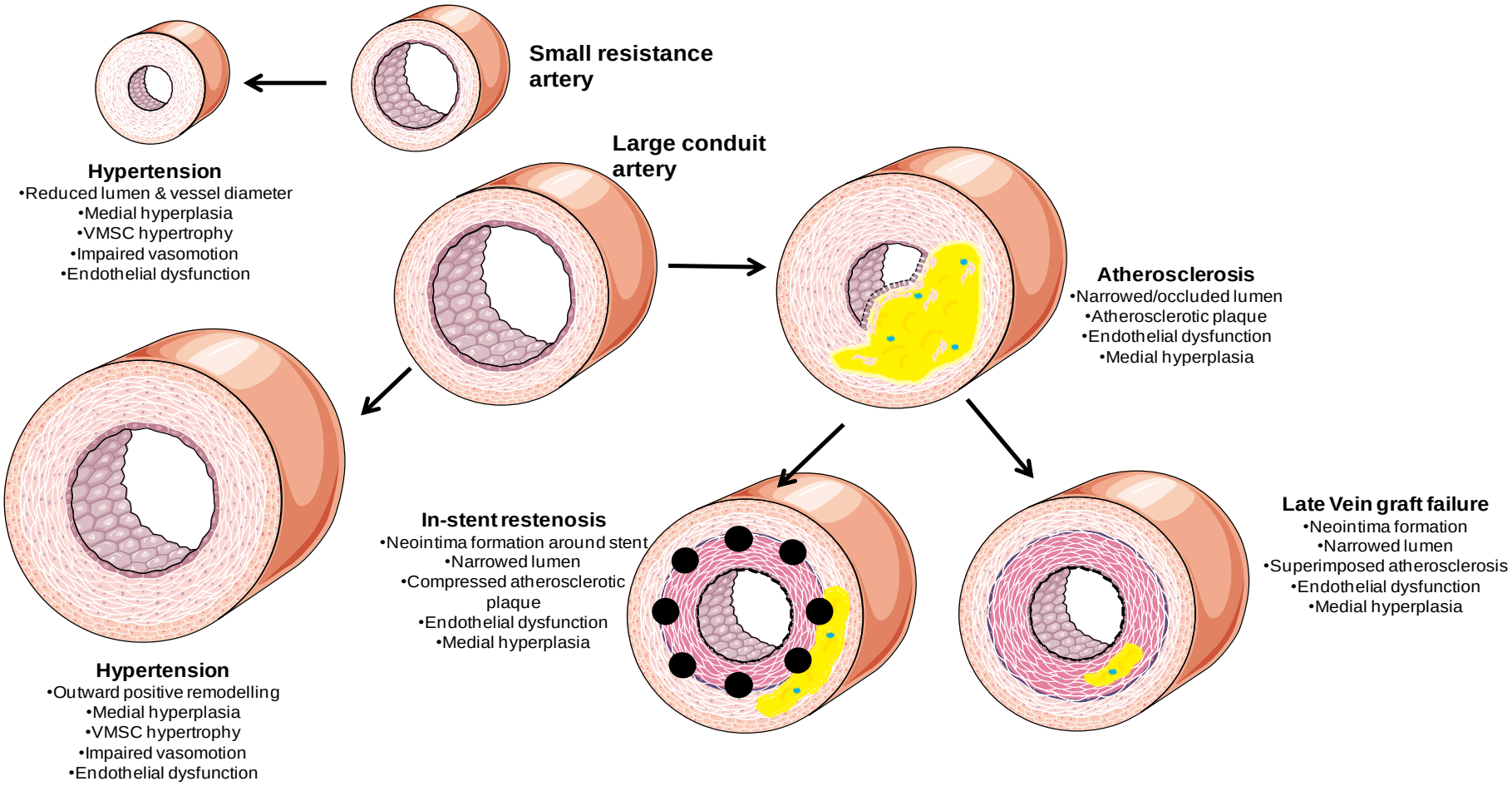

Figure 1.1 Schematic overview of vascular remodelling
Diagram outlining the remodelling that occurs in small resistance and large conduit arteries as a result of hypertension, and remodelling of large conduit arteries in atherosclerosis and following revascularisation due to in-stent restenosis and late vein graft failure.

1.1.3 Endothelial dysfunction

While vascular remodelling largely involves changes in the structure and function of VSMC, the process by which pathological vascular remodelling occurs begins with damage to the endothelial layer. In the vasculature the endothelium exists as a cell monolayer on the luminal surface of the vessel wall. The importance of the endothelium was first recognised by its effect on vascular tone, achieved by the production and release of several vasoactive molecules as well as by response to circulating vasoactive mediators, resulting in vessel relaxation or contraction. The pioneering experiments of Furchgott and Zawadzki first demonstrated an endothelium-derived relaxing factor that was subsequently shown to be nitric oxide (NO) (Furchgott and Zawadzki, 1980). NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) and diffuses to the VSMC where guanylate cyclase becomes activated, leading to cGMP-mediated vasodilation. The endothelium also mediates hyperpolarization of VSMC via an NO-independent molecule, endothelial derived hyperpolarizing factor (EDHF), which increases potassium conductance and subsequent propagation of depolarization of VSMC, to maintain vasodilator tone (Feletou and Vanhoutte, 1988). During states of reduced NO bioavailability EDHF can compensate for loss of NO-mediated vasodilator tone, particularly in the microcirculation (Feletou and Vanhoutte, 1988, Shimokawa *et al.*, 1996). The endothelium also promotes vasoconstriction via the local generation of endothelin and angiotensin II (Ang II) [via the actions of endothelial angiotensin converting enzyme (ACE)] (Saye *et al.*, 1984, Kinlay *et al.*, 2001).

In addition to regulating vascular tone, NO plays a key role in maintaining the vascular wall in a quiescent state through inhibition of inflammation, cellular proliferation, and thrombosis [reviewed by (Deanfield *et al.*, 2007)]. This is in part achieved by s-nitrosylation of cysteine residues in a wide range of target proteins, including the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), cell cycle–controlling proteins, and proteins involved in generation of tissue factor, which reduces their biological activity (Stamler *et al.*, 2001, Ghosh and Karin, 2002). However, when the endothelium is damaged, NO bioavailability is reduced leading to impaired vasorelaxation and a switch in the state of the cells from the quiescent state to the active state, termed endothelial cell activation (Willms-Kretschmer

et al., 1967). Endothelial activation leads to a change in the endothelium to a pro-inflammatory and pro-thrombotic phenotype (Deanfield *et al.*, 2007). Increased endothelial inflammation occurs as a result of expression of adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which promote inflammatory cell adhesion to the endothelium and migration to VSMC (Tummala *et al.*, 1999, Gauthier *et al.*, 1995, de Graaf *et al.*, 1992, Kaplanski *et al.*, 1998). Additionally, synthesis of various cytokines including interleulin-6 (IL-6) and IL-8, and chemoattractants such monocyte chemoattractant protein-1 (MCP-1) further enhances a pro-inflammatory state (Porreca *et al.*, 1997, Mantovani *et al.*, 1997). The prothrombotic phenotype occurs due to a loss of surface anti-coagulant molecules such as thrombomodulin and herparan sulphate, reduced fibrinolytic effects due to enhanced plasminogen activator inhibitor type 1 (PAI-1) release, loss of the platelet anti-aggregatory effects of ecto-ADPases and prostacyclin, and production of platelet activating factor (Cines *et al.*, 1998). Increased vascular inflammation and thrombosis is a key process that contributes to remodelling of the vasculature, particularly in the setting of atherosclerosis.

Reduced NO bioavailability occurs as a result of increased NO degradation or reduced NO production, both of which are largely caused by oxidative stress, which is caused by a number of CVD risk factors including as hypertension, hypercholesterolaemia and smoking (Deanfield *et al.*, 2007). During oxidative stress there is an enhanced production of ROS, particularly superoxide (O_2^-), which rapidly reacts with NO reducing NO bioavailability and producing peroxynitrite ($ONOO^-$), a potent ROS that nitrosylates cellular proteins and lipoproteins, thereby increasing vascular inflammation (White *et al.*, 1994, Darley-Usmar *et al.*, 1992, Gryglewski *et al.*, 1986). Additionally, O_2^- directly stimulates VSMC growth and reduces eNOS expression and activity in endothelial function, further contributing to reduced NO levels and vascular disease (Suh *et al.*, 1999, Peterson *et al.*, 1999). Nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H) oxidase has been shown to be the main source of O_2^- production within the vasculature (Griendling *et al.*, 1994, Rajagopalan *et al.*, 1996). Importantly, Ang II is a potent activator of NAD(P)H, demonstrating a key role for this peptide in the generation of oxidative stress (Griendling *et al.*, 2000, Rajagopalan *et al.*, 1996, Yan *et al.*, 2003, Lassegue *et al.*, 2001).

Furthermore, vascular ROS can also be produced via eNOS (Vasquez-Vivar *et al.*, 2003). eNOS generates NO through oxidation of L-arginine, and this reaction requires the co-factor tetrahydrobiopterin (BH_4) (Schmidt *et al.*, 2001, Vasquez-Vivar *et al.*, 2003). However, in the absence of L-arginine or BH_4, eNOS is uncoupled resulting in the production of O_2^- and hydrogen peroxide (H_2O_2) (Vasquez-Vivar *et al.*, 2003). Uncoupling of eNOS contributes to enhanced oxidative stress and endothelial dysfunction through diminished NO production and increased production of ROS. Additionally, it has been demonstrated that eNOS can be partially uncoupled, resulting in the simultaneous production of NO and O_2^-, thereby enhancing the production $ONOO^-$ (Cai and Harrison, 2000). In summary, alterations in NO levels due to vascular injury and oxidative stress promotes changes in the vascular endothelium leading to reduced endothelial function, increased VSMC growth and increased vascular inflammation, all processes which underlie the pathology of vascular remodelling and disease.

1.1.4 Hypertension

Hypertension has been identified as one of the most important risk factors for CVD and is defined as a systolic blood pressure of 140mmHg or above, or a diastolic blood pressure of 90mmHg and above. In very simplistic terms, hypertension can either be caused as an increase in fluid volume or an increase in peripheral resistance. Hypertension is a risk factor for various other CVD inclusing atheroscelrosis, myocardial infarction and stroke and induces long term changes in the haemodynamic conditions within the vasculature, thereby contributing to vascular remodelling. Hypertension induced remodelling can occur in both large conduit arteries and small resistance arteries.

1.1.4.1 Remodelling of large conduit arteries

During hypertension, the large arteries exhibit an increased lumen size, a thickened media with increased collagen deposition, and decreased vascular compliance (Folkow, 1982, Lee *et al.*, 1995, Schiffrin, 2001b, Schiffrin, 2001a) (Figure 1.1). The thickening of the arterial wall is considered to be one of the main compensatory mechanisms to preserve circumferential wall stress (Girerd *et al.*, 1994). The outward hypertrophic vascular remodelling is thought to be progressive as it increased with age in the carotid arteries of patients with

untreated hypertension (Sasaki *et al.*, 2002). Hypertension-induced vascular hypertrophy it thought to lead to increased atherosclerotic disease in vessels such as the carotid artery (Frohlich and Susic, 2007).

1.1.4.2 Remodelling of small resistance arteries

In contrast to the outward hypertrophic remodelling of the large arteries during hypertension, small resistance arteries exhibit a smaller lumen and external diameter, a normal or increased media thickness and an increased media-to-lumen ratio (Schieffer *et al.*, 2000) (Figure 1.1). VSMC hypertrophy or hyperplasia may be present depending on the species, vascular bed, or severity of the disease. For example, in hypertensive patients, rearrangement of VSMCs around a smaller lumen has been reported, however, in patients with renovascular hypertension, remodelling of resistance arteries resulting from VSMC hypertrophy was also present (Korsgaard *et al.*, 1993, Rizzoni *et al.*, 2000). Changes in ECM content are also involved in this remodelling, as collagen and fibronectin deposits are observed in the resistance arteries of experimental models and patients with hypertension (Intengan and Schiffrin, 2000). Remodelling of the resistance arteries during hypertension may be one of the first signs of organ damage found in mild hypertension in humans. It appears to precede the development of left ventricular hypertrophy and thickening of the intima–media of the large conduit arteries (Park and Schiffrin, 2001). Small artery remodelling is also involved in the clinical complications of hypertension, such as stroke and myocardial infarction (Schiffrin, 2001a).

1.1.5 Atherosclerosis

Atherosclerosis is a progressive disease of the vasculature that affects large (aorta) and medium-sized (carotid, coronary, etc) arteries of the cardiovascular system (Ross, 1999a). The narrowing or complete occlusion of the luminal area of these arteries results in reduced blood flow resulting in ischaemia due to a reduction in oxygen supply (Figure 1.1). It is the most common underlying cause for many CVDs and various risk factors associated with atherosclerosis, such as hyperlipidaemia, hypercholesterolaemia, hypertension, smoking and obesity, are common within the UK (British Heart Foundation, 2012). The pathology of atherosclerosis matures over many years from an initial fatty streak to the

formation of an advanced complex lesion. The first clinical manifestations of atherosclerosis usually occur when the lumen diameter is reduced by 70 % and most often manifest as angina-like symptoms such as chest, shoulder or neck pain, alongside shortness of breath (Brown and Dodge, 1982).

Atherosclerosis is widely accepted as an inflammatory disease with the first step in disease progression identified as endothelial dysfunction (Ross, 1999b), which can be caused by several factors including hypertension, the presence of free radicals from tobacco smoke and modified low density lipoproteins (LDL) (Ross, 1999a, Bassiouny *et al.*, 1994). Compensatory mechanisms that change the homeostatic control of the endothelium subsequently occur to overcome the initial damage (Ross, 1999a). Damage to the endothelium and the presence of high plasma levels of circulating LDL leads to the deposition of these lipids in the subendothelial space of the arterial wall (Davignon and Ganz, 2004). The cholesterol rich LDL is then subject to oxidation, leading to the activation of endothelial cells and expression of inflammatory cell adhesion molecules, including P-selectin, VCAM-1 and ICAM-1, and the recruitment of blood borne monocytes (Shih *et al.*, 1999, Glass and Witztum, 2001). This in turn facilitates the adherence of leukocytes to the damaged endothelium and results in the transmigration of these cells into the intimal layer by the actions of chemokines such as MCP-1. Once inside the arterial wall, migrated monocytes mature into activated intimal macrophages by the action of macrophage colony-stimulating factor, which also results in the upregulation of scavenger receptors (Yan and Hansson, 2007). Macrophages internalise oxidised LDL particles in an uncontrolled manner giving rise to lipid-rich foam cells which secrete a variety of pro-inflammatory cytokines such as IL 1ß and tumour necrosis factor-α (TNF-α), which worsen the inflammatory response and initiate VSMC proliferation and migration (Bevilacqua *et al.*, 1984). These cytokines also transform the surface of the endothelium from an anti- to pro-thombotic state by reducing the production of tissue plasminogen activator and protein-S, and increasing production of matrix metalloproteinases (MMPs), endothelin 1, ICAM-1 and VCAM-1 (Isoda *et al.*, 2006, Tedgui and Mallat, 2006). Accumulation of foam cells results in the formation of fatty streaks, the earliest form of atherosclerotic plaque which consist mainly of inflammatory cells (Stary *et al.*, 1994). The artery can then undergo positive vascular remodelling causing the lumenal

diameter to be unaffected. However, these fatty streaks continue to grow with the accumulation of foam cells, lipids and VSMCs forming advanced atherosclerotic plaques. Additionally, macrophage apoptosis within advanced plaques increases inflammation within the plaque, contributing to the development of a necrotic core (Seimon and Tabas, 2008). Atherosclerotic lesions contain fibrous connective tissue which forms a fibrous cap, which initially offers protection to the vessel by stabilising the plaque. As the disease advances and cells accumulate further within the plaque the fibrous cap becomes thinner and more vulnerable (Ross, 1999a). Fibrous cap erosion exposes the plaque contents and necrotic core to the circulating blood causing thrombosis formation, potentially inducing a fatal cardiovascular event.

1.1.5.1 The role of vascular smooth muscle cells

While it is clear that atherosclerosis is an inflammatory disease, with inflammatory cells being integrally involved at all stages of disease development, VSMC also play an important role and become more dominant as atherosclerosis advances (Raines and Ross, 1993). The phenotype of intimal VSMC within atherosclerotic plaques differs from that of those in the medial area (Mosse *et al.*, 1985). Medial VSMC usually express high levels of smooth muscle myosin heavy chain (SM-MHC) and α-smooth muscle actin (α-SMA), proteins that are involved in the contractile function of the cell (Owens *et al.*, 2004). In contrast, intimal VSMC have a high proliferative index and express much lower levels of the contractile proteins. VSMC exhibit this plasticity as they are not terminally differentiated and can therefore undergo phenotypic switching from a quiescent contractile state to an active synthetic state (Campbell and Campbell, 1994). This can occur in response to numerous pro-inflammatory and atherogenic stimuli including MMPs, cytokines and modified lipids (Thyberg and Hultgardh-Nilsson, 1994, Hautmann *et al.*, 1997, Pidkovka *et al.*, 2007). In addition to macrophages, VSMC are responsible for stimulating foam cells generation in atherosclerosis due to increased expression of scavenger receptors, which promote lipid uptake and foam cell formation (Stary *et al.*, 1994, Rong *et al.*, 2003). Synthetic VSMC also promote remodelling as they are able to migrate and proliferate more readily than those in a contractile state. In addition to proliferation and migration, apoptosis plays a critical role in vascular remodelling and contributes to the development of atherosclerosis (Gibbons and

Dzau, 1994). VSMC apoptosis in atherosclerotic lesions occurs via a number of pathways but most commonly through activation of the caspase cascade (Bennett and Boyle, 1998, Mallat and Tedgui, 2000), leading to increased inflammation within the plaque and thinning of the fibrous cap (Clarke *et al.*, 2008, Mallat *et al.*, 2007). Therefore, VSMC, as well as inflammatory cells, are integral to the development and progression of atherosclerosis.

1.1.6 Neointimal formation and vein graft failure

One of the main interventions employed to treat atherosclerosis is coronary artery bypass graft surgery (CABG), where a healthy segment of blood vessel from another part of the body is engrafted to bypass the obstructed portion of the diseased vessel, thereby restoring blood flow to the heart. Despite the fact that arterial conduits, such as the radial artery or internal mammary artery, have more successful long term patency than the saphenous vein, with 10 year patency rates of approximately 85% compared to 61% respectively, the saphenous vein is most commonly used due to the need to bypass multiple occlusions (Goldman *et al.*, 2004). Approximately 25,000 CABG procedures are performed per year in the UK and this form of revascularisation has been associated with reduced morbidity and re-occurrence of incapacitating angina, and increased quality of life, particularly in patients with three-vessel or left main coronary artery disease, in comparison with medical therapy (British Heart Foundation, 2012, Mayou and Bryant, 1987, Herlitz et al., 2001, European Coronary Surgery Study Group, 1982, CASS Principal Investigators, 1983). While the benefits of this procedure are evident, surgical revascularization presents a significant limitation for a large proportion of patients as approximately 50% require further intervention to alleviate the symptoms of graft occlusion within 10-15 years due to neointimal thickening and superimposed atherosclerosis (Hata *et al.*, 2007, Parang and Arora, 2009) (Figure 1.1).

Various cellular processes are initiated immediately following engraftment of the vein to the arterial system that underlie the pathogenesis of vein graft failure, both at an early and late stage. Early vein graft failure can occur within the first few days as a result of thrombosis, initiated by endothelial damage which exposes the blood to a thrombogenic surface (Bryan and Angelini, 1994). The process of late failure begins immediately following implantation when the

vein is exposed to a period of ischaemia followed by reperfusion, resulting in the generation of ROS, which in turn trigger an inflammatory response within the graft (Shi *et al.*, 2001, West *et al.*, 2001). Due to the damaged endothelium and cytotoxic environment within the graft VSMC migrate from the media to the intima and proliferate, and deposit ECM components, contributing to the formation of a neointimal area (Lerner *et al.*, 1986, Newby, 1997). This neointimal area is highly susceptible to an accelerated form of atherosclerosis due to the infiltration of inflammatory cells, which creates a cytotoxic environment within the plaque encouraging the uptake of lipids (Schwartz *et al.*, 1995). The atherosclerotic plaques that form within the graft are more diffuse and unstable due to poorly developed or absent fibrous caps, and are therefore more prone to rupture (Shelton *et al.*, 1988, Virmani *et al.*, 1988).

1.1.7 Restenosis

A non-surgical alternative treatment for atherosclerosis is percutaneous coronary intervention (PCI) where a balloon catheter is advanced from an accessible vessel such as the femoral or radial artery to the occluded area, then the balloon inflated at a controlled rate to expand the vessel, followed by the deployment of a stent, a metal mesh tube that acts as a scaffold to help maintain the resultant increase in lumen diameter. PCI is the most common form of revascularisation in the UK, with approximately 80,000 procedures performed per year, and while in comparison with pharmacological therapies, PCI has been shown to reduce reoccurrence of angina and improve quality of life, evidence to suggest this form of revascularisation improves morbidity is conflicting and varies depending on the stability of the patients CAD (King, 2005, Schomig et al., 2008, RITA-2 trial participants, 1997). Furthermore, the long-term success of PCI is limited by restenosis, which is thought to be an exaggerated form of wound healing after injury. Restenosis is characterized by a recurrence of luminal narrowing resulting from neointimal hyperplasia and remodelling of the injured vessel (Inoue and Node, 2009) (Figure 1.1). The formation of neointima is largely due to excessive VSMC migration and proliferation in addition to remodelling of the ECM and an increased inflammatory response at the site of injury (Libby and Clinton, 1992, Inoue and Node, 2009).

In an attempt to circumvent the issue of restenosis following bare metal stent employment drug eluting stents were developed, which were coated with polymers to elute cell cycle inhibitory drugs, such as paclitaxel or sirolimus (Htay and Liu, 2005). Drug eluting stents have been shown to inhibit restenosis in comparison to bare metal stents, confirming that inhibition of VSMC proliferation is beneficial in this setting, however they do not come without complications of their own (Schampaert *et al.*, 2006, Stone *et al.*, 2009, Caixeta *et al.*, 2009). Several studies have reported that drug eluting stents are associated with an increased risk of late stent thrombosis as the drugs they are coated with also inhibit endothelial cell proliferation, thereby inhibiting regeneration of the endothelial layer, increasing the incidence of late in-stent thrombosis (Luscher *et al.*, 2007, Karha *et al.*, 2006, Joner *et al.*, 2007). Therefore therapies that specifically target VSMC growth and migration, without preventing re-endothelialisation are optimal in vein graft failure and in-stent restenosis (Inoue and Node, 2009).

1.2 The renin-angiotensin system

The renin angiotensin system (RAS) is a key component of cardiovascular physiology, playing an important role in the regulation of vascular tone, blood pressure and volume, and electrolyte balance. However, dysregulation of the system largely contributes to the pathogenesis of cardiovascular remodelling and the development of various CVDs. The extent to which chronic dysregulation of the RAS contributes to cardiovascular disease is demonstrated by the fact that inhibitors of this system, including ACE inhibitors and angiotensin II type 1 receptor (AT_1R) antagonists are among the most effective treatments for CVD. Clinical trials blocking the RAS have widely demonstrated a reduction in target organ damage in the heart and vasculature, associated with reduced morbidity and mortality (The SOLVD Investigators, 1991, The SOLVD Investigators, 1992, Yusuf et al., 2000, Mancini et al., 1996, Fox, 2003).

The link between renal disease and CVD was first reported by Bright *et al* in the early 1800's where it was shown that cardiac hypertrophy was associated with increased resistance to blood flow within small renal vessels (Bright, 1836). This relationship between systemic hypertension and pathological alterations in the kidney was explored for a number of years, leading to the discovery of renin by

Tigerstedt and Bergman in 1898 (Tigerstedt and Bergman, 1898). Renin was identified as a pressor compound in renal tissue of the rabbit and through a series of studies it was demonstrated that the association between renal disease and cardiac hypertrophy was due to the release of a vasoactive compound that acted directly on blood vessels to induce vasoconstriction (Tigerstedt and Bergman, 1898). To further investigate this relationship a number of attempts were made to develop an experimental model of arterial hypertension by manipulation of renal function, however it wasn't until 1934 when Goldblatt *et al* linked renal ischaemia with hypertension (Goldblatt *et al.*, 1934), and using this experimental protocol the presence of a pressor agent in the venous blood of the ischaemic kidney was detected in 1938 (Fasciolo *et al.*, 1938). This pressor protein, originally named hypertensin and now known as angiotensin, was then isolated from the blood in 1939 (Braun-Menendez *et al.*, 1939) and described as a potent short duration pressor protein. In order to investigate the relationship between renin and hypertension, semi-purified kidney extract was incubated with plasma and a vasoconstrictor agent similar to hypertensin, but different from renin and other known pressor agents such as epinephrine and vasopressin, was identified (Braun-Menendez *et al.*, 1939). Based on these results renin was, for the first time, described as a protease acting on a plasma protein to release hypertensin as the final product. Subsequently, it was demonstrated that renin was secreted by the kidney, and hypertensin was formed in the plasma from a protein substrate that was initially named hypertensinogen, and later angiotensinogen (Leloir *et al.*, 1940). The hepatic origin of the renin substrate was first suggested by Page *et al* however conclusive experiments were performed by Leloir *et al* (Page *et al.*, 1941, Leloir *et al.*, 1942). Further investigation into the actions of angiotensin revealed that there were in fact two forms of angiotensin, one being the product of the action of renin on angiotensinogen, designated angiotensin I (Ang I) (Skeggs *et al.*, 1954b, Skeggs *et al.*, 1954a). Ang I was shown to be a decapeptide which can then be converted by a plasma enzyme to the octopeptide, named Ang II, by cleavage of the histidyl-leucine from the C terminus (Skeggs *et al.*, 1954a, Lentz *et al.*, 1956). Initially both peptides were thought to elicit pressor responses however it was later shown that only Ang II was able to achieve this (Bumpus *et al.*, 1957). Since these findings over 50 years ago the depth of our knowledge of the RAS has greatly expanded and continues to do so.

1.3 The classical RAS

Traditionally, the RAS was viewed as a linear enzymatic cascade initiated by the release of renin from the juxtaglomerular cells of the kidney in response to sympathetic nerve activation of ß1 adrenoreceptors, renal artery hypotension or decreased sodium delivery to the distal tubules of the kidney. Once secreted, circulating renin promotes the hydrolysis of the prohormone angiotensinogen, a 453 amino acid glycoprotein produced by the liver, of which the first 12 amino acids are the most important for its function. Cleavage of angiotensinogen by renin results in the production of the decapeptide Ang I. Ang I is then converted to the octapeptide Ang II by cleavage of the N-terminal amino acid residue via ACE (Skeggs *et al.*, 1956). ACE is a membrane bound peptidyl dipeptidase and was first discovered by Skeggs *et al* in 1956 (Skeggs *et al.*, 1956). The ACE gene is located on chromosome 17 and encodes for two different isoforms, the somatic and the germinal form (Soubrier *et al.*, 1988, Rigat *et al.*, 1992). Somatic ACE is expressed in the endothelium of various tissues including the lung, heart, vasculature and kidney, while the germinal form is expressed mainly in the testes. The overall action of ACE results in an increase in blood pressure as ACE not only promotes the production of Ang II, it also inactivates bradykinin, a potent vasodilator (Turner and Hooper, 2002, Erdos and Skidgel, 1987) (Figure 1.2).

1.3.1 Angiotensin II

Ang II is the main active peptide of the RAS and has an important role in blood pressure homeostasis via its effects on the kidney to conserve sodium, stimulation of aldosterone release, actions on smooth muscle to mediate vasoconstriction, and sympathetic activation in the central nervous system (CNS), all actions which maintain blood pressure. Ang II also has important roles in individual tissues which promote cell signalling leading to growth and cell differentiation. Once generated, Ang II has a half life of 30 seconds in the circulation and 15 to 30 minutes in tissues (van Kats *et al.*, 1997). Ang II levels have been described as 2.4 ± 1.2 µg/100mL in arterial blood under normal physiological conditions (Catt *et al.*, 1969). The effects of Ang II are mediated by two transmembrane G protein-coupled receptors (GPCRs), the AT_1R and angiotensin type 2 receptors (AT_2R), that tend to have opposing actions (Figure

1.2). The AT_1R is mainly responsible for the classical actions of Ang II as described above, whereas Ang II stimulation of the AT_2R is reported to promote vasodilation and reduce cellular growth, as discussed in sections 1.3.2 and 1.3.3.

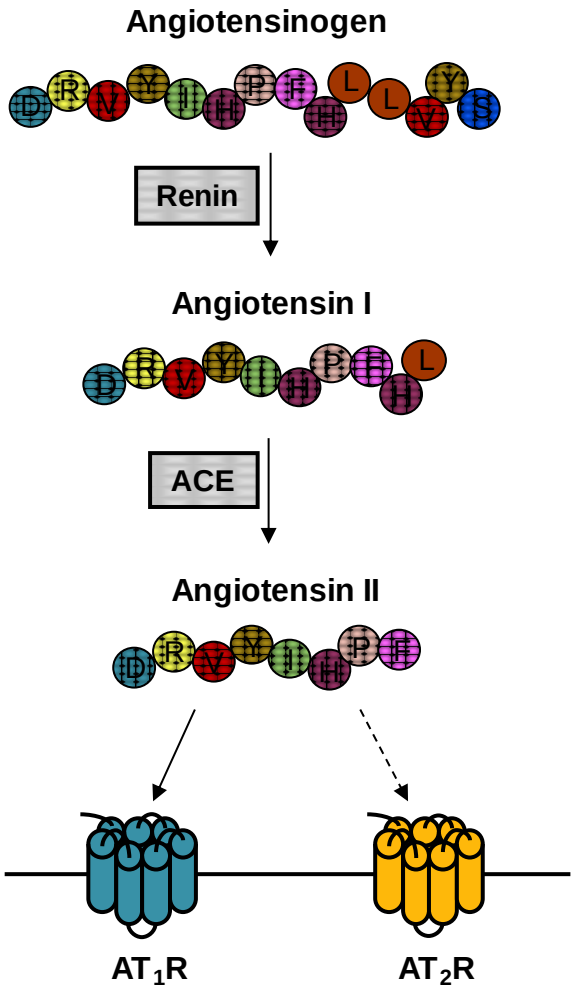

Figure 1.2 The renin angiotensin system
The traditional view of the RAS is as a linear enzymatic cascade beginning with the release of renin, the enzyme responsible for the conversion of angiotensinogen to Ang I. Ang I is then converted to Ang II, the main active peptide of the RAS, via the action of ACE. Ang II signals via two GPCRs, the AT_1R and AT_2R. ACE: Angiotensin converting enzyme; AT_1R: Angiotensin type 1 receptor; AT_2R: Angiotensin type 2 receptor

1.3.2 The angiotensin type 1 receptor

Ang II classically mediates the majority of its effects via the AT_1R, a 40 kDa receptor which is composed of 359 amino acids and belongs to the GPCR superfamily. The AT_1R is ubiquitously expressed throughout the body and found in a number of tissues of the cardiovascular system including the heart and both vascular endothelial and smooth muscle cells. Upon stimulation with Ang II the AT_1R interacts with multiple heterotrimeric G-proteins, including $G_{q/11}$, G_i, G_{12} and G_{13} (Griendling *et al.*, 1997), resulting in the production of various second messengers and initiation of downstream signalling cascades. Once activated the AT_1R is rapidly desensitised, internalised by endocytosis, and then recycled to the cell surface (Griendling *et al.*, 1987). Desensitisation of the AT_1R involves phosphorylation of the AT_1R by G-protein related kinases (GRK), which mediate receptor desensitisation by uncoupling of the receptor from its activated G protein (Mehta and Griendling, 2007). Following this the receptor is internalised into clathrin-coated pits via the action of ß-arrestins, a group of multifunctional proteins that not only initiate receptor internalization, but also promote activation of various downstream signalling pathways such as mitogen-activated protein kinases (MAPK) and c-Jun terminal kinases (*JNK*) (Gaborik *et al.*, 2001, Kim *et al.*, 1997, Lefkowitz, 1998). It has also been suggested that the AT_1R can be internalised via caveolae, specialised noncoated vesicles associated with caveolin (Ishizaka *et al.*, 1998). Recycling of the AT_1R has been suggested to be mediated via Ras-related GTPases that regulate intracellular vesicular transport (Somsel Rodman and Wandinger-Ness, 2000, Li *et al.*, 2008). Alternatively, it has been shown that AT_1R recycling may also be mediated via the type 1 Ang II receptor-associated protein (ARAP1) (Guo *et al.*, 2001).

As well as mediating recycling of receptors, AT_1R associated proteins have also been shown to influence receptor signalling, via interaction with the C-terminal cytoplasmic domain of the AT_1R. In addition to ARAP1, two other AT_1R associated proteins have been identified, namely AT_1R -associated protein (ATRAP) and GEF-like protein (GLP) (Guo *et al.*, 2003, Guo *et al.*, 2004). ARAP1 has functionally been implicated in the control of blood pressure as well as receptor recycling, as mice that overexpress ARAP1 in the proximal tubules have hypertension, suggesting that renal ARAP1 increases blood pressure through enhanced AT_1R signalling (Guo *et al.*, 2006). Conversely, ATRAP has been

suggested to function as a negative regulator of AT_1R signalling (Lopez-Ilasaca *et al.*, 2003, Tsurumi *et al.*, 2006). Overexpression of ATRAP in VSMC increased AT_1R internalization upon Ang II stimulation, resulting in reduced proliferation associated with reduced signal transducer and activator of transcription (STAT) 3 and Akt activation (Cui *et al.*, 2000). Furthermore following vascular injury, neointimal formation was attenuated in ATRAP overexpressing mice compared to wild-type controls and this was associated with reduced extracellular-signal-related-kinase1/2 (ERK1/2), STAT1 and STAT3 activity (Oshita *et al.*, 2006).

In addition to ligand mediated GPCR signalling, there is mounting evidence to suggest that receptors can interact directly to form heteromeric complexes and that these interactions could be important for receptor function and signalling. In this respect the AT_1R is the most widely studied of the RAS receptors and was one of the first GPCRs shown to form heteromers with other receptors (Monnot *et al.*, 1996). A number of studies since have shown that the formation of such heterodimers alters AT_1R signalling. For example, the AT_1R forms heterodimers with receptors from the kinin-kallikrein-(AbdAlla *et al.*, 2001b, AbdAlla *et al.*, 2001a, AbdAlla *et al.*, 2005), adrenergic- (Barki-Harrington, 2004), dopaminergic- (Zeng *et al.*, 2003) system and the cannabanoid family (Rozenfeld *et al.*, 2011) resulting in enhanced Ang II potency at the AT_1R, contributing to the progression and pathology of cardiovascular disease. Conversely, other AT_1R dimers have been shown to reduce signalling via the AT_1R and the effects of Ang II. For example, the AT_2R has been reported to act as a functional antagonist of the AT_1R via formation of constitutive heterodimers between the two receptors (AbdAlla *et al.*, 2001a). This was shown in cells transiently transfected with receptors, foetal fibroblasts and human myometrial biopsies (AbdAlla *et al.*, 2001a). Importantly, this functional antagonism was independent of ligand binding at the AT_2R, as confirmed by the use mutant forms of the AT_2R, which were unable to bind ligand or activate intracellular protein phosphatases, and which produced similar levels of antagonism of the AT_1R as wild type AT_2R (AbdAlla *et al.*, 2001a). This interaction has also been shown to have functional consequences *in vivo* as in human female myometria it has been reported that AT_2R expression is reduced during pregnancy and this is associated with increased AT_1R signalling (AbdAlla *et al.*, 2001b). Another receptor of the RAS, Mas, also acts as a functional antagonist at the AT_1R via direct interaction

(Kostenis *et al.*, 2005, Canals *et al.*, 2006). Co-expression of the Mas receptor with the AT_1R in Chinese hamster ovary (CHO)-KI cells antagonized Ang II mediated calcium signalling via the AT_1R (Kostenis *et al.*, 2005). This was shown to be independent of Mas activation by its endogenous ligand Ang-(1-7). To further investigate this interaction, a series of bioluminescence resonance energy transfer (BRET) experiments were performed, with the conclusion that Mas can hetero-oligomerize with the AT_1R to act as a physiological antagonist (Kostenis *et al.*, 2005). Finally, it was shown that Mas knockout mice have increased vasoconstriction in response to Ang II, indicating that his functional antagonism can be observed *in vivo* (Kostenis *et al.*, 2005). This functional interaction between the AT_1R and Mas was further confirmed by Canals *et al* who demonstrated that co-expression of Mas with the AT_1R reduced AT_1R signalling while simultaneously increasing AT_1R expression (Canals *et al.*, 2006). However, the results of this study indicated that the effects of Mas at the AT_1R were not necessarily a result of dimerization but potentially a result of constitutive activity of Mas, leading to PKC dependent AT_1R phosphorylation and concurrent receptor-desensitisation (Canals *et al.*, 2006).

Furthermore, Ang II effects mediated via the AT_1R have also been shown to involve transactivation of other receptors such as the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR), resulting in the activation of various cell growth pathways (Heeneman *et al.*, 2000, Du *et al.*, 1996, Eguchi *et al.*, 1996, Eguchi *et al.*, 1998).

1.3.3 The angiotensin type 2 receptor

The AT_2R is also a member of the 7 transmembrane receptor family (Mukoyama *et al.*, 1993). The AT_2R is a 41 kDa protein consisting of 363 amino acids and shares 34% sequence homology with the AT_1R (Mukoyama *et al.*, 1993). Various rodent studies have demonstrated that the AT_2R is highly expressed in foetal tissue, including foetal aorta, intestine, brain and adrenal medulla; however its expression declines rapidly after birth, suggesting that it may play an important role in foetal development and cellular growth and differentiation (Shanmugam *et al.*, 1996, Akishita *et al.*, 1999). In adults the AT_2R is less expressed than the AT_1R, yet had been shown to be expressed in the heart, kidney, brain, adrenal gland and both vascular endothelial and VSMC (Viswanathan *et al.*, 1992, Leung

et al., 1997, Wang *et al.*, 1999, Roulston *et al.*, 2003). Furthermore, AT_2R expression is increased in a number of cardiovascular pathologies such as hypertension, atherosclerosis and neointimal formation (Savoia *et al.*, 2006, Touyz *et al.*, 1999c, Widdop *et al.*, 2008, Johansson *et al.*, 2005, Zulli *et al.*, 2006, Nakajima *et al.*, 1995, Suzuki *et al.*, 2002, Wu *et al.*, 2001).

The AT_2R is generally thought to counteract the effects of Ang II at the AT_1R, for which signalling pathways have been well delineated, however, signalling via the AT_2R remains poorly understood. Various AT_2R signalling pathways have been suggested, including regulation of the NO cyclic guanosine monophosphate (cGMP) system, stimulation of phospholipase A2 (PLA2) and release of arachidonic acid, and activation of protein phosphatases and protein dephosphorylation (Siragy and Carey, 1996, Hannan *et al.*, 2003, Stoll *et al.*, 1995, Tsuzuki *et al.*, 1996, Lokuta *et al.*, 1994) however further work is required to fully investigate signalling pathways initiated by activation of the AT_2R.

While the AT_2R is a member of the GPCR superfamily and displays the hallmark motifs and residues of a GPCR it is well recognised that AT_2R signal transduction does not always occur via classical G-protein-dependent pathways. Furthermore, the AT_2R fails to demonstrate most of the classic features of GPCR signalling such as typical second messenger responses and rapid desensitization or downregulation of the receptor following ligand binding (Porrello *et al.*, 2009). These non-classical GPCR characteristics of the AT_2R have contributed to our lack of understanding of its signalling mechanisms however, recent studies of ligand independent GPCR modulation and function, particularly within areas such as constitutive activity, receptor dimerisation, and interaction with receptor-associated proteins have highlighted novel insights into GPCR signalling and provide avenues of research that may assist in delineating signalling via the AT_2R (Miura and Karnik, 2000, Miura *et al.*, 2005, Jin *et al.*, 2002, Nouet *et al.*, 2004, Bockaert *et al.*, 2003).

It has been demonstrated that the AT_2R can induce cell signalling effects independent of ligand binding, indicating that it may possess constitutive activity. For example, overexpression of AT_2R in cultured fibroblasts, CHO cells or VSMC, triggers apoptosis via p38 MAPK and caspase-3 signalling pathways, independent of receptor activation (Miura and Karnik, 2000). Additionally,

overexpression of AT_2R in VSMC has also been shown to downregulate AT_1R expression and signalling, again in a ligand-independent manner (Jin *et al.*, 2002). While in these studies the AT_2R was overexpressed to levels which would far exceed those observed under both physiological and pathophysiological conditions, this work has demonstrated that the AT_2R may function through some degree of constitutive activity, providing further information about the signalling of this receptor.

The AT_2R has also been shown to form both homo- and hetero-dimers independent of ligand binding. Homodimerisation of AT_2R via disulphide bonding was shown to occur in CHO cells overexpressing this receptor, resulting in apoptosis via increased caspase-3 activity (Miura *et al.*, 2005). While this effect was independent of ligand binding, it was prevented by inhibition of disulphide bonding, suggesting that this was not an effect mediated by expression of the receptor, but by the formation of homo-dimers (Miura *et al.*, 2005). In addition to hetero-dimerisation with the AT_1R, the AT_2R has also been reported to form hetero-dimers with the bradykinin B_2 receptor (BK_2R) (Abadir *et al.*, 2006). The formation of AT_2R-BK_2R hetero-dimers was independent of ligand binding and resulted in an increase in NO production, demonstrating that this interaction results in functional signalling processes (Abadir *et al.*, 2006).

Accessory proteins that bind to the carboxyl terminal of the AT_2R and modulate receptor trafficking to the cell membrane and signalling have been identified (Bockaert *et al.*, 2003). AT_2R-interacting protein 1 (ATIP1), also known as mitochondrial tumour suppressor-I (MTUSI), has been shown to mediate transactivation of tyrosine kinases via binding to the C-terminal tail of the AT_2R (Mogi *et al.*, 2007). Overexpression of AT_2R has also been shown to inhibit ERK2 through interaction with ATIP1, an effect which was found to occur in the absence of stimuli but was potentiated by ligand mediated activation (Nouet *et al.*, 2004). A similar protein, AT_2R-binding protein of 50 kDa (ATBP50) was also found to associate with the AT_2R resulting in increased surface expression of the AT_2R and reduced ERK1/2 activation, suggesting an anti-mitotic role for this interaction (Wruck *et al.*, 2005). However, the AT_2R has also been shown to promote cellular growth through interaction with accessory proteins. Upon stimulation with Ang II, the AT_2R has been shown to interact with the transcription factor promyelocytic zinc finger protein (PLZF), leading to

increased expression of phosphatidylinoistol-3 kinase p85α and activation of $p70^{S6}$ kinase activation, which has an important role in protein synthesis (Senbonmatsu *et al.*, 2003). Taken together these findings indicate that the AT_2R may have different effects on cell growth depending on what accessory protein it interacts with and provides further insight into the cellular effects of the AT_2R.

1.4 Angiotensin cell signalling

In the vasculature, Ang II activates various signalling pathways via the AT_1R resulting in both acute responses such as vasoconstriction and increased blood pressure, and longer term responses such as cell proliferation and structural remodelling.

1.4.1 G-protein coupled pathways

Upon activation by Ang II, the AT_1R couples predominantly to the $G\alpha_{q/11}$ complex, activating the second messenger phospholipase C (PLC) (Heineke and Molkentin, 2006, Inagami, 1995, Ohtsu *et al.*, 2008). However, the AT_1R has also been shown to couple to both $G\alpha_{12/13}$ and $G_{\beta\gamma}$ complexes, activating PLA2 and phospholipase D (PLD), respectively (Macrez-Lepretre *et al.*, 1997). Activation of PLC leads to the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), both of which have direct effects on calcium handling, resulting in VSMC contraction and vasoconstriction (Heineke and Molkentin, 2006, Griendling *et al.*, 1997). Additionally, DAG also activates protein kinase C (PKC), which as well as participating in the contractile response, also activates the Ras/Raf/MAPK pathway, which is integrally involved in VSMC proliferation and migration (Liao *et al.*, 1996).

1.4.2 Mitogen-activated protein kinases

Ang II interaction with the AT_1R also initiates activation of various MAPK, such as ERK1/2, JNK and p38 MAPK, which play important roles in VSMC proliferation, migration and differentiation (Sugden and Clerk, 1997, Mehta and Griendling, 2007).

The ERK1/2 pathway in VSMC is well characterised. Activation of the AT_1R results in Src and proline-rich tyrosine kinase 2 (Pyk2) phorphorylation of the EGFR, leading to the formation of the Shc/growth factor receptor-bound protein 2 (Grb2) complex, which in turn promotes activation of Raf through association with the small G protein Ras or PKC. Raf activation leads to phosphorylation of the mitogen-activated protein kinase-kinase (MEK), which in turn phosphorylates ERK1/2 on threonine/tyrosine residues (Sugden and Clerk, 1997, Liao *et al.*, 1996, Liao *et al.*, 1997). ERK1/2 activation promotes VSMC contraction, proliferation, differentiation, migration, and inhibits apoptosis (Touyz *et al.*, 1999d, Touyz, 2004, Allen *et al.*, 2005).

In addition to activation of ERK1/2, Ang II also promotes phosphorylation of stress-related kinases such as JNK and p38MAPK which effect cell survival and induce vascular inflammation (Force *et al.*, 1996). During oxidative stress, ROS are produced, which through various signalling cascades, activate MEK4/7 and MEK3/6, leading to phosphorylation of JNK and p38MAPK, respectively (Tobiume *et al.*, 2001, Seko *et al.*, 2003, Touyz *et al.*, 2004, Ohtsu *et al.*, 2005).

1.4.3 Tyrosine kinases

Ang II signalling is also mediated via cross-talk between the AT_1R and various non-receptor tyrosine kinases and receptor tyrosine kinases. Non-receptor tyrosine kinase signalling includes activation of the cSrc pathway, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, and the focal adhesion kinase (FAK) pathway. c-Src is a tyrosine kinase that has been shown to be activated by $G_{\beta\gamma}$ via ROS signalling, and is involved in a variety of downstream pathways, including Ras, PLC, JAK/STAT, and FAK, leading to cell growth, adhesion, migration and ECM formation (Berk and Corson, 1997, Sabri *et al.*, 1998, Ishida *et al.*, 1999, Taniyama *et al.*, 2004).

In addition to activation downstream of the cSrc pathway, Ang II activates JAK/STAT signalling through the association of JAK2 with the AT_1R via Src homology domain containing tyrosine phosphatase 2 (SHP-2) (Marrero *et al.*, 1995, Marrero *et al.*, 1998). JAK then activates STAT, leading to dimerisation of STAT proteins and translocation to the nucleus, resulting in transcription of early

growth response genes such as c-fos and c-myc (Ishida *et al.*, 1999, Berk and Corson, 1997).

Ang II promotes reorganisation of cytoskeletal structure, leading to cell adhesion and ECM remodelling, through interaction with the FAK pathway (Okuda *et al.*, 1995). AT_1R mediated increase in intracellular calcium induces phosphorylation of FAK, thereby enabling activation of cytoskeletal proteins including Pyk2, p130Cas, paxillin and talin, all of which interact to regulate cell shape and movement (Eguchi *et al.*, 1999, Leduc and Meloche, 1995, Sabe *et al.*, 1997).

Ang II also promotes VSMC proliferation and migration via AT_1R mediated transactivation of tyrosine kinase receptors, such as the EGFR and PDGFR, which exhibit the intrinsic kinase activity that the AT_1R lacks. Transactivation of EGFR is a major mechanism by which Ang II influences growth-related signalling pathways and occurs via calcium-dependent and -independent pathways (Eguchi *et al.*, 1998). These pathways lead to activation of a disintegrin and metalloproteinase (ADAM), mainly ADAM 17, causing release of heparin-binding EGF (HB-EGF) (Andreev *et al.*, 2001, Blobel, 2005, Mifune *et al.*, 2005, Ohtsu *et al.*, 2006). HB-EGF induces conformational changes the EGFR, allowing dimerization and autophosphorylation (Prenzel *et al.*, 1999). Once activated, EGFRs interact with Shc/Grb2 complexes, inducing activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway, leading to cellular growth, survival, and remodelling, and the Ras/Raf/ERK pathway which leads to cell growth and inflammation.

Ang II has been shown to transduce growth-related signalling, independent of platelet-derived growth factor (PDGF), via the PDGF receptor. While the exact mechanism of PDGFR transactivation has yet to be identified a number of signalling events have been suggested. For example, it has been shown that Ang II stimulates PDGF-ß-receptor phosphorylation via formation of the Shc/Grb2 complex, an effect which is independent of calcium and blocked by losartan (Heeneman *et al.*, 2000, Linseman *et al.*, 1995). It has also been demonstrated that AT_1R-mediated PDGFR transactivation requires activation of a ROS-sensitive tyrosine kinase, distinct from Src or JAK (Heeneman *et al.*, 2000). Transactivation of the PDGFR has been linked to increased ERK activity downstream (Linseman *et al.*, 1995, Kim *et al.*, 2000).

1.4.4 Reactive oxygen species

ROS, generated by oxidative stress, have been implicated in the regulation of various signalling cascades resulting in increased inflammation, VSMC proliferation and migration and impaired endothelial function. Ang II is widely accepted as a potent mediator of oxidative stress and has been shown to produce ROS such as O_2^- and H_2O_2 via activation of NAD(P)H oxidases (Griendling and Ushio-Fukai, 2000, Rajagopalan *et al.*, 1996, Touyz *et al.*, 2004, Zafari *et al.*, 1998, Taniyama and Griendling, 2003, Yan *et al.*, 2003, Lassegue *et al.*, 2001). Ang II mediated activation of of NAD(P)H oxidases involves induction of various signalling pathways, including those discussed above such as G-protein dependent signalling, cSrc signalling, and EGFR transactivation. In VSMC NAD(P)H oxidase subunit (NOX) 1 and NOX4 are the main NAD(P)H subunits involved in ROS production (Lambeth *et al.*, 2000).

1.5 The counter-regulatory axis of the RAS

Until recently, the RAS was considered a linear process with Ang II as the major active peptide. However, the conventional view of the RAS has undergone significant change based on two fundamental discoveries. The first was the expression of the RAS in specific tissues, highlighting the existence of a local RAS in which Ang II could be generated within individual tissues, without recruitment from the systemic circulation. Genes coding for various components of the RAS, including ACE, Ang I, Ang II and the angiotensin receptors, are expressed in various tissues such as the heart, blood vessels, kidney, and brain, suggesting the presence of a functionally active RAS at the tissue level (Bader *et al.*, 2001, Paul *et al.*, 2006). However, the role of the tissue-specific RAS in humans is still unclear. For example, elements of the RAS are present in the vasculature, in veins and arteries, suggesting that Ang II can be synthesised within and act locally on vessels to regulate blood pressure and cell growth (Oliver and Sciacca, 1984, Paul *et al.*, 1993). In fact, the concept of the a vascular-specific RAS was first suggested in 1986 when it was shown in the rat two clip, one kidney model of hypertension, that there was an increase in vascular ACE activity during the chronic phase of hypertension and increased vasoconstriction of isolated vessels to Ang II (Okamura *et al.*, 1986). Importantly, plasma renin levels were unchanged in these rats, indicated that elevated vascular ACE activity increases

local production of Ang II, which results in vasoconstriction by acting directly on the vessels (Okamura *et al.*, 1986). It has since been shown that Ang II generated in the vasculature can stimulate synthesis of prostaglandins, endothelin-1 and aldosterone in the endothelium and vessel wall, which in turn influences vascular function (Paul *et al.*, 1995, Paul *et al.*, 2006). In addition to influencing vascular tone, increased activity of RAS specifically in the vasculature has also been implicated in the progression and development of atherosclerosis (Ribichini *et al.*, 2006, Diet *et al.*, 1996, Schieffer *et al.*, 2000, Fukuhara *et al.*, 2000). Furthermore, elements of the RAS have been identified in the eye, which may be important in physiological maintenance of ocular pressure but also in vascular pathologies associated with ocular vessel damage, as observed in hypertension and diabetes (Fletcher *et al.*, 2010, Wilkinson-Berka *et al.*, 2011).

The second discovery was the identification of novel components of the RAS, most importantly the enzyme angiotensin converting enzyme 2 (ACE2), a homologue of ACE which differs in its substrate specificity (Donoghue *et al.*, 2000, Harmer *et al.*, 2002, Vickers *et al.*, 2002). The discovery of ACE2 led to the recognition of functionally active Ang-derived peptides, particularly angiotensin 1-7 [Ang-(1-7)], which was previously described in the 1990s, and more recently peptides such as angiotensin-(1-9) [Ang-(1-9)] (Santos *et al.*, 1992, Donoghue *et al.*, 2000). Further investigation into the actions of ACE2 and the alternative angiotensin peptides, primarily Ang-(1-7), has revealed that they generally antagonise the actions of Ang II, forming the basis for alternative pathways within the RAS known as the counter-regulatory axis (Santos *et al.*, 1992, Santos *et al.*, 2004, Donoghue *et al.*, 2000, Nagata *et al.*, 2006). Since the discovery of the tissue-specific, counter-regulatory RAS many studies have been performed in an attempt to elucidate the mechanisms of action of their components and characterise their roles within both physiological and pathophysiological settings. Together this has greatly expanded our knowledge of the RAS leading to the current understanding of this system as a cascade with multiple mediators, multiple receptors and multifunctional enzymes (Figure 1.3).

1.5.1 Angiotensin converting enzyme 2

ACE2 is a zinc metalloproteinase that shares about 42% homology with ACE at its active site, and is highly expressed in a number of tissues including the heart, vasculature and kidney (Donoghue *et al.*, 2000, Harmer *et al.*, 2002). Due to structural differences ACE acts mainly as a peptidyl dipeptidase, while ACE2 acts as a carboxypeptidase (Vickers *et al.*, 2002). ACE2 functions in the RAS by cleaving the C terminal residues from Ang I and Ang II, producing Ang-(1-9) and Ang-(1-7), respectively (Donoghue *et al.*, 2000) (Figure 1.3). The actions of ACE2 leads to reduced production of Ang II thereby lessening its deleterious effects, and also increased production of Ang-(1-7) and Ang-(1-9), both of which have been shown to protect against the actions of Ang II (Donoghue *et al.*, 2000). Additionally, while ACE can also break down the potent vasodilator bradykinin, ACE2 has no effect on bradykinin (Donoghue *et al.*, 2000). The expression and activity of ACE2 in the heart and blood vessels has also been shown to be protective in various models of CVD (Crackower *et al.*, 2002, Lovren *et al.*, 2008, Sluimer *et al.*, 2008, Dong *et al.*, 2009, Kassiri *et al.*, 2009, Patel *et al.*, 2012). These protective effects were associated with both reduced levels of Ang II and increased levels of Ang-(1-7), suggesting a key role for ACE2 in balancing the activity of both axes of the RAS in CVD. However, it has also been shown that ACE2 expression in the heart worsens the disease phenotype; therefore further work is required to fully investigate the role of ACE2 expression, particularly in the heart (Masson *et al.*, 2009, Donoghue *et al.*, 2003).

1.5.2 Angiotensin-(1-7)

Ang-(1-7) is a seven amino acid peptide first described to be generated in the endothelium (Santos *et al.*, 1992) and is one of the most well studied peptides of the counter-regulatory axis of the RAS. The majority of Ang-(1-7) is produced via the actions of ACE2 and it is the main product since ACE2 has approximately 400-fold greater affinity for Ang II than Ang I (Tipnis *et al.*, 2000). Ang-(1-7) can also be produced less efficiently via hydrolysis of Ang-(1-9) by ACE or via the actions of alternative enzymes including prolyl endopeptidase (POP), neutral endopeptidase (NEP) or thimet oligopeptidase (TOP) (Donoghue *et al.*, 2000, Chappell, 1994, Rice *et al.*, 2004) (Figure 1.3). The half life of Ang-(1-7) in the circulation is approximately 10 seconds (Chappell *et al.*, 1998) and circulating

levels of Ang-(1-7) are reported to be 20 pg/mL (Vilas-Boas *et al.*, 2009). Ang-(1-7) is then converted to the inactive metabolite Ang-(1-5) by ACE (Chappell *et al.*, 1998). Since its discovery, there has been a vast amount of research into its actions as it has been shown to antagonise many of the effects of Ang II. This was first demonstrated by Roks *et al* when it was shown that in human blood vessels Ang-(1-7) inhibited Ang II induced vasoconstriction (Roks *et al.*, 1999). Since then Ang-(1-7) has been shown to oppose the actions of Ang II in a number of tissues, mainly by inhibiting cell growth, migration, and inflammation that occur as a result of Ang II, ultimately preventing adverse remodelling and subsequent dysfunction of the cardiovascular system.

Originally the mechanism by which Ang-(1-7) exerted its effects was unknown. It was first suggested that Ang-(1-7) mediated its effects via the classical angiotensin receptors, but research by Rowe *et al* suggested that due to low affinity for both angiotensin receptors, the effects of Ang-(1-7) were unlikely to be mediated by signalling via either of these receptors (Rowe *et al.*, 1995). Receptor binding studies later showed that Ang-(1-7) could bind to the orphan GPCR Mas and further research in Mas-deficient mice identified Ang-(1-7) as the endogenous ligand for this receptor (Santos *et al.*, 2003). The gene encoding Mas has been mapped to chromosome 6q and encodes for a 325 amino acid protein (Alenina *et al.*, 2008). Mas is expressed in a number of tissues involved in cardiovascular physiology including the heart, blood vessels, brain and kidney (Metzger *et al.*, 1995) and is largely accepted to be the receptor through which Ang-(1-7) exerts its effects [reviewed by (Gironacci *et al.*, 2013, McKinney *et al.*, 2014, Passos-Silva *et al.*, 2013)]. However, despite the wealth of evidence that indicates Ang-(1-7) is a Mas ligand, there is also more recent evidence that despite its low affinity for the AT_2R Ang-(1-7) may also elicit certain biological effects via the AT_2R. For example, in stable cell lines generated to express either AT_1R or AT_2R, Ang-(1-7) was found to bind the AT_2R with higher affinity than the AT_1R (Bosnyak *et al.*, 2011). The Ang-(1-7)/AT_2R interaction has also been observed *in vivo*. In isolated mouse hearts exposed to the AT_2R antagonist PD123, 319, Ang-(1-7) increased perfusion pressure, an effect not observed following Ang-(1-7) infusion alone and which was independent of both the AT_1R and Mas. It was also observed that in the presence of AT_1R blockade, Ang-(1-7) reduced blood pressure in both normotensive and spontaneously hypertensive

stroke prone rats (SHRSP), an effect mediated via the AT_2R (Walters *et al.*, 2005). This effect was preserved in aged normotensive rats under similar experimental conditions; however, the vasodepressor effect was via both the AT_2R and Mas receptor in the aged rats (Bosnyak *et al.*, 2012). In addition to inhibiting the effects of Ang II via engagement with Ang-(1-7), the Mas receptor also acts as a functional antagonist at the AT_1R via direct receptor interaction (Kostenis *et al.*, 2005, Canals *et al.*, 2006).

1.5.3 Angiotensin-(1-9)

Ang-(1-9) is a 9 amino acid peptide member of the counter-regulatory axis of the RAS formed from Ang I, mainly via cleavage of the terminal amino acid of Ang I by ACE2 (Donoghue *et al.*, 2000) (Figure 1.3). However, it has also been shown that following incubation of Ang I with human heart homogenates Ang-(1-9) is formed through the activity of carboxypeptidase A (CPA2) (Kokkonen *et al.*, 1997). This was further confirmed in ACE knockout ($ACE^{-/-}$) and ACE2 knockout ($ACE2^{-/-}$) mice where Ang-(1-9) was the main product of carboxypeptidase mediated cleavage of Ang I (Garabelli *et al.*, 2008). Furthermore, in human heart extracts it was shown that Ang-(1-9) generation from Ang I was mediated via cathepsin A (CpA) (Jackman *et al.*, 2002). These reports provide conclusive evidence that Ang-(1-9) is generated by Ang I by a number of different enzymes. Once formed Ang-(1-9) can be further converted to Ang-(1-7) via ACE. Additionally, it has recently been demonstrated that Ang-(1-9) can be converted to angiotensin-(2-9) [Ang-(2-9)] by aminopeptidase A (AmpA) in glomerular podocytes, however, a biological role for this novel peptide has yet to be identified (Schwacke *et al.*, 2013). Analysis of Ang-(1-9) levels has revealed that while in healthy subjects circulating levels of Ang-(1-9) have been reported to be around 2-6 fmol/mL, these levels are thought to increase in pathological states (Campbell *et al.*, 1993, Kokkonen *et al.*, 1997, Ocaranza *et al.*, 2006), suggesting that in pathological conditions the heart functions to increase levels of Ang-(1-9). For example, in human heart failure patients, Ang-(1-9) is formed at a rate of 1nM/min/mg in the myocardium and a large proportion of available Ang I is rapidly converted to equal levels of Ang-(1-9) and Ang II (Kokkonen *et al.*, 1997). While the half life of Ang-(1-9) within the circulation has yet to be identified, it has been demonstrated that in cells stably transfected with human ACE Ang-(1-9) was hydrolysed 18 times slower than Ang I and 30 % slower than

Ang-(1-7), suggesting that Ang-(1-9) may have a longer half-life compared with other RAS peptides (Chen *et al.*, 2005).

There is currently little known about the biological effects of Ang-(1-9) in the cardiovascular system. Originally it was thought to be biologically inactive, contributing indirectly to counteregulate actions of Ang II by competing with Ang II for the ACE active site, resulting in reduced Ang II and increased Ang-(1-7) levels (Snyder, 1986). Additionally, Ang-(1-9) has been shown to stimulate bradykinin release in cardiac endothelial cells and to enhance the effects of bradykinin by augmenting NO and arachidonic acid release (Erdos *et al.*, 2002, Jackman *et al.*, 2002). Importantly, it was shown that not only was Ang-(1-9) an active peptide but that it more potent than Ang-(1-7) in achieving these results (Jackman *et al.*, 2002). Recent research has demonstrated that Ang-(1-9) exerts direct biological effects in the cardiovascular system, and these effects may be via the AT_2R (Flores-Munoz *et al.*, 2011) (Figure 1.3). Using radioligand binding assays it was demonstrated that Ang-(1-9) could bind to both the AT_1R and AT_2R and in cardiomyocytes Ang-(1-9) mediated anti-hypertrophic effects via the AT_2R as PD123,319, an AT_2R antagonist, blocked these effects (Flores-Munoz *et al.*, 2011). This suggests that despite having approximately 100 fold lower affinity for the AT_2R than Ang II, Ang-(1-9) may elicit functional effects via this receptor. Further work is required to elucidate Ang-(1-9)-mediated cell signalling. Moreover, selective functional activity at the AT_2R is possibly due to the pharmacological concept of functional selectivity where ligands may induce unique, ligand specific conformations resulting in differential activation of signalling pathways (Clarke and Bond, 1998, Galandrin *et al.*, 2007, Smith *et al.*, 2011, Nagata *et al.*, 2006). While the AT_1R is reported to exist in a constrained conformation, the AT_2R exists in a relaxed state (Miura and Karnik, 1999). Therefore, it has been postulated that the additional histidine present in Ang-(1-9) may stabilise the AT_2R in a distinct conformational state, leading to its enhanced activation, and hence counter-regulation of Ang II actions at the AT_1R (Flores-Munoz *et al.*, 2011). However, this remains to be demonstrated experimentally. Alternatively, Ang-(1-9) may be metabolized to Ang-(1-7) or another peptide, and act at the AT_2R or an alternative receptor which is sensitive to PD123, 319, as has previously been shown for the recently reported

counter-regulatory RAS receptor, Mas related gene D receptor (MrgD) (Lautner *et al.*, 2013).

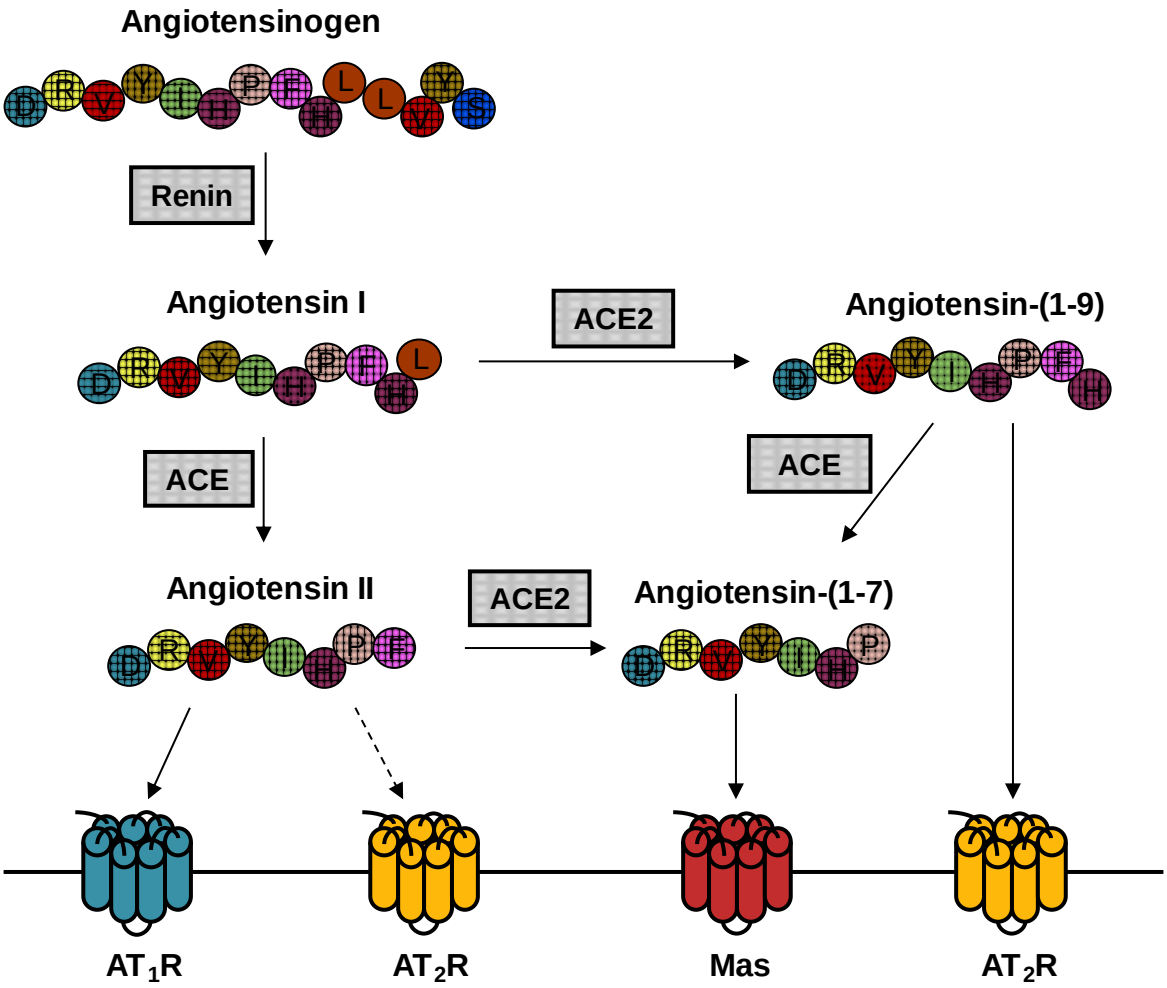

Figure 1.3 The counter-regulatory axis of the RAS
With the discovery of the counter-regulatory axis of the RAS, the view of this system has changed to that of a cascade of interconverted peptides that are mainly generated by the actions of ACE and ACE2. The traditional axis of the RAS is via ACE-mediated generation of Ang II which signals mainly via the AT_1R and also the AT_2R. The counter-regulatory axis of the RAS is centered around the actions of ACE 2 and production of Ang-(1-7) and Ang-(1-9) from Ang II and Ang I, respectively, counteracts pathological effects of Ang II at the AT_1R. Ang-(1-7) exerts protective effects via Mas while Ang-(1-9) has recently been reported to act via the AT_2R. ACE: Angiotensin converting enzyme; AT_1R: Angiotensin type 1 receptor; AT_2R: Angiotensin type 2 receptor.

1.5.4 Other angiotensin peptide metabolites

1.5.4.1 Angiotensin-(1-12)

Although it is now apparent that the RAS contains multiple functional peptides, until recently it was thought that angiotensinogen acted as a precursor for the production of Ang I, from which all other peptides are derived either directly or indirectly. However, this concept has since been challenged with the discovery of angiotensin-(1-12) [Ang-(1-12)], a 12 amino acid peptide containing an additional two amino acids at the C terminal of Ang I (Nagata *et al.*, 2006) (Figure 1.4). Ang-(1-12) was initially isolated and identified from the rat small intestine and has since been found to be present in concentrations comparable with those of Ang I and Ang II in other tissues such as the aorta, heart and kidneys (Nagata *et al.*, 2006, Nagata *et al.*, 2010). Importantly, tissue levels of Ang-(1-12) have been shown to be unaltered in response to manipulation of the systemic RAS, indicating that it may function as part of the local RAS (Nagata *et al.*, 2006, Nagata *et al.*, 2010, Trask *et al.*, 2008). While it is currently unknown how Ang-(1-12) is formed from angiotensinogen, previous studies have shown that renin is not involved in either the formation or metabolism of Ang-(1-12), suggesting that it might serve as an alternative substrate for local production of angiotensin peptides by circumventing the classical renin dependent conversion of angiotensinogen to Ang I (Bujak-Gizycka *et al.*, 2010, Ahmad *et al.*, 2011, Ahmad *et al.*, 2013). Functionally, Ang-(1-12) induces a vasoconstrictor response in the rat aorta that could be blocked by either an ACE inhibitor or an AT_1R antagonist, suggesting that Ang-(1-12) may act as a peptide precursor of Ang II (Nagata *et al.*, 2006, Bujak-Gizycka *et al.*, 2010). However, it is not clear if this conversion to Ang II in the vasculature is direct (as has been shown in the heart via the actions of chymase) or via initial generation of Ang I (by the actions of ACE or CPA2) (Bujak-Gizycka *et al.*, 2010, Ahmad *et al.*, 2011, Ahmad *et al.*, 2013).

1.5.4.2 Angiotensin-(2-10)

Angiotensin-(2-10), also known as Des Asp^1 Ang I, is an octapeptide that was first identified as a metabolite of Ang I, formed by aminopeptidase-mediated metabolism of the N-terminal residue of Ang I, in the cat adrenal gland (Ackerly *et al.*, 1976) (Figure 1.4). Since its discovery few studies have attempted to fully

elucidate its action in the cardiovascular system and existing studies supporting a role for this peptide in the vasculature are conflicting. For example, one of the earliest studies on the effects of Ang-(2-10) demonstrated that in conscious rats, Ang-(2-10) acted as a pressor agent, largely via conversion to angiotensin III (Ang III) (Campbell and Pettinger, 1976). Conversely, in normotensive and hypertensive rats Ang-(2-10) attenuated the vasoconstriction of mesenteric and renal arteries induced by the pressor peptide Ang III and it has been shown to block Ang II-induced proliferation of rat aortic VSMC, suggesting a protective role for Ang-(2-10) in the vasculature (Mustafa *et al.*, 2004, Min *et al.*, 2000). It has been suggested that Ang-(2-10) may also play an important role in the kidneys, functioning as part of the 'intrarenal RAS'. A functional intrarenal RAS was first discovered in 1977 (Kimbrough *et al.*, 1977) and has since been demonstrated to be important in the control of renal sodium excretion and blood pressure (Navar *et al.*, 2011). The intrarenal RAS is one part of the 'tissue specific RAS' which functions independent of changes in circulating RAS components. However, similar to the circulating RAS, overactivation of the intrarenal RAS has been shown to play a pivotal role in glomerular injury which is implicated in progressive kidney diseases. It has recently been shown that Ang-(2-10) is one of the main products of Ang I metabolism, generated via the action of aminopeptidase A, suggesting that it may play an important, but yet undefined, role in the intrarenal RAS (Velez *et al.*, 2009).

1.5.4.3 Angiotensin III

Ang III is generated from cleavage of the N terminus amino acid of Ang II by AmpA (Zini *et al.*, 1996) (Figure 1.4). Ang III has been shown to act in a similar manner to Ang II in that its physiological effects are similar to those elicited by Ang II and it acts at the AT_1R, albeit with 10 times lower affinity for this receptor than Ang II (Pendleton *et al.*, 1989). Ang III has been shown to have similar effects on vasoconstriction and blood pressure as Ang II both centrally and peripherally (van Esch *et al.*, 2008, Li *et al.*, 1995).

1.5.4.4 Angiotensin IV

Angiotensin IV (Ang IV) is generated from cleavage of the N terminus amino acid by aminopeptidase N (AmpN) (Figure 1.4). Originally Ang IV was considered to be

biologically inactive however a wide range of physiological effects have now been identified. Ang IV has been shown to have an important role in the CNS, including regulation of blood flow, and cognitive and sensory functions in the brain such as learning and memory (Braszko *et al.*, 1988, Wright *et al.*, 1993, Albiston *et al.*, 2001). Additionally, Ang IV has been shown to be involved in proliferation of cardiac fibroblasts, endothelial cells and VSMC (Wang *et al.*, 1995, Hall *et al.*, 1993). Ang IV binds with high affinity to the angiotensin II type 4 receptor (AT_4R), which has recently been identified as the enzyme insulin-related aminopeptidase, with Ang IV functioning to inhibit this enzyme (Swanson *et al.*, 1992, Albiston *et al.*, 2001). Ang IV specific binding sites have been identified in various tissues and cells including the brain, blood vessels and heart, consistent with the fact that Ang IV elicits effects in all of these tissues (Miller-Wing *et al.*, 1993, Kerins *et al.*, 1995, Hall *et al.*, 1993, de Gasparo *et al.*, 2000, Chai *et al.*, 2000). While Ang IV is widely accepted to have functional effects in the vasculature, current evidence as to whether it contributes to or protects against vascular disease is conflicting. For example, in cultured VSMC Ang IV via AT_4R activated the transcription factor nuclear factor kappa-light-chain-enhancer of B cells (NF-κB) and upregulates related genes involved in cardiovascular damage, such as the adhesion molecule ICAM-1, the cytokines IL-6 and TNF-α, the chemokine MCP-1, and the prothrombotic factor PAI-1 (Esteban *et al.*, 2005), suggesting that Ang IV may contribute to development of vascular disease. In various rodent models of vascular disease including the rabbit balloon injury model and the mouse model of atherosclerosis (the apolipoprotein E knockout [$ApoE^{-/-}$] mouse), the AT_4R has been shown to be upregulated within the vessel wall indicating that it may play an important role in disease development (Moeller *et al.*, 1999, Vinh *et al.*, 2008). However, it is not clear whether increased expression of AT_4R enhances or retards disease development at this stage. Conversely, Ang IV via the AT_4R has also been linked to an improved vascular phenotype. Ang IV has been shown in a number of studies to activate eNOS, leading to increased NO bioavailability and vasodilation (Patel *et al.*, 1998, Chen *et al.*, 2000). Furthermore, chronic administration of Ang IV to $ApoE^{-/-}$ mice resulted in improved endothelial function associated with increase eNOS activation and reduced oxidative stress, as well as reduced atherosclerotic lesion size (Vinh *et al.*, 2008). Therefore, while it is evident that Ang IV plays an

important role in the vasculature, its exact function is still unclear and requires further investigation.

1.5.4.5 Angiotensin-(3-7)

Angiotensin-(3-7) [Ang-(3-7)] can be generated from Ang II, Ang-(1-7) or Ang IV via the action of both aminopeptidases and carboxypeptidases (Greene *et al.*, 1982, Chappell *et al.*, 1990, Welches *et al.*, 1991) (Figure 1.4). Little is known about the effects of Ang-(3-7) in the cardiovascular system. Ang-(3-7) can be generated in the heart and kidney, however it is unclear whether it has any functional activity or acts as a waste product. It has been suggested that some of the actions of Ang-(1-7) in the kidney might depend on metabolism to Ang-(3-7) and subsequent activation of AT_4R (Handa, 1999). It was found that Ang-(1-7) mediated generation of Ang-(3-7) inhibited nystatin-stimulated proximal tubule O_2 consumption, indicative of reduced basolateral Na^+,K^+-ATPase activity. This effect was abolished by AT_4R blockade (Handa, 1999). However, it has also been shown that Ang-(1-7) does not affect basolateral Na^+,K^+-ATPase activity, therefore further studies are required to fully understand the interaction between both Ang-(1-7) and Ang-(3-7), and angiotensin receptors within the kidney (Caruso-Neves *et al.*, 2000). In fact, the majority of research on Ang-(3-7) has focussed on its role in the brain, particularly within the area of the brain that is involved in the central control of blood pressure, the rostral ventrolateral medulla (RVLM) (Ferreira *et al.*, 2007). It has been shown that micro-injection of Ang-(3-7) into the RVLM induces an increase in mean arterial pressure and heart rate independent of AT_1R, AT_2R or Mas (Ferreira *et al.*, 2007). While further work is required to identify the receptor for Ang-(3-7) in the RVLM it is possible it may initiate its effects via the AT_4R as has been suggested in the kidney and through *in vitro* receptor binding studies (Handa, 1999, Albiston *et al.*, 2001, Wright *et al.*, 1993).

1.5.4.6 Angiotensin A

Angiotensin A (Ang A) was first identified in human plasma by Jankowski *et al* in 2007 and is an octapeptide that differs from Ang II by the presence of an alanine residue in place of aspartate at its N-terminal residue (Jankowski *et al.*, 2007) (Figure 1.4). Ang A is synthesised from Ang II by enzymatic decarboxylation of

aspartate in the presence of mononuclear leukocytes (Jankowski *et al.*, 2007). Initially it was shown that Ang A possessed similar affinity to Ang II for the AT_1R but higher affinity for the AT_2R, however subsequent studies have revealed that the functional effects of Ang A are mediated via the AT_1R (Jankowski *et al.*, 2007, Yang *et al.*, 2011). Ang A was shown to promote renal vasoconstriction both *ex vivo* and *in vivo* in an AT_1R dependent manner (Jankowski *et al.*, 2007, Yang *et al.*, 2011). Ang A has been shown to be elevated in human plasma of patients with end-stage renal failure compared to healthy patients, indicating that it may play a role in renal pathophysiology. However, the role of Ang A in the heart and vasculature, especially in pathophysiology, is poorly explored. Recently Coutinho *et al* demonstrated that in anaesthetised rats Ang A elevated systemic blood pressure via the AT_1R in a concentration-dependent manner, to similar levels to those achieved by Ang II (Coutinho *et al.*, 2013). Similarly, using an isolated heart preparation Ang A resulted in similar effects to those achieved by Ang II, as both peptides reduced cardiac flow, myocardial relaxation and contraction, and heart rate (Coutinho *et al.*, 2013). However, while the effects of Ang II on cardiac function were fully blocked by losartan, the effects of Ang A were only partially blocked, indicating that Ang A may function at a different receptor in the heart (Coutinho *et al.*, 2013). Furthermore, Ang II, but not Ang A, resulted in an increase in cardiac arrhythmias in the isolated heart and altered calcium handling in isolated cardiomyocytes, suggesting that these peptides may also function via separate receptors (Coutinho *et al.*, 2013). Together these data provide further evidence as to the complexity of the RAS and identifies a potential new peptide, Ang A. However, more work is required to fully elucidate its role in the cardiovascular system.

1.5.4.7 Alamandine

The most recently discovered component of the RAS is alamandine (Lautner *et al.*, 2013). Alamandine is a hectapeptide that differs from Ang-(1-7) only by the presence of an alanine residue in place of an aspartate residue at the amino terminus (Lautner *et al.*, 2013). Alamandine can be formed either from catalytic hydrolysis of Ang A by ACE2 or via decarboxylation of the aspartate radical group of Ang-(1-7), as has previously been shown for Ang II (Lautner *et al.*, 2013, Jankowski *et al.*, 2007) (Figure 1.4). In addition to being structurally similar to Ang-(1-7), alamandine was also shown to produce several physiological actions

that resemble those induced by Ang-(1-7). For example, alamandine induces endothelium-dependent vasodilation in aortic rings of FVB/N mice and Wistar rats, an effect attenuated by pretreatment with the NOS-inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) (Lautner *et al.*, 2013). Alamandine also reduced blood pressure when administered into the caudal ventrolateral medulla of anaethetised Fisher rats, as well as systemically in spontaneously hypertensive rat (SHR) (Lautner *et al.*, 2013). Furthermore, an orally available form of alamandine produced an anti-hypertensive effect in the SHR and reduced cardiac remodelling in isoprotenelol treated rats (Lautner *et al.*, 2013). However, despite being structurally and functionally similar to Ang-(1-7), alamandine was demonstrated to act independently of both Mas and the AT_2R suggesting a different mechanism of action to that of Ang-(1-7) (Lautner *et al.*, 2013). While the vasodilator effects of alamandine in aortic rings were unaltered by the Mas antagonist A779 and in Mas receptor deficient mice, they were blocked by another Ang (1–7) antagonist, D-Pro^7-Ang (1–7), suggesting that alamandine may act at an alternative receptor that is also sensitive to Ang-(1-7). Ang-(1-7) has previously been reported to act as a weak agonist of the MrgD therefore Lautner *et al* then investigated whether this could be the receptor through which alamandine acts (Lautner *et al.*, 2013, Gembardt *et al.*, 2008). Indeed, pre-incubation of aortic rings with ß-alanine, an MrgD agonist, attenuated alamandine induced vasodilation. Furthermore, alamandine was shown to bind to MrgD-transfected cells, an effect which was competitively inhibited by D-Pro^7-Ang (1–7) but not A779 (Lautner *et al.*, 2013). Finally, in cells expressing MrgD, alamandine induced NO release, an effect which was not observed in cells expressing Mas. Although alamandine was shown to be present in human plasma and increased in patients with end-stage renal disease, the potential role of this peptide in humans requires further investigation (Lautner *et al.*, 2013). Together, this data suggest that the novel peptide alamandine is a functionally active peptide within the RAS that acts via the MrgD receptor and indicates a novel interaction within the RAS that required further exploration.

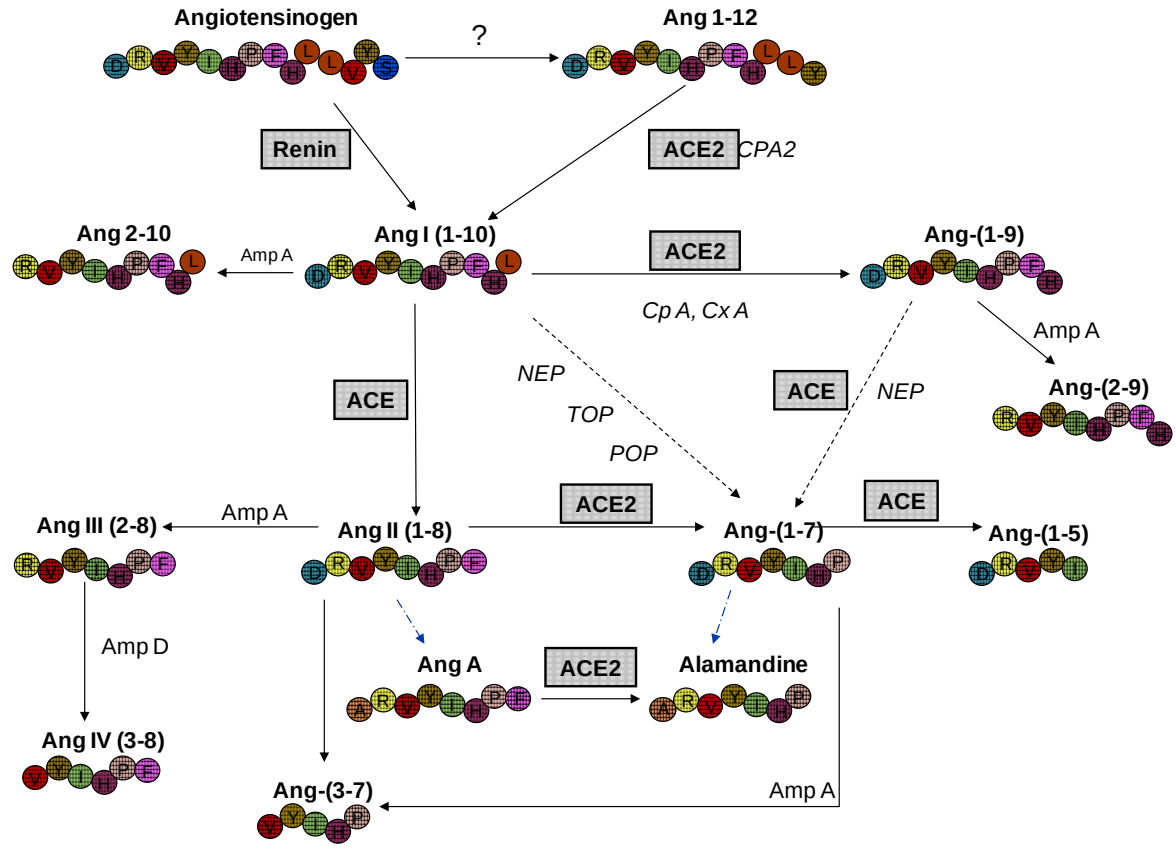

Figure 1.4 Angiotensin peptide metabolites
The discovery of the tissue specific and counter-regulatory axis of the RAS has resulted in the view of the RAS as a system of interconverted peptides, generated by a number of multifunctional enzymes. Black arrows indicate peptide formation via enzymatic metabolism. Blue dashed arrows indicate peptide formation via amino acid decarboxylation. ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2 Amp A: aminopeptidase a; Amp N: aminopeptidase N; Ang: angiotensin; CpA: cathepsin A; CPA2: carboxypeptidase A2; Cx A: carboyxpeptidase A; NEP:Neutral endopeptidase; POP: Prolyl endopeptidase; TOP: Thimet oligopeptidase.

1.6 The renin angiotensin system and vascular remodelling

Dysregulation of the RAS, resulting in increased Ang II activity via the AT_1R, largely contributes to pathological remodelling of the vasculature in both hypertension and atherosclerosis, and contributes to the failure of revascularisation in coronary artery disease.

1.6.1 Hypertension-induced remodelling

The RAS has been shown to be one of the key pathways involved in hypertension-induced vascular remodelling. In large, conduit arteries, Ang II has been shown to stimulate VSMC hypertrophy largely via interaction with NAD(P)H oxidases, resulting in ROS generation (Griendling *et al.*, 1994). Additionally, Ang II mediates activation of various growth factors such as transforming growth factor-ß (TGF-ß) and PDGF (Naftilan *et al.*, 1989a, Gibbons *et al.*, 1992). Ang II–induced VSMC hypertrophy and collagen deposition has been observed in various hypertensive animal models (Albaladejo *et al.*, 1994, Levy *et al.*, 1988).

Similarly, various signalling pathways have been implicated for Ang II-induced resistance vessel remodelling in hypertension. In the SHR, ACE inhibitors or AT_1R antagonists reduced blood pressure and vascular activity of ERK1/2, leading to improved endothelial function and Ang II-induced contractility of mesenteric resistance arteries, suggesting that Ang II induced changes in vascular function may be mediated by MAPK signalling (Kim *et al.*, 1997, Touyz *et al.*, 2002). Additionally, in Ang II–infused mice, inhibition of NAD(P)H oxidase by apocynin, attenuated blood presure elevation and prevented structural alterations, endothelial dysfunction, and collagen deposition in the media of small mesenteric arteries, indicating that NAD(P)H oxidase activity is also involved in Ang II–induced functional and structural alterations of the vascular wall (Virdis *et al.*, 2004).

1.6.2 Pro-atherogenic effects of angiotensin II

It is largely accepted that Ang II is one of the key players involved in the development and progression of atherosclerosis; in fact ACE inhibitors are among the most commonly prescribed pharmacological therapies for atherosclerosis and

have been shown to reduce cardiovascular events in patients with multiple risk factors for atherosclerosis (Yusuf *et al.*, 2000). Ang II regulates many processes implicated in atherogenesis, including ROS generation, VSMC growth, inflammatory responses, and ECM remodelling through activation of NAD(P)H oxidases, MAPK signalling and interaction with both receptor- and non receptor-tyrosine kinase signalling pathways (reviewed by (Touyz and Schiffrin, 2000, Mehta and Griendling, 2007). Furthermore, in human atherosclerotic lesions the local RAS is activated, as evidenced by high lesion levels of ACE, Ang II and the AT_1R (Schieffer *et al.*, 2000). Additionally, inflammatory cells present in atherosclerotic lesions express high ACE activity (Diet *et al.*, 1996).

Ang II also influences the expression of a number of pro-inflammatory molecules in the vessel wall, contributing largely to the recruitment of inflammatory cells and ultimately the development of atherosclerosis. Through the AT_1R, Ang II activates the transcription factor NF-κB, a protein that controls networks of chemokine-modulating, growth factor–modulating, translational control, and cellular survival genes (Ruiz-Ortega *et al.*, 2000). In endothelial cells, Ang II upregulates VCAM-1, ICAM-1, and E-selectin expression (Pueyo *et al.*, 2000, Pastore *et al.*, 1999, Grafe *et al.*, 1997). In VSMCs, Ang II stimulates the production of VCAM-1, MCP-1, and the cytokine IL-6 (Tummala *et al.*, 1999, Hernandez-Presa *et al.*, 1997, Han *et al.*, 1999). MCP-1 specifically attracts monocytes and memory T lymphocytes expressing the C-C chemokine receptor 2 (CCR2) receptor, cell types that are present at all stages of the atherosclerotic lesion. MCP-1 is thought to function locally in the vessel wall by establishing a chemical gradient to attract adherent monocytes and T lymphocytes to the site of injury in the vessel media. IL-6 promotes VSMC proliferation involving the local production of PDGF (Ikeda *et al.*, 1991). The ability of Ang II to induce coordinated expression of adhesion molecules and chemokines then ensures the recruitment of inflammatory cells into the vessel wall (Pastore *et al.*, 1999). Furthermore, Ang II has been shown to be involved in cellular deposition of ECM, an important component in VSMC remodelling, migration and adhesion. Ang II-induced EGFR- and MAPK-dependent pathways may participate in matrix formation and regulation (Matsubara *et al.*, 2000, Touyz and Schiffrin, 2000). Ang II has also been shown to promote collagen synthesis within VSMC (Kato *et al.*, 1991). Besides regulating structural components such as collagen, Ang II has

also been implicated in adhesive remodelling. Moriguchi *et al* reported that Ang II-mediated EGFR transactivation regulates fibronectin and TGF-ß synthesis (Moriguchi *et al*., 1999). Furthermore, production of MMPs, for example, MMP-2, and breakdown of collagen IV is also modulated by Ang II (Libby and Lee, 2000). Thus, Ang II acts on several different components of ECM formation and deposition to influence matrix turnover.

Furthermore, Ang II has also been reported to promote of macrophage mediated uptake of oxidised LDL via increased IL-6 production and expression of the scavenger receptor CD36 on macrophages (Keidar *et al*., 2001). Additionally, Ang II upregulated endothelial cell expression of the lectin-like oxidised LDL receptor 1, which may result in increased oxidised LDL entry into the atherosclerotic lesion (Morawietz *et al*., 1999). While the exact mechanisms are unknown, these studies suggest that Ang II enhances the uptake of LDL and generation of foam cells.

1.6.3 Vein graft failure

Ang II is known to mediate a number of pathways involved in vein graft failure and through the use of animal models of vein grafting, has been directly implicated in its pathology. For example, administration of the ACE inhibitor captopril reduced neointimal formation following vein grafting in the rabbit (O'Donohoe *et al*., 1991). In a canine model, it was found that ACE activity was increased in grafted veins compared with control veins at 4 weeks post-engraftment and this was associated with an increase in neointimal formation (Yuda *et al*., 2000). Furthermore, the AT_1R antagonist L-158, 809, a non-peptide imidazopyridine derivative, significantly reduced neointimal formation and VSMC proliferation in comparison to control groups (Yuda *et al*., 2000). L158, 809 has also been shown to reduce neointimal formation in grafted veins in the rabbit when administered either systemically or directly to the vessel (Fulton *et al*., 1998). Taken together, these findings suggest that local production of Ang II via increased ACE expression and activity enhances neointimal formation following vein grafting.

1.6.4 Restenosis

Ang II is known to mediate a number of the cellular effects involved in restenosis, it has been directly implicated in the pathogenesis of restenosis and neointimal formation through the use of various rodent models of vascular injury. Increased levels of ACE have been observed in the neointimal area of balloon injured rat carotid arteries and in mouse femoral arteries following cuff induced injury, indicative of increased Ang II production within the remodelled vessel (Rakugi *et al.*, 1994, Akishita *et al.*, 2001). In line with these findings, ACE inhibitors have been shown to reduce neointima formation in response to vascular injury in several models and species, such as balloon catheter induced injury in rats, rabbits and guinea pig, and cuff induced injury in various strains of mice (Powell *et al.*, 1989, Chen *et al.*, 2003, Akishita *et al.*, 2001). Increased expression of AT_1R, indicative of increased Ang II signalling, has been observed in the neointimal following vascular injury of the rat carotid artery (Eto *et al.*, 2003). The role of the AT_1R was further confirmed in this study as AT_1R inhibition reduced neointimal thickening, associated with reduced collagen and elastin accumulation (Eto *et al.*, 2003). In monkeys and rabbits, AT_1R blockade also reduced in-stent restenosis, oxidative stress, proinflammatory factors (MCP-1, IL-1β, and TNF-α) and NAD(P)H oxidase expression suggesting that AT_1R signaling is involved in neointimal formation (Ohtani *et al.*, 2006).

1.6.5 The counter-regulatory RAS and vascular remodelling

While Ang II mediated effects via the AT_1R are widely accepted to contribute to pathological remodelling of the vasculature, the components of the counter-regulatory axis of the RAS, namely ACE2, Ang-(1-7) and Ang-(1-9) have been shown to exert protective effects on the vasculature.

1.6.5.1 Vascular ACE2

The expression and activity of ACE2 in the vasculature has beneficial effects, particularly in the setting of atherosclerosis, via reducing Ang II levels and increasing levels of Ang-(1-7). In atherosclerotic human carotid arteries, ACE2 activity was found to be increased in early stage atherosclerosis or in unstable lesions in comparison to stable lesions, demonstrating differential activity of ACE2 at different stages of the disease (Sluimer *et al.*, 2008). While it is

currently unclear how this differential activity is regulated, it may be that ACE2 is increased in the early stages of atherosclerosis and in unstable lesions in an attempt to control the disease pathology and lessen injury by both reducing Ang II production and increasing production of Ang-(1-7) and Ang-(1-9). Additionally, over-expression of ACE2 using an adenoviral vector has been shown to reduce atherosclerotic lesion size in ApoE$^{-/-}$ mice (Lovren *et al.*, 2008). Moreover, ACE2 overexpression also stabilizes existing atherosclerotic lesions, inhibiting progression of lesion development at early stages of atherosclerosis, but not at advanced stages of the disease (Dong *et al.*, 2009). These effects were associated with both reduced levels of Ang II and increased levels of Ang-(1-7), suggesting a key role for ACE2 in balancing the activity of both axes of the RAS in atherosclerosis.

1.6.5.2 Effects of Ang-(1-7) on vascular remodelling

To date a large proportion of the protective effects of the counter-regulatory axis of the RAS in the vasculature have been attributed to the production of Ang-(1-7), and its resulting interaction with Mas (Figure 1.5). Numerous *in vitro* studies have shown that Ang-(1-7) signaling via Mas inhibits VSMC proliferation and migration via two main mechanisms: (1) inhibition of MAPK signaling pathways, for example reduced activity and expression of ERK1/2 (Tallant *et al.*, 1999, Zhang *et al.*, 2010b) and (2) release of prostaglandins including prostacyclin (PGI2) and prostaglandin E2 (PGE2), resulting in increased cyclic adenosine monophosphate (cAMP) levels and inhibition of cyclo-oxygenases (Jaiswal *et al.*, 1993b).

The anti-proliferative and anti-migratory role of Ang-(1-7) is also observed in numerous rodent models of vascular disease. Ang-(1-7), via Mas, reduces neointimal formation following balloon injury (Strawn *et al.*, 1999), stent implantation in rats (Langeveld *et al.*, 2005), and angioplasty in rabbits (Zeng *et al.*, 2009), and has been associated with reduced atherosclerotic lesion size in Apo E$^{-/-}$ mice (Tesanovic *et al.*, 2010, Yang *et al.*, 2013). Additionally, a non-peptide agonist of Mas, AVE0991, which mimics the actions of Ang-(1-7) (Wiemer *et al.*, 2002), inhibits rat VSMC proliferation *in vitro* (Sheng-Long *et al.*, 2012).

Ang-(1-7) increases NO release, thereby acting as a vasodilator and improving vascular endothelial function (Brosnihan *et al.*, 1996, Faria-Silva *et al.*, 2005). Increased NO is achieved directly via Mas-mediated stimulation of eNOS and sustained Akt phosphorylation, or indirectly via production of bradykinin and receptor cross talk with the BK_2R (Jackman *et al.*, 2002, Sampaio *et al.*, 2007a). Additionally, Ang-(1-7) has been shown to promote vasodilation via the AT_2R in the SHRSP during AT_1R blockade. This was shown to involve interaction between the AT_2R and BK_2R as vasodilation in response to Ang-(1-7) was prevented by both PD123,319 and HOE 140 (a BK_2R antagonist) (Walters *et al.*, 2005). In addition to promoting vasodilation, this increase in NO release was also demonstrated to inhibit platelet aggregation, demonstrating an anti-thrombotic role for Ang-(1-7) (Kucharewicz *et al.*, 2002).

An Ang-(1-7)-mediated increase in NO bioavailability has also been linked to reduced ROS production, thereby promoting improved vascular function and reduced atherosclerosis. In Apo $E^{-/-}$ knockout mice, chronic administration of Ang-(1-7) via osmotic mini pump restored renal endothelial function which was associated with increased NO bioavalibility (Stegbauer *et al.*, 2011). To investigate the relationship between ROS levels and NO bioavailability in this setting, the ROS scavenger Tempol was used. While Tempol improved endothelial function in untreated Apo $E^{-/-}$, it had no effect on Ang-(1-7) infused mice, indicating that these animals already have reduced ROS levels (Stegbauer *et al.*, 2011). This was further suggested by reduced levels of H_2O_2 and NAD(P)H oxidase subunit expression in Ang-(1-7)-infused animals. In addition to reduced ROS activity, increased levels of eNOS were observed following treatment with Ang-(1-7), providing a further mechanism to support the findings of increased NO bioavailability (Stegbauer *et al.*, 2011).

In addition to Ang-(1-7)-mediated NO release from vascular endothelial cells (Sampaio *et al.*, 2007a, Heitsch *et al.*, 2001) it has recently been shown that Ang-(1-7) signalling mediates NO release from platelets, enhancing its anti-thrombotic activities (Fraga-Silva *et al.*, 2008). *In vivo* the anti-thrombotic effect of Ang-(1-7) was blocked by pharmacological Mas inhibition and is absent in Mas knockout ($Mas^{-/-}$) mice (Fraga-Silva *et al.*, 2008). It was not established whether this was due to the actions of Ang-(1-7) in endothelial cells or platelets, although it is likely to be a combination of both (Fraga-Silva *et al.*, 2008). While

further work is required to fully dissect the mechanisms involved in the anti-thrombotic properties, these findings have identified the Ang-(1-7)/Mas axis as a potential therapeutic target for the treatment of thrombotic events. The recent development of an orally available form of Ang-(1-7) (Ang-(1-7)-CyD), in which Ang-(1-7) has been incorporated into a cyclodextrin (CyD), a cyclic oligosaccharide that enhances drug stability, absorption across biological barriers and provides gastric protection (Uekama, 2004), has greatly increased the potential of utilising Ang-(1-7) in a therapeutic setting (Fraga-Silva *et al.*, 2011). This compound has been shown to be of particular use as an anti-thrombotic intervention as it exerts antithrombotic effects *in vivo,* associated with increased plasma levels of Ang-(1-7) (Fraga-Silva *et al.*, 2011).

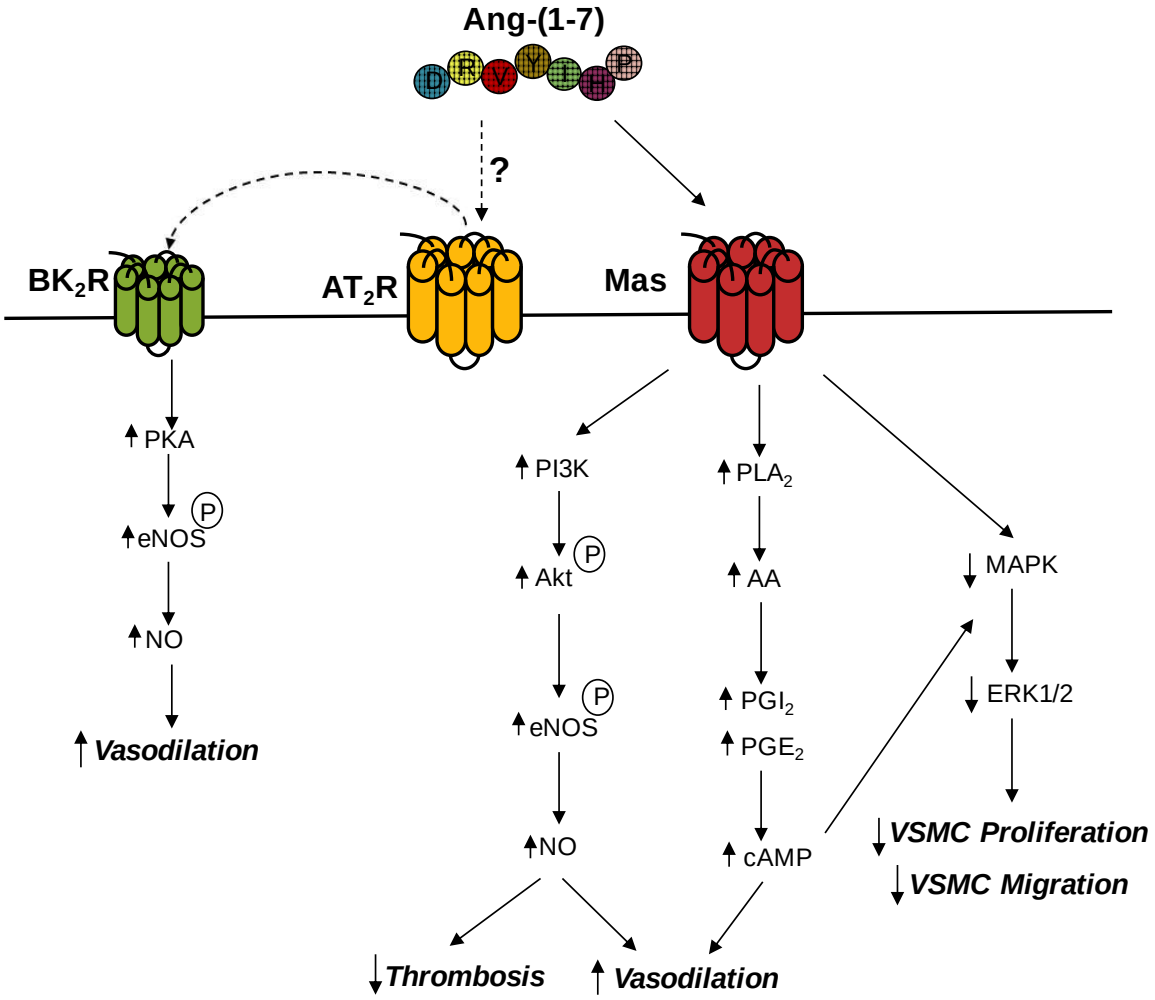

Figure 1.5 Angiotensin-(1-7) signalling in the vasculature
Ang-(1-7) via Mas increases NO release, thereby acting as a vasodilator and reducing thrombosis either 1) directly via activation of the PI3K/Akt signaling pathway resulting in increase phosphorylation of eNOS or 2) indirectly via cross talk with the BK_2R, which increases PKA mediated phosphorylation of eNOS-this pathway may involve interaction with the AT_2R also. Ang-(1-7) via Mas also promotes vasodilation by stimulating PLA2 leading to increased release of AA, which in turn produces the prostanoids PGI2 and PGE2, both of which increase levels of cAMP. cAMP also reduces MAPK signaling and VSMC proliferation and migration. Ang-(1-7) via Mas also directly inhibits MAPK activity. AA: Arachidonic acid; AT_2R: Angiotensin type 2 receptor; BK_2R: Bradykinin 2 receptor; cAMP: Cyclic adenosine monophosphate; eNOS: endothelial nitric oxide synthase; NO: Nitric oxide; PI3K: Phosphoinositide 3-kinase; PGI2: Prostacyclin: PGE2: Prostaglandin E2; PKA: Protein kinase A.

1.6.5.3 Effects of Ang-(1-9) on vascular remodelling

The role of Ang-(1-9) in the vasculature is relatively unexplored; however, as Ang-(1-9) can also exert biological effects, some of the previously described vasculoprotective effects of the counter-regulatory axis of the RAS may be attributed to production of both Ang-(1-9) and Ang-(1-7) (Figure 1.6). However, signalling via the AT_2R in the vasculature is poorly defined and further work is required to delineate a role in this setting (Figure 1.6). Ang-(1-9) has been previously shown to indirectly contribute to improved vascular function through a number of pathways. For example, Ang-(1-9) promotes bradykinin release from endothelial effects and enhances the effects of bradykinin by augmenting NO and arachidonic acid release (Jackman *et al.*, 2002). Additionally, inhibition of the RhoA/Rho-associated, coiled-coil containing protein kinase (ROCK) signaling pathway results in increased activity and expression of ACE2 in the aorta, and increased Ang-(1-9) plasma levels, associated with reduced blood pressure and vascular remodelling (Ocaranza *et al.*, 2011). These effects were coupled with reduced ACE activity, Ang II levels and increased expression of eNOS, identifying that the ROCK pathway may interact with the ACE2/Ang-(1-9) axis as a novel, protective interaction in the vasculature.

Ang-(1-9) has recently been demonstrated, for the first time, to have a direct beneficial effect on vascular function. In this study continuous infusion of Ang-(1-9) improved aortic vasorelaxation and NO bioavailability in the SHRSP; importantly these effects were blocked by PD123,319, suggesting that these effects are due to Ang-(1-9) signalling via the AT_2R (Flores-Munoz *et al.*, 2012). Furthermore, Ang-(1-9) has recently been shown to induce relaxation of rat aortic rings in a concentration- and endothelium-dependent manner, an effect which was unaltered by A779 or losartan, but blocked by PD123,319 and L-NAME suggesting that Ang-(1-9) induces vasodilation via the AT_2R and NO signalling (Ocaranza *et al.*, 2014). While the mechanisms involved are currently unknown it is possible that Ang-(1-9) may increase NO bioavailability by stimulating bradykinin release, as previously documented in cardiac endothelial cells (Jackman *et al.*, 2002), or by enhancing the activity of eNOS, as has been shown for Ang-(1-7) (Sampaio *et al.*, 2007a).

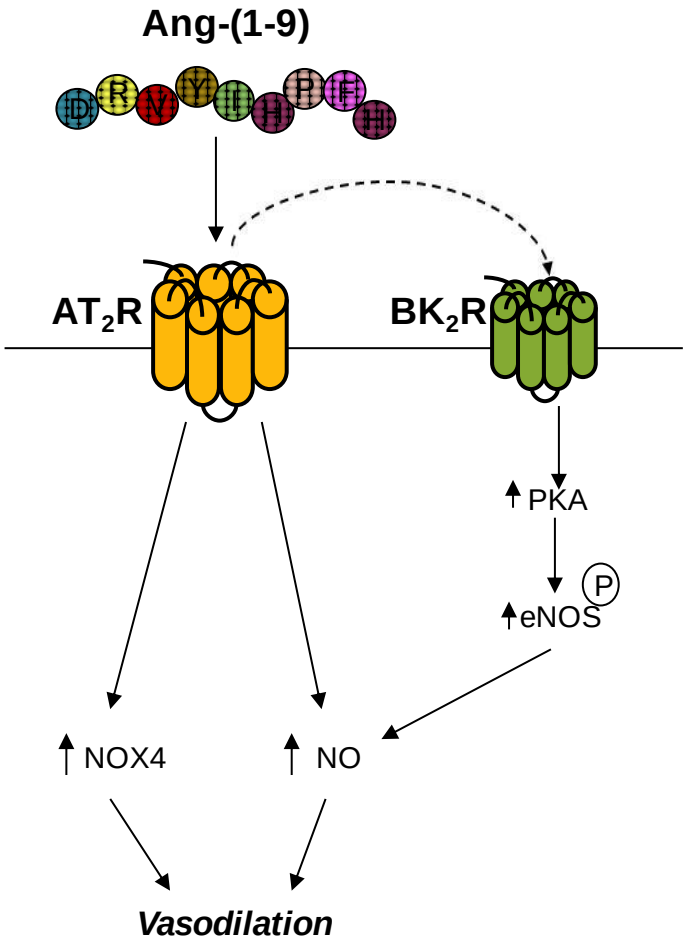

Figure 1.6 Angioteinsin-(1-9) signalling in the vasculature
Ang-(1-9) via the AT_2R promotes vasodilation through increased NO either directly or via crosstalk with the BK_2R. Ang-(1-9) mediated vasodilation is also associated with increased expression of NOX4. AT_2R: Angiotensin type 2 receptor; BK_2R: Bradykinin 2 receptor; eNOS: endothelial nitric oxide synthase; NO: Nitric oxide; NOX4: NADPH oxidase 4; PKA: protein kinase A. (Adapted from (McKinney *et al*., 2014)).

1.7 Hypothesis and aims

Numerous studies *in vitro* and *in vivo* studies have demonstrated a vasculoprotective role for Ang-(1-7) signaling via Mas. The majority of this work has relied on the use of rodent cells and these effects have yet to be demonstrated in human cells. Furthermore, the cell signaling mechanisms involved in the effects of Ang-(1-7) have still to be fully delineated. We hypothesized that Ang-(1-7) also elicits protective effects in human cells relevant to vascular disease.

While Ang-(1-9) has previously been demonstrated to have protective effects within the heart, the role of Ang-(1-9) in the vasculature remains undefined and the effects of this peptide in the setting of vascular remodeling have not previously been explored. However, as Ang-(1-9) has been demonstrated to antagonize the effects of Ang II, which contributes greatly to remodeling of the vasculature in a number of disease states, it is possible that Ang-(1-9) may also have protective effects within the vasculature. Furthermore, as ACE2, the enzyme responsible for the production of Ang-(1-9), and the AT_2R, the receptor through which Ang-(1-9) has been demonstrated to act, have both previously been reported to be protective in the setting of vascular remodeling, it is possible that some of these previously described vasculoprotective effects of the counter-regulatory axis of the RAS could be attributed in part to Ang-(1-9). Therefore, we hypothesized that in addition to Ang-(1-7), Ang-(1-9) may also have protective effects in the setting of vascular remodeling.

Therefore, the principal research aim of this thesis was to investigate and compare the effects of Ang-(1-7) and Ang-(1-9) in vascular remodeling. This was achieved through the following experimental study aims:

- Characterise the effects of Ang-(1-7) and Ang-(1-9) on human saphenous vein SMC proliferation and migration, and assess the cell signalling pathways involved.

- Investigate the effects of Ang-(1-7) and Ang-(1-9) on human saphenous vein on endothelial cell growth, migration and function.

- *In vivo* assessment of the effects of Ang-(1-7) and Ang-(1-9) on vascular remodelling and neointimal formation following vascular injury in mice.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals, unless otherwise stated were purchased from Sigma-Aldrich (Poole, UK). Angiotensin peptides were purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany). Receptor antagonists were purchased from Sigma-Aldrich (losartan and PD123,319) or Bachem (Rhein, Germany) (A779). All tissue culture reagents were obtained from Gibco (Paisley, UK), unless otherwise stated. All transfection, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) reagents were purchased from Life Technologies (Paisley, UK), unless otherwise stated.

2.2 Human vascular tissue

Saphenous veins were obtained from patients undergoing CABG surgery and stored in sterile saline solution by the surgical team at the Golden Jubilee National Hospital (Glasgow, UK). Saphenous veins were also obtained from patients undergoing elective varicose vein removal and were stored in the same way by the surgical team at Gartnavel General Hospital (Glasgow, UK). At the laboratory vessels were cleaned of any excess tissue under sterile tissue culture conditions. Endothelial cells were isolated from the vessels on the day of surgery and VSMC were isolated from the vessels within 24 hrs of the surgery.

2.3 Cell and tissue culture

All cell culture was performed under sterile conditions using class II biological safety vertical laminar flow cabinets (Holton Safe 2010). All cells were cultured in a humidified incubator at 37ºC with a constant supply of 5% carbon dioxide (CO_2). Cells were cultured in 25cm^2, 75cm^2 or 150cm^2 flasks with vented lids (Corning, Poole, UK). CHO cells used at the Federal University of Minas Gerais (UFMG) were gifted by Prof Robson. A Santos and cultured in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% (volume/volume [v/v]) foetal calf serum (FCS), 100 international units (I.U)/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine.

2.3.1 Isolation of primary endothelial cells from human saphenous veins

Human saphenous vein endothelial cells (HSVEC) were isolated on the day of surgery by enzymatic collagenase digestion using a protocol based on the technique described by Jaffe *et al* (Jaffe *et al.*, 1973). Briefly, saphenous veins were flushed of any remaining blood using wash media (DMEM supplemented with 100 I.U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine and 25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Veins were then filled with a solution of filter-sterilised collagenase (2 mg/mL in wash medium) and clamped at both ends to prevent any leakage of the solution. The vein was placed in a sterile petri dish and incubated in the presence of 5% CO_2 for 15 minutes at 37ºC. Endothelial cell suspensions were collected by flushing the vein with wash medium and collecting the eluate in a separate sterile petri dish. This process was repeated with fresh collagenase solution and the vein incubated for 10 minutes at 37ºC. The cells and eluate were collected and pelleted by centrifugation at 12,000 g for 5 minutes at room temperature. Cells were resuspended in complete Large Vessel Endothelial Cell Basal Medium (TCS Cellworks Ltd, Botolph, Claydon, Bucks, U.K), supplemented with 20% (v/v) FCS, 100 I.U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 90 µg/mL heparin, 1 µg/mL hydrocortisone, 1 ng/mL basic fibroblast growth factor (bFGF) and 0.1 ng/mL epidermal growth factor (EGF), and then incubated for 24 hours before a complete medium change. Cells were cultured in complete growth medium and used below passage 7.

2.3.2 Isolation of primary smooth muscle cells from human saphenous veins

HSVSMC were isolated within 24 hours of surgery using the explant technique as described by Southgate and Newby (Southgate and Newby, 1990). Briefly, the vein was cut longitudinally and pinned lumenal side up. Remaining endothelium was removed by gentle rubbing of the lumenal surface. The medial layer was scored with a sterile scalpel, separated from the adventitial layer and then chopped into 1 mm^2 segments using a McIlwain Tissue Chopper (Ted Pella Inc., California, USA). Explants were washed with media and incubated at 37ºC in 5% CO_2 in minimal medium until they had adhered to the bottom of the flask (approximately 24 hours). Explants were cultured at 37ºC in the presence of 5%

CO_2 in 5 mL complete Smooth Muscle Cell Growth Medium 2 supplemented with 15% (v/v) FCS, 100 I.U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 0.5 ng/mL EGF, 2 ng/mL bFGF and 5 µg/mL insulin. Medium was changed once a week until HSVSMC migrated from the explants and then every 3 days once passaged. Cells were cultured in complete growth medium and used below passage 7.

2.3.3 Cell passage

Cells were grown as a monolayer and media was replenished every 2-3 days. Cells were routinely passaged when approaching confluence to prevent overgrowth. To passage cells were washed twice with sterile phosphate buffered saline (PBS) and incubated at 37°C in 3mL trypsin-ethylenediamine tetra-acetic acid (trypsin-EDTA) (0.05% trypsin, 0.02% EDTA) for approximately 5 minutes or until the majority of the cells had detached from the flask. The action of trypsin-EDTA was neutralised by the addition of an equal volume of complete growth media. Cells were harvested by centrifugation at 1,500 g for 5 minutes and resuspended in complete growth media for passaging or plating. Before plating cells were counted using a haemocytometer to ensure the required seeding density was met.

2.3.4 Cryopreservation

Cells were harvested as described in section 2.3.3. Following centrifugation cells were resuspended in 1mL of complete growth media supplemented with 10% dimethyl sulphoxide (DMSO) per culture flask of cells. Cell suspensions were collected in cryo-preservation vials and cooled at -80ºC in a freezer storage box containing isopropanol for 24 hours. Vials were then stored in the vapour phase of liquid nitrogen. Cryo-preserved cells were recovered by thawing at 37ºC and then adding drop wise to 15 mL of complete growth media. Cell suspensions containing primary cells were transferred directly to a 75cm^2 flask. Cell suspensions from cell lines were harvested by centrifugation at 1,500 g for 5 minutes and resuspended in complete growth media before being added to the relevant sized cell culture flask. Cells were incubated overnight at 37ºC in the presence of 5% CO_2 and the media changed the following day.

2.4 Stimulation of HSVSMC and HSVEC

In all *in vitro* experiments using HSVSMC or HSVEC cells were stimulated with Ang II, Ang-(1-7) or Ang-(1-9) at 200 nM, unless otherwise stated. Cells were incubated with Ang-(1-7) and Ang-(1-9) 30 minutes prior to the addition of Ang II. In experiments where receptor antagonists were used, cells were incubated with the AT_1R antagonist losartan at 10 μM, the AT_2R antagonist PD123,319 at 500 nM or the Mas antagonist A779 at 100 μM for 15 minutes prior to the addition of Ang-(1-7) or Ang-(1-9). The concentrations of peptides and antagonists, and stimulation protocol were chosen based on previous experiments within our group and from the literature (Flores-Munoz *et al.*, 2011, Sampaio *et al.*, 2007a, Sampaio *et al.*, 2007b, Zhang *et al.*, 2010b, Freeman *et al.*, 1996, Tallant and Clark, 2003, Zhu *et al.*, 2002).

2.5 Proliferation assay

CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Southampton, UK), also known as the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) assay was used to measure cell proliferation. The MTS assay is a colorimetric method for determining the number of viable cells and is based on the conversion of the novel tetrazolium compound MTS to a soluble formazan product by viable cells. The absorbance of the formazan product can be measured and this is directly proportional to the number of viable cells.

HSVEC or HSVSMC were seeded at $5x10^3$ cells/well in 96 well plates and incubated overnight at 37ºC in the presence of 5% CO_2. The cells were then rendered quiescent by incubation in serum free medium (HSVEC: 24 hours in DMEM, 100 I.U/mL penicillin and 100 μg/mL streptomycin; HSVSMC 48 hours in DMEM GlutaMAX, 100 I.U/mL penicillin and 100 μg/mL streptomycin). To induce proliferation, cells were then exposed to fresh media containing different concentrations of FCS (0%-20% (v/v) FCS in quiescing media) for 24 hours (HSVEC) or 48 hours (HSVSMC) at 37ºC in the presence of 5% CO_2. A working solution containing MTS and phenazine sulphate (PMS, an electron coupling reagent) was prepared at a ratio of 20:1, respectively, and added to the cells in 100 μL media.

Cells were then incubated at 37ºC for 3 hours. Finally, absorbance was read at 490 nm using the Wallac 1420 Victor^2 plate reader (Wallac, Turku, Finland).

To assess the effect of Ang II, Ang-(1-7) and Ang-(1-9) (all 200 nM) on cell proliferation, cells were incubated in either serum free medium or 5% FCS containing medium for 24 hours (HSVEC) or 48 hours (HSVSMC). To assess the role of angiotensin receptors in HSVSMC were incubated with losartan, PD123,319 or A779 as decribed in Section 2.4. Proliferation was then assessed as described above.

2.6 Migration assay

Cell migration was assessed in the form of a scratch assay as described by Liang *et al* (Liang *et al* 2007). Briefly, cells were seeded in 6 well plates at a density of $3x10^5$ cells per well and grown in complete culture medium at 37ºC in the presence of 5% CO_2 until fully confluent. The cells were rendered quiescent by incubating in serum free medium as described in section 2.5. Horizontal lines were drawn on the outside bottom surface of each well to act as a guide for measurements. Three straight, vertical scratches were induced in the cell monolayer of each well using a sterile 200 µL pipette tip. Cellular debris was removed by gently washing cells once with 1 mL of PBS. Cells were incubated in serum free media with Ang II at 200nM to induce cell migration. To assess the effects of Ang-(1-7) and Ang-(1-9) on cell migration, cells were incubated in serum free media with Ang-(1-7) or Ang-(1-9) (200nM) alone for the duration of the experiment and 30 minutes before stimulation with Ang II. To assess the role of AT_1R, AT_2R and Mas, cells were incubated with losartan (10 µM), PD123, 319, 500 nM or A779 (100 µM) for 15 minutes before Ang-(1-7) or Ang-(1-9), and 45 minutes before Ang II. Cells in serum free media alone or complete growth medium were used as control samples. Images of the scratch were taken directly above the guide line using a Nikon Eclipse TS1000 microscope and imaged on QICAM Fast1394 camera (QImaging, Maidenhead, UK) at various time points between 0-30 hours post scratch. Image analysis was performed using Image J software where, to ensure unbiased measurements were made, a grid composed of 12 horizontal lines were placed over the image. The distance between the edges of the wound were measured along the grid lines and migration was expressed as a percentage of the original scratch width (0 hour).

In experiments which were performed to assess changes in gene and miRNA expression and protein activity, cells were subject to 8 scratches per well in order in increase the proportion of migrating cells and lysed as described in section 2.8 for RNA extraction or section 2.12 for protein extraction at 24 hours post scratch.

2.6.1 Real time analysis of HSVSMC migration

HSVSMC migration was also assessed in real-time using the xCELLigence system (Roche, Mannheim, Germany). The xCELLigence system monitors cellular events in a label free environment and provides quantitative information about the biological status of cells in real time (Atienza *et al.*, 2006). This is achieved by measuring cellular impedance using specialised micro-titre plates, E-Plates, which have integrated gold electrodes covering 80% of the bottom of each well that measure impedence; changes in cellular impedance can be correlated to changes in cell morphology or growth (Atienza *et al.*, 2006). In the absence of cells, background impedance is determined by the ionic environment of the culture media at the electrode-media interface and the media itself. When cells attach to the electrodes this alters the ionic environment at the electrode-media interface resulting in an increase in impedance (Yu *et al.*, 2006). As the cells begin to spread and proliferate, the impedance further increases. Short term changes in cell impedance can be correlated to changes in cell morphology while longer term changes in cell impedance are indicative of growth and movement (Atienza *et al.*, 2006, Yu *et al.*, 2006). Impedance measurements are expressed as an arbitrary unit, Cell Index (CI). CI is defined as (Rn-Rb)/15Ω, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the media alone (Atienza *et al.*, 2006).

To assess migration, HSVSMC were seeded at a density of $1x10^4$ cells/well in an E-plate to create a confluent monolayer and incubated at 37ºC overnight to allow cells to adhere to the electrodes, resulting in a gradual increase in CI. Cells were rendered quiescent in serum free medium for 48 hours causing the CI to reach a stable plateau, indicating the cells had changed from a proliferative to a quiescent state. A scratch was induced in the cell monolayer as described in section 2.6 causing a rapid drop in CI due to the reduced electrode coverage. As changes in temperature can affect CI readings the cells were allowed to

equilibrate for 30 minutes then stimulated in serum free media as described in section 2.4. CI was measured every 15 minutes for 24 hours. CI was normalised at the time of stimulation with Ang II (referred to as 0 hour) and migration quantified as the fold change in normalised CI relative to 0 hour.

2.7 Transfection of HSVSMC

RNA interference (RNAi) techniques were used to assess the involvement of miRNA in HSVSMC migration. HSVSMC were transfected with a short interfering RNA (siRNA) designed to target human DICER or a specific miRNA-132 inhibitor (both Life Technologies). A siRNA designed to target GAPDH was used as a positive control and assess transfection efficiency. A non targeting Cy3 labelled siRNA or miR-inhibitor was used as a negative control. A list of all RNAi assays used can be found in Table 2.1.

Table 2.1 List of RNAi assays used in HSVSMC

RNA i	Assay ID
Silencer® Select DICER1 antisense RNA 1	n269596
Silencer® Select GAPDH siRNA	4404024
Silencer® Cy™3 Labeled Negative Control No. 1 siRNA	AM4621
Cy3™ Labeled Anti-miR™ Negative Control	AM17011
mirVana™ miRNA Inhibitor, Negative Control	4464076
mirVana™ miR-132 Inhibitor	MH10166

2.7.1 Transfection efficiency

Cells were transfected using siPORT^{TM} neoFX^{TM} (Life Technologies) according to manufacturer's instructions. Briefly, cells were plated at 3x10^5 cells/well in 6 well plates and cultured in complete media at 37ºC in the presence of 5% CO_2 until 90% confluent. For each well, 5 µL siPORT^{TM} neoFX^{TM} was diluted in 100 µL Opti-MEM® and incubated at room temperature for 10 minutes. SiRNA or miR inhibitors were diluted to 10, 30 or 60 nM in 100 µL Opti-MEM® and then combined with the diluted siPORT^{TM} neoFX^{TM} and incubated for a further 10 minutes. The RNA/siPORT^{TM} neoFX^{TM} transfection complexes were added to culture media to a total volume of 2.0 mL, which was then added to the cells. A mock transfection control was also included where cells were treated with siPORT^{TM} neoFX^{TM} but not exposed to any RNA. The cells were incubated with the transfection reagents at 37ºC in the presence of 5% CO_2 for 10 hours, after which cells were washed then incubated for a further 48 hours in serum free media. The media was changed to fresh serum free media and cells were incubated at 37ºC in the presence of 5% CO_2 for a further 24 hours. Transfection efficiency was first confirmed by visualisation of the Cy3 labelled control transfected well using a fluorescence microscope and then cells were lysed for RNA extraction as described in section 2.9 to allow for subsequent inhibition of target gene expression using q-RT-PCR as described in section 2.11.

2.7.2 RNA interference in HSVSMC migration

Cells were transfected as described with the optimal concentration of siRNA or miR-132 inhibitor as determined in section 2.7.1. Following a 48 hours quiescence period outlined cells were subject to the migration assay protocol as described in section 2.6. RNA was isolated at 24 hours post induction of scratch and stimulation as described in section 2.8.

2.8 RNA and miRNA extraction from HSVEC and HSVSMC

Total and miRNA was extracted using the miRNeasy mini kit (Qiagen, Manchester, UK), including on-column DNAse treatment to remove any DNA contamination, as per manufacturer's instructions. Briefly, cells were washed in PBS to remove any residual media and lysed using 700 µL QIAzol lysis reagent. Lysates were briefly vortexed to homogenize prior to the addition of 140 µL of

chloroform to facilitate separation of the sample. Protein remains in the lower organic phase and DNA partitions to the interphase while the RNA partitions to the upper aqueous phase. The sample was partitioned fully by centrifugation at 12,000 g for 15 minutes at 4ºC. The RNA containing aqueous phase was transferred to a fresh RNAse free microcentrifuge tube (Applied Biosystems) and 1.5 volumes of 100 % ethanol added to facilitate binding to the RNeasy mini spin column. Samples were immediately added to the spin column and subjected to ultracentrifugation at 8000 g for 15 sec to bind the RNA to the spin column. To remove any contamination, columns were washed with 350 µL of buffer RWT for 15 sec and 8000 g and the flow through discarded. Digestion of residual DNA was performed by incubating the spin columns with DNAse for 15 minutes at room temperature. The column was further washed with 350 µL buffer RWT, followed by two washes of 500 µL buffer RPE by centrifugation at 8000 g for 15 sec to remove any traces of salts from the sample. To dry, spin columns were subjected to centrifugation at 13,100 g for 1 minute. RNeasy spin columns were transferred to RNAse free microcentrifuge tubes and RNA was eluted using 50 µL of RNAse free water by centrifugation at 8000 g for 1 minute. This elution step was repeated using the original volume of RNAse free water to maximise the RNA yield. RNA samples were stored at -80ºC.

2.9 Quantification of RNA

Total RNA was quantified using a NanoDrop 1000 Spectrophotometer (ThermoScientific, Loughborough, UK). The NanoDrop measures the concentration of nucleic acid in solution by exposing the sample to a pulse of light at 260 nm and then measuring absorption. The relationship between the absorbance and the concentration of the sample is based on the Beer-Lambert law:

$$c=A/(\Theta \times l)$$

Where:

c= nucleic acid concentration (ng/µL)
A= absorbance
Θ= molar absorptivity (constant for given solution)
l= path length of light passing through (constant for given instrument

The purity of the sample was assessed by calculating the ratio of absorbance at 260 and 280 nm. A 260/280 ratio of <2.0 was accepted as pure.

2.10 Complementary deoxyribonucleic acid (cDNA) synthesis

mRNA extracted from cells was reverse transcribed to cDNA to allow for analysis of gene or miRNA expression via qRT-PCR. For both gene expression and miRNA cDNA synthesis two negative control reactions were prepared: one containing water instead of mRNA and the other containing no reverse transcriptase, the enzyme responsible for cDNA synthesis.

2.10.1 Gene expression

mRNA extracted from cells was reverse transcribed to cDNA for gene expression analysis using the Taqman Reverse Transcription Reagents (Applied Biosystems, Warrington, UK) as per manufacturer's instruction. A maximum of 1 µg (the same concentration of RNA used in each experimental repeat) was reversed transcribed in a reaction containing: 5.5 mM $MgCl_2$, 2mM deoxyribonucleotide triphosphate (dNTP) mix (0.5 mM each), 2.5 µM random hexamers, 1x RT buffer, 0.4 U/µL RNAse inhibitor and 1.25 U/µL multiscribe reverse transcriptase. Cycling conditions used were as follows: 25ºC for 10 minutes to allow annealing, 48ºC for 30 minutes to allow for reverse transcription, followed by 95ºC for 5 minutes to inactivate the reverse transcriptase. cDNA was stored at -20ºC.

2.10.2 miRNA expression

mRNA was reverse transcribed to cDNA for the detection of miRNA using specific stem-loop reverse transcription primers as per the Taqman miRNA Reverse Transcription kit (Applied Biosystems, Paisley, UK). Briefly, a 7.5 µL reaction was prepared containing: 1 mM dNTP mix (0.25 mM each), 1x RT buffer, 1x RT primer, 0.25 U/µL RNase inhibitor, 3.3 U/µL multiscribe reverse transcriptase and 2.5 µL of total RNA diluted to 2 ng/µL. The cycling conditions used were as follows: 16ºC for 30 minutes to anneal, 42ºC for 30 minutes to allow for reverse transcription to occur, followed by 95ºC for 5 minutes to inactivate the reverse transcriptase. cDNA was stored at -20ºC.

2.11 TaqMan® quantitative real-time polymerase chain reaction (qRT-PCR)

TaqMan® qRT-PCR was used to quantify relative expression levels of genes or miRNAs of interest. TaqMan® assays contain forward and reverse primers specific to the target DNA sequence between which a probe can anneal. The probe is labelled with a reporter fluorophore at the 5' end and a non fluorescent quencher at the 3' end, and when intact the quencher suppresses any fluorescence emitted by the reporter. In the presence of the target sequence the probe anneals and is then cleaved upon amplification of the target sequence via forward and reverse primers. This results in separation of the quencher from the reporter and therefore a detectable increase in fluorescence. Further reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the concentration of the target product produced. Data is acquired whilst PCR is in the exponential phase and is measured when the reporter dye emission reaches a threshold, known as the cycle threshold (Ct). In all experiments a housekeeping gene is used, the expression of which remains stable to correct for any errors in RNA content. Results are shown relative quantification (RQ) to the experimental control using the $-2^{\Delta\Delta Ct}$ method as described by Livak and Schmittgen (Livak and Schmittgen, 2001).

2.11.1 Gene expression

For gene expression, q-RT-PCR was performed using inventoried TaqMan gene expression assays (Table 2.2). Expression of each gene was normalised to the housekeeping gene GAPDH (in experiments where GAPDH was the gene of interest, GAPDH expression was normalised to the housekeeping gene 18s). A reaction mixture of 12.5 µL was prepared for each sample containing 6.25 µL TaqMan Universal MasterMix II, 0.625 µL probe, 3.125 µL RNAse free water and 2.5 µL cDNA, and then added in technical duplicate to a 384 well plate. For each probe tested the negative reverse transcription control was run in addition to a water only control. qRT-PCR was performed in simplex using the 7900HT sequence detection system (Applied Biosystems) using the following cycling conditions: 10 minutes at 95ºC for enzyme activation, 40 cycles of 15 seconds at

95ºC for denaturing of cDNA and then 60 sec at 60ºC for primer and probe annealing, and primer extension.

2.11.2 miRNA expression

For miRNA expression, qRT-PCR was performed using miR probes complementary to the mature miRNA sequence (Table 2.3). Expression of each miRNA was normalised to the housekeeper RNU48. A reaction mix of 10 µL was prepared for each sample containing 5 µL of TaqMan Universal MasterMix II, 0.5 µL probe, 3.835 µL RNAse free water and 0.67 µL of miRNA RT product, and then added to a 384 well plate in technical duplicate. Controls were performed as above and the experiment performed using the 79000HT sequence detection system as in section 2.11.1.

Table 2.2 List of TaqMan Gene Expression Assays used in qRT-PCR

Gene	Assay ID	RefSeq Gene ID
AT_1R	Hs00258938_m1	NM_004835.4
AT_2R	Hs01564134_g1	NM_000686.4
Mas	Hs00267157_s1	NM_002377.2
PTEN	Hs02621230_s1	NM_000314.4
MCP-1	Hs00234140_m1	NM_002982.3
RASA1	Hs00243115_m1	NM_002890.2
MMP2	Hs01548727_m1	NM_001127891.1
MMP9	Hs00234579_m1	NM_004994.2
DICER1	Hs01651834_g1	NR_015415.1
GAPDH	Hs02758991_g1	NM_001256799.1
18s	Hs03003631_g1	

Table 2.3 List of TaqMan miRNA Expression Assays used in qRT-PCR

miRNA	Assay ID	MIRBASE ID
hsa-miR-132	000457	hsa-miR-132-3p
hsa-miR-212	000515	hsa-miR-212-3p
RNU48	001006	NR_002745 (NCBI accession)

2.12 Protein extraction

To investigate early changes in protein activity or expression in response to peptide stimulation, HSVSMC were plated at $3x10^5$ cells/well in 6 well plates and incubated at 37ºC in the presence of 5% CO_2 overnight. Cells were then serum starved as described in section 2.5. The media was then changed to fresh serum free media and cells stimulated with Ang II alone or in combination with Ang-(1-7) or Ang-(1-9) (all peptides 200 nM) for 5, 15, 30 or 60 minutes. Cells in serum free media alone or complete growth medium were used as control samples. Following stimulation, cells were washed using PBS and lysed for protein extraction.

Total protein was extracted from cells grown in 6 well plates using 300 µL radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8) containing a 1x dilution of commercially available Complete Protease Inhibitor Cocktail (Roche Diagnostics, West Sussex, UK) and Phosphatase Inhibitor Cocktail (Sigma, Poole, UK). Cells were scraped using a cell scraper and frozen at -20°C overnight. The following day cells were thawed and then agitated on ice for 45 minutes at 4°C. Cell lysates were collected in a sterile microcentrifure tube and centrifuged at maximum speed at 4°C for 10 minutes. The supernatant containing protein was then transferred to a fresh microcentrifuge tube and stored at -20°C for short-term storage or -80°C for long-term storage.

2.12.1 Protein quantification

Protein concentration was determined using bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, USA), according to manufacturer's instructions. This assay relies on a temperature dependent reduction of Cu^{2+} ions by protein peptide bonds, which then is chelated by the BCA to generate a purple coloured product. The absorbance of the developed colour is proportional to the protein content of the sample.

Briefly, a standard curve was generated using the following bovine serum albumin (BSA) dilutions: 2000 µg/mL, 1500 µg/mL, 2000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 25 µg/mL. Working reagent, which contains

copper sulphate and BCA, was prepared by mixing reagent A and reagent B at a ratio of 50:1, respectively. In a clear well 96 well plate, 200 µL of working reagent was added to 25 µL of each standard or sample in duplicate and the plate was covered to protect from light and incubated at 37ºC for 30 minutes. Absorbance was measured at 560 nm using the Wallac 1420 Victor^2 plate reader (Wallac). Protein concentration was calculated using the linear equation based on the standard curve generated from the BCA protein standards.

2.13 Western immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting was performed to detect phosphorylation and expression of specific proteins.

First, 15 µg of protein lysate was mixed with 4x LDS Sample Loading buffer (Pierce) supplemented with 2% (v/v) ß-mercaptoethanol, and then heated at 95°C to denature the protein. Samples were fractioned via SDS-PAGE using 10% polyacrylamide gels which were either purchased precast (10% Mini-PROTEAN® TGX™ Precast Gel; BioRAD, Hemel Hempstead, UK) or prepared within the laboratory (consisting of a non-restrictive 4% stacking gel containing 13.3% (v/v) N,N'-methylene-bis-acrylamide (polyacrylamide 30%), 25% tris(hydroxymethyl)aminomethane) (Tris) pH 6.8 (3.75 mM), 0.1% (v/v) SDS, 1% ammonium persulphate (APS) and 0.1% Tetramethylethylenediamine (TEMED) and a 10% resolving gel containing 33.3% (v/v) of polyacrylamide 30%, 25% of Tris pH 8.8 (11.25 mM), 0.1% (v/v) SDS, 1% APS and 0.1% TEMED). Samples and 10 µL of rainbow ladder (RPE 800 NE Amersham Bioscience UK Ltd, Buckingham, UK), a marker of protein size, were loaded to each well.

Gels were electrophoresed in running buffer (25 mM Tris, 0.2 M glycine, 0.1% (v/v) SDS) at 80 V till samples left the well (and stacking gel of manually prepared gels), then switched to 120 V until the dye front was at the bottom of the gel.

Proteins were transferred onto methanol charged Hybond-P membrane (GE Life Sciences, Buckingham, UK) overnight at 4°C at 90 mA in transfer buffer (25mM Tris, 0.2 M glycine, 0.1% (v/v) SDS, 20% (v/v) methanol). Membranes were then

blocked in 5% (w/v) BSA in TBS-T (150 mM w/v NaCl, 50 mM w/vTris, 0.1% v/v Tween-20) (blocking buffer) for at least 2 hours at room temperature with constant shaking. Membranes were then incubated with the primary antibody diluted in blocking buffer as outlined in Table 2.4, overnight with shaking at 4°C. The following day membranes were washed 3 times in blocking buffer, 5 minutes each wash, at room temperature prior to incubation with the secondary antibody. Swine anti-rabbit horse radish peroxidase (HRP) secondary antibody (DAKO, Cambridge, UK) was used at a 1:1000 dilution in blocking buffer and incubated with membranes for 1 hour at room temperature, with shaking. Membranes were then washed 3 times in blocking buffer, followed by 3 washes in TBS-T; all washes 15 minutes at room temperature. Protein bands were visualised using Amersham Enhanced Chemiluminescence (ECL) western blotting detection reagents (GE Life Sciences) as per manufacturer's instructions. Briefly equal volumes of reagent A and reagent B were mixed, added to the membranes and incubated for 5 minutes at room temperature. Excess ECL was removed from the membranes and Kodak general purpose medical X-ray film was exposed for varying lengths of time and then developed using a Kodak X-Omat 1000 developer.

In all cases the membrane was incubated with a primary antibody to detect the phosphorylated protein first, and then stripped by incubation at 50°C for 30 minutes in stripping buffer (62.5 mM Tris pH 6.8, 2% (v/v) SDS, 100mM 2-mercaptoethanol). Membranes were washed 3 times, 10 minutes each wash, then incubated in blocking buffer for at least 2 hours and re-probed using a primary antibody for GAPDH for normalisation purposes.

Films were scanned using Molecular Imager Chemidoc XRS+ System and the band intensity quantified using densitometry with Quantity One software. Bands were normalised to GAPDH loading control signal and presented as a ratio of the protein:GAPDH signal.

Table 2.4 List of primary antibodies used for Western Blotting

Protein	Host species	Molecular Weight (kDa)	Dilution	Company (Catalogue number) *(clone ID)*
Phospho ERK1/2	Rabbit	42, 44	1:1000	CST (9101s)
Akt	Rabbit	60	1:1000	CST (4691) *(C67E7)*
GAPDH	Rabbit	37	1:1000	CST (2118s) *(14c10)*

2.14 Detection of cellular nitric oxide release

Release of NO was detected from cells using 4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate (DAF-FM diacetate, Life Technologies), a non fluorescent compound that reacts with NO to form a fluorescent benzotriazole which is detectable upon excitation at 488nm. DAF-FM diacetate enters the cell via passive diffusion and is then deacetylated by intracellular esterases to become DAF-FM, the compound that reacts with NO.

2.14.1 NO release from HSVEC

HSVEC were seeded at $3x10^5$ cells/well in 6 well plates containing glass coverslips (22 mm diameter, #0 thickness) coated with Poly-l-lysine and incubated at 37ºC with 5% CO_2 overnight. To diminish basal NO signalling cells were starved in phenol free, serum free medium (DMEM No Phenol Red, 100 I.U/mL penicillin and 100 µg/mL streptomycin) for 2 hours at 37ºC. Cells were then incubated for 30 minutes at 37ºC in serum free, phenol free medium containing 5 µM DAF-FM diacetate. The media was then refreshed and cells stimulated with Ang-(1-9) (0.2 µM or 1 µM) or PD123, 319 (500 nM), alone or in combination for 15 minutes. When Ang-(1-9) and PD213,319 were used in combination, PD123,319 was was incubated with cells for 5 minutes prior to Ang-(1-9) stimulation. Flourescent images were obtained using a Nikon TE2000-E inverted microscope (Nikon Instruments, Melville, NY) excited at 488 nm which was equipped with 63x oil immersion Plan Fluor lens and a cooled digital CoolSNAPHQ charge-coupled device camera (Photometrics, Tucson AZ, USA). Images were taken of at least 5 fields of view per coverslip and exported into Metamorph (version 7.7), then Image J for final image processing.

2.14.2 NO release from transfected CHO cells

CHO cells were cultured as described in section 2.3, and seeded at $3x10^5$ cells/well in 6 well plates containing glass coverslips (22mm diameter, #0 thickness) coated with Poly-l-lysine, then incubated at 37ºC with 5% CO_2 overnight. To assess the involvement of the AT_2R in Ang-(1-9) mediated NO release, cells were transfected with plasmid containing the human AT_2R gene (generated by Daniel C. Villela, Federal University of Minas Gerais, Brazil) using Lipofectamine 2000 as per manufacturer's instructions. Briefly, for each well, 10

µL lipofectamine 2000 was diluted in 250 µL Opti-MEM® and incubated for 5 minutes at room temperature. Plasmid DNA (2 µg) was then diluted in 250 µL Opti-MEM®, mixed with the diluted Lipofectamine 2000 reagent and incubated for 20 minutes at room temperature. The DNA/Lipofectamine 2000 complexes were then added to 1.5 mL cell culture media and the total volume added to the cells. Untransfected cells were used as a control and were incubated with 5 µL Opti-MEM®, 500 µL Opti-MEM® and 1.5 mL culture media. Following 24 hours incubation cells were washed in PBS and incubated with 5 µM DAF-FM diacetate as described in section 2.14.1. Cells were then stimulated with Ang-(1-9), PD123,319 (as described in section 2.14.1) or the AT_2R agonist compound 21 (1 µM). Fluorescent images were then obtained using a Zeiss LSM 510 Meta laser-scanning confocal microscope excited at 488 nm with an argon-ion laser (63x oil-immersion lens; Carl Zeiss, Oberkochen, Germany). Images were taken of at least 4 fields of view per coverslip and exported to Image J software for image processing.

2.15 Small vessel wire myography

Wire myography experiments using vessels isolated from $AT_2R^{-/-}$ mice were performed at the Federal University of Minas Gerais. Krebs-Henseleit buffer (Krebs buffer) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4, 11 mM glucose and 2.5 mM $CaCl_2$ was prepared and gassed continuously through the experiment with 95% O_2. Male $AT_2R^{-/-}$ mice of 8-10 weeks old were sacrificed by decapitation, and the aorta and mesenteric arteries immediately excised and placed in ice cold Krebs buffer. All vessels were cleaned of any fat and connective tissue, and if required, denuded of the endothelial layer by gentle rubbing. The vessels were then cut into 2 mm segments and mounted on two stainless steel 40 µm stainless steel wires in a 4 channel small vessel wire myograph (Danish Myo Technology, Aarhus, Denmark), with one of the wires connected to a force transducer and the other an adjustable jaw (Figure 2.1).

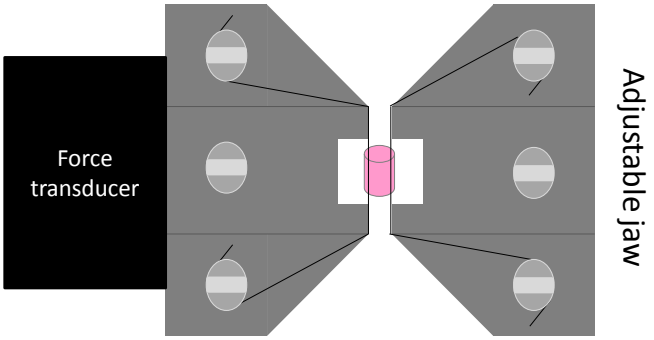

Figure 2.1 Schematic overview of a vessel segment mounted in a small vessel wire myograph
Vessel segments were mounted on two 40 µm stainless steel wires with one connected to a force transducer to record changes in tension of exerted by the artery. The other wire was connected to an adjustable jaw to allow for application of tension to the vessel.

Vessels were gradually warmed to 37ºC in Krebs buffer at resting tension. Due to the small diameter of mesenteric arteries a normalization step was then performed to calculate the internal circumference of the vessel and thereby determine the optimal active tension required. The internal circumference of the vessel was calculated based on the tension exerted by the vessel at rest and under transmural pressure of 100 mmHg; only vessels found to have an internal circumference of >150 µm were used. The optimal active tension required was calculated as the tension required to stretch the vessel to 90% of its internal circumference. A predetermined active tension of 0.6 g was applied to aortic rings. Active tension was applied to all vessels for 30 minutes. To establish the viability of each segment and to sensitise the vessel prior to further pharmacological stimulation, the contractile response to 0.6 mM KCl was assessed. Once a stable plateau was reached the vessels were washed in Krebs buffer till the tension returned to the applied active tension. This was then repeated twice, with 30 minutes between each repeat. To assess the presence of functional endothelium vessels were contracted to 0.1 µM phenylephrine (Phe) until a stable plateau was reached and then treated with 10 µM acetylcholine. Endothelium was deemed viable if a relaxation of at least 70% was observed in response to acetylcholine. Vessels were then washed at least 3 times in fresh Krebs buffer till the applied active tension was achieved and incubated for 30 minutes prior to beginning each experimental protocol.

To assess the effects of Ang-(1-9) on vascular tone, vessels were first contracted to 0.1 µM Phe until a stable plateau was achieved. Cumulative concentration

response curves were then performed to Ang-(1-9) using a range of concentrations ($1x10^{-10}$ M to $1x10^{-6}$ M). This concentration range was chosen based on previous experiments conducted in Prof. Robson Santos laboratory and published reports (Ocaranza *et al.*, 2014). In experiments where antagonists were used, $1x10^{-6}$M A779 or PD123, 319 was added to the organ bath 5 minutes prior to the first stimulation with Ang-(1-9). Lab Chart™ 5 Pro software (ADInstruments, Chalgrove, UK) was used to record and measure all responses. Data were expressed as a percentage of relaxation of the Phe induced contraction.

2.16 *In vivo* experimentation

All animal experiments were performed in accordance with the Animals Scientific Procedure Act 1986 under the project license, 60/4114, held by Dr Simon Kennedy (University of Glasgow, UK) and the personal license, 60/12207. Mice were housed at the Central Research Facility at the University of Glasgow and maintained on 12 hour cycles of light and dark, at ambient temperature. Mice were fed a standard chow diet and water provided *ab libitum*.

2.16.1 Mouse carotid artery injury model

Prior to surgery, 8-10 week old male C57BL/6 mice (Harlan, Oxon, UK) received an intraperitoneal injection of the analgesic buprenorphine (0.1 mg/kg) and 2.5 mg of the antiplatelet therapy dipyridamole (Persantin). Sterile saline (0.5 mL, subcutaneous injection) was administered to prevent dehydration during the surgical procedure. General anaesthesia was induced by 3% (v/v) isoflurane supplemented with oxygen (0.5 L/minute) and maintained at 1.5% (v/v) isoflurane via a face mask throughout the procedure. Depth of anaesthesia was monitored throughout the surgery by assessment of hind limb reflex. Carotid artery injury was performed following an adapted method described by Lindner *et al* (Lindner *et al.*, 1993). An incision was made in the ventral side of the neck and blunt dissection of the muscle and connective tissue was performed to expose the left common carotid artery. The vagus nerve was detached from the artery and two silk ligatures (size 6.0, Fine Science Tools, Heidelberg, Germany) were positioned at the proximal and distal ends of the vessel. The distal ligature was tightly tied and an arterial clip (Fine Science Tools, Heidelberg, Germany)

was positioned at the most proximal end to temporarily occlude blood flow. A small incision was made in the artery and a piece of modified flexible nylon wire, adapted by melting the end to create a blunt spherical tip, was inserted into the incision site and held loosely in place by tightening of the proximal ligature. The arterial clip was removed and the wire was advanced, while rotating, down the carotid artery into the thoracic aorta. This was repeated 3 times to ensure the removal of the endothelium. The nylon wire was removed, the artery clip reapplied and the proximal ligature was tied and secured just below the incision site. Sham operated mice were used as controls, these animals were subjected to the same procedure except the ligatures were not tied, only secured in place, and blood flow occluded for 5 minutes (the approximate time required to perform the wire injury) using the distal ligature and an artery clip at the proximal end. No incision was made in the artery and the wire was not inserted. The ligatures were removed prior to wound closure. A continuous line of subcutaneous sutures (size 5.0, Vircyl, Ethicon, Edinburgh, UK) was used to close the skin incision. For post-operative care the mice were transferred to a heating mat and maintained at 37^{o}C overnight.

2.16.2 Osmotic mini pump implantation

Ang-(1-7) and Ang-(1-9), alone or alongside A779 or PD123,319 (Ang-(1-9) only) were delivered subcutaneously via osmotic mini pump (Model 2004, Alzet, CA, USA) and the effects on neointimal formation assessed. Mini pumps filled with water were implanted into control animals. Osmotic mini pumps operate due to a difference in osmotic pressure between a compartment within the pump known as the salt sleeve and the tissue where the pump has been implanted. The salt sleeve has a high molarity and this causes water to enter the pump via a semipermeable membrane which forms the outer surface of the pump. As the water enters the salt sleeve, it compresses the reservoir containing the peptide, causing it to be released at a controlled rate predetermined by the water permeability of the pumps outer membrane (Theeuwes and Yum, 1976).

Osmotic mini pumps secreted Ang-(1-7), Ang-(1-9) and A779 at a concentration of 48 µg/kg/hour and PD123,319 at a concentration of 200 ng/kg/minute, at a rate of 0.25 µL/hour for 4 weeks following implantation. Minipumps were primed by incubation in an isotonic solution at 37^{o}C for 48 hours to ensure immediate

delivery of the peptides following vascular injury. Prior to implantation, the prepared mini pumps were assigned a code by an independent researcher to blind the study. To implant mini pumps a small incision was made in the flank and a subcutaneous pocket created to allow insertion of the pump. The wound site was closed using a continuous line of sutures (size 5.0, Vircyl, Ethicon, Edinburgh, UK).

2.16.3 Pluronic gel studies

To assess the effectiveness of local delivery of Ang-(1-7) or Ang-(1-9), the peptides were applied directly to the carotid artery immediately following injury using Pluronic F127 gel (BASF, Ludwigshafen, Germany). Pluronic F127 is a copolymer that when dissolved in aqueous solution displays the unique characteristic of reverse thermal gelation, as at room temperature Pluronic F127 solution is a viscous liquid which is transformed to a semisolid gel at body temperature (Schmolka, 1972). Therapies can therefore be dissolved in the Pluronic F127 solution when aqueous then be delivered locally to the animal to form a gel based depot of the therapeutic at the site of administration.

Pluronic F127 was made to 22% (w/v) in sterile PBS and stored at 4°C at a liquid state. Immediately before administration, peptides were added to the gel to create a solution of 2.5 µg/µL and kept on ice. Gel only was used as a control. Following completion of wire injury, 100 µL of the pluronic gel was applied to the carotid artery using a pipette. Once it was observed that the pluronic solution had changed from a liquid state to gel state the wound was closed as described in section 2.5.1.

Prior to assessing therapeutic effects of local delivery of Ang-(1-7) or Ang-(1-9), a pilot study was performed to confirm delivery of the peptides to the vessels. To do this, 9 animals received either gel only, Ang-(1-7) or a custom made biotinylated Ang-(1-9) as described above. Three animals per group were sacrificed as described in section 2.16.4 at 7, 14 and 28 days following injury and peptide application. Peptide delivery to the vessel was assessed via immunohistochemistry (IHC), using a specific antibody for Ang-(1-7) and streptavidin for the biotinylated Ang-(1-9) as described in sections 2.17.4 and 2.17.4.2, respectively.

Following this a blinded study was performed to assess the effects of local delivery of Ang-(1-7) and Ang-(1-9) on neointimal formation with 8 animals per group.

2.16.4 Termination of procedure and tissue harvesting

Mice were sacrificed 28 days and transcardially perfused by gravity flow with 0.9% saline to exsanguinate. To ensure that no damage was done to the wire injured vessels, mice were perfused at 120 mmHg to represent physiological conditions. This was achieved by placing the perfusate 1.63 m above the animal, based on the fact that 1 mmHg equals 13.6 mm H_20. For perfusion, animals were firstly deeply anaethetised using 4-5% isoflurane supplemented with oxygen (0.5 L/minute) via face mask. A vertical midline incision was made through the skin and peritoneal cavity to expose the rib cage and internal organs, which were moved to expose the femoral artery of the left leg. The diaphragm was incised and the heart exposed. To begin perfusion, a butterfly needle (23 g) connected to the perfusate was inserted into the left ventricle and held in position using mosquito clips and the left femoral artery cut to allow the blood to drain from the animal. Once the perfusate ran clear the heart was excised to confirm termination of the procedure. Tissues and vessels were collected and stored in 10% (v/v) formalin for 24 hours followed by transfer to 70% ethanol.

2.17 Histology

2.17.1 Tissue fixation

Tissue samples were dehydrated through an ethanol gradient to xylene and finally to paraffin wax using the Shandon Excelsior tissue processor (Thermo Scientific, Leicestershire, UK) in the sequence outlined in Table 2.5.

Following the tissue processing sequence, vessels were held vertically in biopsy cassettes and embedded in paraffin wax using a Shandon Histocenter 3 (ThermoFisher Scientific, Leicestershire, UK). Paraffin blocks were cut at 4 µm using a Leica microtome (ThermoFisher Scientific, Leicestershire, UK) to expose transverse sections of the embedded vessel and transferred to a 45^{o}C water bath where they were mounted onto slides and then baked overnight at 60^{o}C.

Table 2.5- Tissue processing sequence for embedding tissues in paraffin

Solution	Incubation Period
70% Ethanol	30 min
95% Ethanol	30 min
100% Ethanol	30 min
100% Ethanol	30 min
100% Ethanol	45 min
100% Ethanol	45 min
100% Ethanol	60 min
Xylene	30 min
Xylene	30 min
Xylene	30 min
Paraffin wax	30 min
Paraffin wax	45 min
Paraffin wax	45 min

2.17.2 Elastin staining

Elastin van Gieson (EVG) staining was performed to visualize the elastic lamina and facilitate neointimal measurements. Briefly, paraffin was removed from the tissue sections by immersion in HistoClear (Fisher Scientific Ltd, Leicestershire, UK) for 10 minutes (changed to fresh HistoClear after 5 minutes) and rehydrated through an alcohol gradient of 100%, 95% and 70% ethanol for 5 minutes each and washed in distilled water for 5 minutes. Tissue sections were oxidized by incubation with 0.5% (w/v) potassium permanganate for 10 minutes, washed in running tap water for 3 minutes followed by rinsing in dH_2O for 30 sec. Sections were decolourised by placing in 1% (w/v) oxalic acid for 10 minutes, running tap water for 2 minutes and dH_2O for 30 sec. Next, sections were immersed in 70% ethanol, and incubated for 3 hours with Miller's elastin stain (VWR Chemicals, Leicestershire, UK) which stains elastin fibres black. To remove excess stain from the slide, sections were washed by dipping 7 times in 70% ethanol then dH_2O for 5 minutes. Sections were counterstained with Van Gieson solution (0.1% acid fuchsin in saturated picric acid), which stains collagen red and muscle and cytoplasm yellow, for 10 minutes. Slides were dried at 60^{o}C for 30 minutes and then rinsed in 100% ethanol for 10 minutes (changed to fresh ethanol after 5 minutes). Slides were immersed in HistoClear for 10 minutes (changed to fresh HistoClear after 5 minutes) before being mounted with glass cover slips using DPX non-aqueous mounting medium. Sections were photographed using QCapture Pro 6.0 software and analysed using Image-Pro® Analyser 7.0 software (Media Cybernetics, Marlow, UK).

2.17.3 Picrosirius red staining

Picrosirius red staining was performed in order to visualise the collagen content of the injured vessels. Tissue sections were deparaffinised and rehydrated as described in section 2.17.2 and then placed in distilled water for 5 minutes. Weigert's Haematoxylin was prepared by mixing equal parts Solution A (1% (v/v) haematoxylin I ethanol) and Solution B (ferric chloride 1.2% (w/v) and 1% (v/v) hydrochloric acid) and used to stain the nuclei for 10 minutes at room temperature. Slides were washed in running tap water for 10 minutes and incubated in 0.1% picrosirius red solution (0.1% (w/v) Sirius red F3B in saturated picric acid) for 90 minutes under dark conditions at room temperature. Slides

were then washed in acidified water (0.01N HCL (v/v) in distilled water) twice for 5 minutes each wash. Any remaining moisture was removed by vigorous shaking and then slides were dehydrated through an alcohol gradient of 70%, 90% and 100% ethanol for 5 minutes each. Slides were then immersed in Histoclear for 5 minutes and mounted as described in section 2.17.3.

2.17.4 Immunohistochemistry

Tissue sections were deparaffinised and rehydrated as described in section 2.17.2 and then washed in running tap water for 5 minutes. Following rehydration heat induced antigen retrieval was performed by incubating sections in boiling 10 mM sodium citrate buffer pH 6.0 for 20 minutes. Sections were cooled by incubation at room temperature for 30 minutes followed by immersion in running tap water for 10 minutes. To block endogenous peroxidase activity slides were incubated in 20% (v/v) H_2O_2 in methanol for 30 minutes. Slides were then washed twice, 5 minutes each, in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). To reduce non-specific background staining sections were blocked using 20% (v/v) of the appropriate serum (all Vector Labs, Peterborough, UK) (Table 2.6) in PBS for 30 minutes at room temperature in a humidified chamber. Sections were then incubated in a humidified chamber with the primary antibody or with an equal concentration of isotype matched IgG non immune control (for primary antibodies whose host species is rabbit, normal rabbit IgG (Invitrogen, product number 10500c) was used, and for antibodies whose host species is rat, normal rat IgG (Invitrogen, product number 10700) was used) diluted in 2% blocking serum for 1 hour at room temperature or 4ºC overnight (Table 2.6). This was the case for all antibodies outlined in Table 2.6 with the exception of the Ang-(1-7) antibody as the concentration of this antibody was not provided by the manufacturer; therefore the correct concentration of IgG could not be calculated. In place of an IgG control, sections were incubated with PBS. Following incubation with the primary antibody sections were washed three times, 5 minutes each, in PBS. Sections were then incubated with the appropriate biotinylated secondary antibody diluted in 2% blocking buffer for 1 hour at room temperature in a humidified chamber as outlined in Table 2.7. Sections were then washed three times, 5 minutes each, in PBS followed by incubation with 1:200 (v/v) ExtraVadin®-peroxidase (Sigma) to facilitate binding of avidin (contained within the ExtraAvadin®-peroxidase) to

the biotinylated secondary antibody. Sections were then washed three times, 5 minutes each, in PBS and the antigen detected using 3, 3'-diaminobenzidine (DAB) chomagen (Vector Laboratories) which produces a brown precipitate in the presence of peroxidase enzyme. Sections were incubated with DAB for 2-5 minutes and placed in distilled water to neutralise the reaction. The nuclei were then counterstained with Mayer's haematoxylin for 1 minute. Slides were then washed in running water for 5 minutes and then dehydrated and mounted as described in section 2.17.2. Sections were photographed using QCapture Pro 6.0 software and analysed using Image J software.

2.17.4.1 α-SMA

Detection of α-SMA was performed using the VECTASTAIN® Elite ABC Kit (Universal) (Vector Labs) according to manufacturer's instructions. Sections were deparaffinised and rehydrated as described in section 2.17.2 and then washed in distilled water for 5 minutes. Endogenous peroxidase activity was quenched by incubating slides in 20% hydrogen peroxidase for 30 minutes at room temperature. The sections were then blocked for 1 hr at room temperature in a humidified chamber with 1% (v/v) blocking serum (normal horse serum) in PBS. The sections were then incubated with primary antibody (Table 2.6) or an equal concentration of isotype matched rabbit IgG non immune control (Invitrogen) diluted in PBS 2% normal horse serum, for 1 hour at room temperature in a humidified chamber. Sections were washed three times, 5 minutes each, to remove any residual unbound primary antibody. Biotinylated horse anti-rabbit secondary antibody diluted 1 in 50 in 2% blocking serum was incubated with sections for 30 minutes at room temperature. VECTASTAIN® Elite avidin biotinylated enzyme complex (ABC), which binds to the biotin of the secondary antibody, was prepared by adding 2 drops reagent A and 2 drops reagent B to 5 mL PBS and incubated for 30 minutes. Sections were washed three times, 5 minutes each, and then incubated with the ABC complex for 30 minutes at room temperature. DAB detection and counterstaining of nuclei was performed as described in section 2.17.4 and then tissue sections were rehydrated and mounted as described in sections 2.17.2.

2.17.4.2 Detection of biotinylated Ang-(1-9)

A custom made biotinylated version of Ang-(1-9) was used to confirm delivery of the peptides to the vessel via Pluronic Gel. As the biotin was already bound to the peptide no primary or secondary antibodies were required to detect peptide expression. To visualise biotin-labelled Ang-(1-9) sections were first deparaffinised and rehydrated as described in section 2.16.2. Heat induced antigen retrieval and quenching of endogenous peroxidase was performed as described in section 2.16.4. Sections were then incubated with ExtrAvadin peroxidase, the peptide detected using DAB and nuclei counterstained as described in section 2.17.4. Finally, tissue sections were then rehydrated and mounted as described in section 2.17.2.

2.18 Statistical analysis

Data are shown as mean ± standard error of the mean (S.E.M) and qRT-PCR data expressed as RQ ± RQmax of experiments which were repeated on at least 3 seperate occasions using cells isolated from different patient samples, unless otherwise stated. Within each experimental repeat, migration assays included 9 technical replicates, proliferation assays included 5 technical replicates, and western immunoblotting included 2 technical replicates. For each experiment the mean of the technical replicates was calculated and this value was used to generate the mean and S.E.M of the biological replicates. Within each experimental repeat, qRT-PCR included 3 technical replicates. The mean dCT of the technical replicates was calculated for each experiment and this value was used to generate the RQ and RQmax of the biological replicates.

A power calculation was performed to assess the required group size for the *in vivo* studies. Using data with analogous experimental protocols, 8 mice per group gives 80% power at 5% significance level to detect a difference in neointimal area of at least 7.2 x 10^3 μm^2 assuming a within group standard deviation of 3.55 x 10^3 μm^2 (Zimmerman *et al,* 2004). Therefore, with the exception of optimisation of peptide delivery via Pluronic gel, all *in vivo* experiments were performed with 8 animals per group and studies were blinded in advance by an independent researcher.

Statistical analysis was performed using GraphPad Prism software (California, USA). Unpaired students t-test was used when comparing two experimental groups. When more than two groups were compared, one way ANOVA (analysis of variance) was performed with Tukeys post-hoc correction for multiple comparisons applied. In all cases a p value of <0.05 was accepted as statistical significance.

Table 2.6 List of primary antibodies used in immunohistochemistry

Protein	Host species	Concentration	Dilution	Incubation Period	Company (Catalogue number, *clone ID*)	Blocking Serum
α-SMA	Rabbit	0. 2 mg/mL	1:75	1 hr at room temperature	Abcam (ab5694)	Normal Horse
CD31	Rat	0.2 mg/mL	1:20	Overnight at 4˚C	Dianova (DIA-310, *SZ31*)	Normal rabbit
MAC-2	Rat	1 mg/mL	1:5000	Overnight at 4˚C	Cedarlane (CL8942AP, *M3/38*)	Normal rabbit
PCNA	Rabbit	0.2 mg/mL	1:200	Overnight at 4˚C	Abcam, (Ab2426)	Normal goat
Active caspase	Rabbit	0.2 mg/mL	1:50	Overnight at 4˚C	Abcam (ab2302)	Normal goat
Ang-(1-7)	Rabbit	n/a	1:100	1 hr at room temperature	Phoenix (H-002-24)	Normal goat

Table 2.7Secondary antibodies used in immunohistochemistry

Protein	Secondary Antibody	Concentration	Dilution	Company(Catalogue number)
α-SMA	Biotinylated Universal Antibody Horse anti rabbit IgG	1.05 mg/mL	1:50	Vector Labs (BA-1400)
CD31	Biotinylated rabbit anti rat IgG	1.5 mg/mL	1:200	Vector Labs (BA-4001)
MAC-2	Biotinylated rabbit anti rat IgG	1.5 mg/mL	1:200	Vector Labs (BA-4001)
PCNA	Biotinylated goat anti rabbit IgG	1.5 mg/mL	1:200	Vector Labs (BA-1000)
Active caspase	Biotinylated goat anti rabbit IgG	1.5 mg/mL	1:200	Vector Labs (BA-1000)
Ang-(1-7)	Biotinylated goat anti rabbit IgG	1.5 mg/mL	1:200	Vector Labs (BA-1000)

Chapter 3

The effects of Ang-(1-7) and Ang-(1-9) on HSVSMC proliferation and migration

3.1 Introduction

Within the vasculature, VSMC in the medial layer normally exist in a quiescent contractile state, responding to various vasoactive substances such as Ang II, endothelin-1 and NO to regulate vascular tone. However, during states of vascular disease or injury, medial VSMC undergo phenotypic switching from the quiescent state to a synthetic state (Campbell and Campbell, 1994). This occurs in response to numerous stimuli involved in the pathogenesis of vascular remodelling, including growth factors such as Ang II and PDGF and pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (reviewed by (Schwartz *et al.*, 1990, Touyz and Schiffrin, 2000, Ross, 1993). The synthetic VSMC largely contribute to the progression and development of atherosclerosis and neointimal formation following stent implantation and vein grafting due to their ability to migrate and proliferate more readily than their quiescent counterparts.

Proliferation of synthetic VSMC occurs through a regulated series of cell-cycle events (Pardee, 1989, Sherr, 1994a, Elledge, 1996). Under normal conditions quiescent VSMC are maintained in G0. After vessel injury, VSMC enter a gap phase (G1) in the cell cycle, leading to the production of various factors necessary for DNA replication in the subsequent synthetic phase (S). After S phase, the cells enter another gap phase (G2), when proteins are synthesized for mitosis (M phase). Restriction points at the G1-to-S and G2-to-M junctions ensure orderly progression through the cell cycle (Pardee, 1989, Sherr, 1994a, Elledge, 1996). Growth factors, such as Ang II, PDGF, and basic fibroblast growth factor (bFGF), stimulate cells to enter the cell cycle and propel them to reach the restriction point in the late G1 phase. Cell cycle progression from the G2 phase to the M phase does not require further growth factor stimulation. On binding to their respective cell surface tyrosine kinase receptors, growth factors trigger cell cycle entry by transactivating nuclear factors such as *c-fos* and *c-myc* (Marx *et al.*, 2011). These nuclear factors act as transcriptional factors which coordinate the expression and activity of a range regulatory proteins, including cyclins and cyclin-dependent kinases (CDK) (Sherr *et al.*, 1994). Cyclins and their respective CDK form distinct complexes and are positive regulators of cell cycle progression. For example, CDK phosphorylation of the retinoblastoma gene product (Rb) at the R point at G1-S junction is an important step in the progression through the cell cycle to enable Rb to bind and inactivate the

transcription factor E2F to maintaining the cell in a quiescent state (Sherr, 1994b). Phosphorylation of Rb in late G1 releases E2F, which in turn enhances the expression of genes encoding regulatory proteins necessary for cell cycle progression through S, G2, and M phases (DeGregori *et al.*, 1995).

Similarly, the process of VSMC migration is well defined and has been reviewed extensively (Gerthoffer, 2007, Schwartz, 1997, Abedi and Zachary, 1995). In the non-injured vessel, VSMC are non-migratory because of a combination of several factors including the relative absence of stimulatory factors, their quiescence from a proliferative standpoint, and because the matrix is highly adhesive (Gerthoffer, 2007). There are many pro-migratory molecules, including peptide growth factors, [such as Ang II, bFGF, and PDGF (Sato *et al.*, 1991, Grotendorst *et al.*, 1981, Jackson *et al.*, 1993, Bell and Madri, 1990)], cytokines (such as IL-1β, IL-6 and TNF-α), and ECM components, [such as collagen, fibronectin and osteopontin (Sibinga *et al.*, 1997, Nelson *et al.*, 1996, Liaw *et al.*, 1994)]. Blood flow, sheer stress, and matrix stiffness can also affect the migration of VSMC (Ward *et al.*, 2001, Li *et al.*, 2003, Peyton and Putnam, 2005). VSMC migration begins with the stimulation of cell surface receptors that activate signal transduction pathways, triggering remodelling of the cytoskeleton, changes in their adhesiveness to the matrix, and activation of the motor proteins (Gerthoffer, 2007). VSMC extend lamellipodia toward the stimulus through actin polymerization (Gerthoffer, 2007). Focal contacts form just behind the leading edge to increase adhesion of the cell membrane to the matrix, and degradation of these focal contacts at the trailing edge is necessary for release of the cell from the matrix and therefore cell migration. The actin cytoskeleton is regulated by numerous signalling pathways and molecules, including trimeric G proteins, small G proteins, lipid kinases, Ca^{2+}-dependent kinases, Rho kinase, and MAPK (Gerthoffer, 2007, Graf *et al.*, 1997, Noma *et al.*, 2006).

Ang II is a powerful mitogen and is known to play an important role in VSMC proliferation and migration in vascular remodelling. One of the most well documented pathways involved in Ang II mediated VSMC migration and proliferation is activation of MAPK signalling, partly due to the fact that Ang II activates the MAPK signalling pathway at various intracellular levels. For example, Ang II directly stimulates tyrosine and threonine phosphorylation of ERK1/2, JNK and p38 MAPK in cultured VSMC from rat, mouse, rabbit and human

(Schieffer *et al.*, 1996, Epstein *et al.*, 1997, Touyz *et al.*, 1999a, Xu *et al.*, 1996, Lee *et al.*, 2007, Kyaw *et al.*, 2004, Yang *et al.*, 2005, Mugabe *et al.*, 2010). Furthermore, Ang II stimulates phosphorylation of various signalling molecules upstream from ERK1/2 such as Ras, Raf, Shc, Src and Pyk2, and it increases activity of MEK kinase (Eguchi *et al.*, 1996, Liao *et al.*, 1996, Griendling *et al.*, 1997, Touyz *et al.*, 1999d). Ang II has also been shown to promote VSMC proliferation and migration through expression of various early response genes such as *c-fos*, *c-jun* and *c-myc* (Naftilan *et al.*, 1989b, Lyall *et al.*, 1992). Expression of these early response genes leads to the production of growth factors including PDGF, EGF, TGF-ß and bFGF that are involved in Ang II mediated-VSMC proliferation and migration [reviewed in (Touyz and Berry, 2002)].

In addition to activation of MAPK signalling, Ang II has also been demonstrated to promote VSMC migration through activation of the FAK-dependent signalling pathway (Leduc and Meloche, 1995). In VSMC, FAK associates with paxillin and talin, and both FAK and paxillin can bind to the cytoplasmic tail of integrins, key molecules involved in attachment of the cell to the ECM (Leduc and Meloche, 1995, Chen *et al.*, 1995). Ang II induced activation of FAK results in its translocation to sites of focal adhesion within the ECM and phosphorylation of paxillin and talin, resulting in VSMC migration (Leduc and Meloche, 1995, Chen *et al.*, 1995). While the upstream signalling pathways involved in Ang II mediated FAK activation are unknown, it has been suggested that Rho GTPases may play an important role (Rozengurt, 1995, Aspenstrom, 1999). Another tyrosine kinase involved in Ang II mediated VSMC migration is Pyk2 (Murasawa *et al.*, 1998). Ang II-mediated stimulation of the AT_1R results in an increase in intracellular calcium concentration, which in turn activates c-Src, leading to activation of Pyk2, which then promotes increased ERK activity, a key process in both VSMC migration and proliferation (Murasawa *et al.*, 1998, Eguchi *et al.*, 1999).

Ang-(1-7) signalling via Mas is widely accepted to oppose the effects of Ang II via the AT_1R, and this is also the case in VSMC proliferation (Freeman *et al.*, 1996, Tallant *et al.*, 1999, Tallant and Clark, 2003, Zhang *et al.*, 2010b). Ang-(1-7) has been shown to exert anti-proliferative effects in VSMC via two main pathways; the first being through inhibition of MAPK signalling pathways (Zhang *et al.*, 2010b, Tallant and Clark, 2003) and the second through release of prostaglandins

such as prostacyclin (PGI2), resulting in increased cAMP levels (Jaiswal *et al.*, 1993b). The first study to identify a direct anti-proliferative role of Ang-(1-7) reported inhibition of proliferation of rat aortic SMC induced by Ang II, FCS or PDGF (Freeman *et al.*, 1996). This anti-proliferative effect was unaltered by antagonism of either the AT_1R or AT_2R and, in the absence of identification of Mas at the time, it was concluded that an unknown receptor was responsible (Freeman *et al.*, 1996). These findings were confirmed in a later study however, it was also established that the effect of Ang-(1-7) was blocked by D-Ala7-Ang-(1-7) (A779), now widely accepted to be a Mas antagonist, suggesting Ang-(1-7) inhibits VSMC proliferation via Mas (Tallant *et al.*, 1999).

Further studies provided a detailed outline of various signalling pathways involved in the anti-proliferative effects of Ang-(1-7) in rat VSMC (Tallant and Clark, 2003). First, Ang-(1-7) blocked Ang II- and PDGF-induced activation of ERK1/2 signalling (Tallant and Clark, 2003). Inhibition of ERK1/2 signalling has also been demonstrated to be important in the anti-proliferative effects of Ang-(1-7) in response to Ang II in mouse VSMC (Zhang *et al.*, 2010b). However, the upstream mechanisms of Ang-(1-7) mediated inhibition of ERK1/2 have still to be established.

Ang-(1-7) has been demonstrated to stimulate PGI2 release from VSMC (Jaiswal *et al.*, 1993a, Jaiswal *et al.*, 1993b, Muthalif *et al.*, 1998, Tallant and Clark, 2003), a prostaglandin which is widely accepted to prevent VSMC proliferation (Uehara *et al.*, 1988, Morisaki *et al.*, 1988). The anti-proliferative effect of Ang-(1-7) in response to Ang II and PDGF was blocked by indomethacin, a cyclo-oxygenase (COX) inhibitor that blocks prostaglandin production, suggesting that COX mediated production of prostaglandins is an important signalling cascade involved in the anti-proliferative effects of Ang-(1-7) in VSMC (Tallant and Clark, 2003, Muthalif *et al.*, 1998).

Furthermore, Ang-(1-7) has been demonstrated to increase the VSMC content of cAMP to block serum-induced VSMC proliferation, an effect blocked by Rp-cAMPS, an inhibitor of the cAMP-dependent protein kinase (Tallant and Clark, 2003). Activated PGI2 receptors on VSMC stimulate adenylate cyclase to increase intracellular cAMP levels (Garg and Hassid, 1989), and as Ang-(1-7) results in an increase in both PGI2 and intracellular cAMP it is possible that these two

signalling events are part of the same pathway. For example, Ang-(1-7) mediated upregulation of PGI2 leads to activation of PGI2 receptors and thereby increased production of cAMP (Tallant and Clark, 2003).

In comparison to its effects on VSMC proliferation, there is less known about the effects of Ang-(1-7) in VSMC migration and the signalling mechanisms involved. An anti-migratory effect for Ang-(1-7) has been demonstrated in rat aortic VSMC, where Ang-(1-7) directly inhibits Ang-II induced VSMC migration (Zhang *et al.*, 2010b). Through the use of the pharmacological antagonists losartan and A779, which block the AT_1R and Mas, respectively, it was confirmed that the anti-migratory effects of Ang-(1-7) were mediated via Mas (Zhang *et al.*, 2010b). Furthermore, it was shown that Ang-(1-7) inhibits Ang II induced ERK1/2 phosphorylation, providing evidence of a potential signalling pathway involved in the effects of Ang-(1-7) in VSMC migration (Zhang *et al.*, 2010b).

Comparatively, there is little known about the role of Ang-(1-9) in the vascular function or remodelling. Ang-(1-9) has been reported to signal via the AT_2R, and while the signalling mechanisms employed by this receptor are currently poorly defined, the AT_2R has been linked to reduced VSMC proliferation *in vitro*. For example, adenoviral-mediated over expression of the AT_2R in rat VSMC resulted in a reduction in Ang II mediated VSMC proliferation via the AT_1R, an effect which was linked to reduced MAPK activity (Nakajima *et al.*, 1995, Stoll *et al.*, 1995). While further work is required to assess the Ang-(1-9)/AT_2R interaction, these findings indicate that the AT_2R may oppose the effects of Ang II in the VSMC.

3.1.1 The role of microRNAs in Ang II-induced VSMC proliferation and migration

In recent years it has also been suggested that Ang II may mediate a number of effects in the cardiovascular system through changes in expression levels of microRNAs (miRNA) (Zhu *et al.*, 2011, Jin *et al.*, 2012, Eskildsen *et al.*, 2013). miRNAs are small non-coding RNA molecules 20~22 nucleotides in length that target the 3′-untranslated region (UTR) of mRNA to negatively regulate gene expression through inhibition of mRNA translation and/or mRNA degradation (van Rooij and Olson, 2007, Bartel, 2009). miRNA biosynthesis and maturation begins

with transcription of the primary miRNA (pri-miRNA) by RNA polymerase II (van Rooij and Olson, 2007). The pri-miRNA is then processed by the Rnase III enzyme Drosha to form the precursor miRNA (pre-miRNA), a ~60-nucleotide stem loop molecule (van Rooij and Olson, 2007, Bartel, 2009). Pre-miRNAs are then transported from the cell nucleus to the cytoplasm via exportin5, and once in the cytoplasm they are cleaved by the endonuclease DICER to produce a miRNA duplex (van Rooij and Olson, 2007, Bartel, 2009). This duplex is then bound to argonaute 2 (Ago 2) and the mature miRNA formed (Meister *et al.*, 2004, Liu *et al.*, 2004b). The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) leading to interaction with target mRNAs, resulting in regulation of a number of genes under a diverse range of patho/physiological conditions (Bartel, 2009). Importantly, various miRNAs have been implicated in the pathogenesis of vascular disease (Robinson and Baker, 2012). For example, miR-143 and miR-145 have been demonstrated to regulate VSMC differentiation and contractility and have been linked to reduced neointimal formation following vascular injury (Cordes *et al.*, 2009, Boettger *et al.*, 2009). Furthermore, miR-21 plays an important role in vascular remodelling and has been demonstrated to be elevated in human, pig and mouse models of vein graft failure (McDonald *et al.*, 2013). Additionally, miR-133 has been shown to negatively regulate VSMC proliferation both *in vitro* and *in vivo* (Torella *et al.*, 2011).

Recently, a role for miRNAs in vascular Ang II signalling has been identified. Through the use of small RNA deep sequencing it was demonstrated that Ang II stimulation of rat aortic VSMC led to increased expression of miR-132 and miR-212 in a time and concentration dependent manner via the AT_1R (Jin *et al.*, 2012). miR-132 and miR-212 have been shown to exist in a 'cluster', characterised by their close proximity to each other in the genome and the fact that they are transcribed together under the regulation of cAMP response element binding protein (CREB), a protein which is itself regulated by Ang II (Neyses *et al.*, 1993, Jeppesen *et al.*, 2011).The miR-132/-212 cluster has additionally been shown to be important in the development of blood vessels, mammary glands and neurons (Anand *et al.*, 2010, Ucar *et al.*, 2010, Wanet *et al.*, 2012). Through the use of various target prediction software packages it was revealed that a number of predicted targets of Ang II-regulated miRNAs within

rat aortic VSMC were involved in proliferation and migration, including phosphatase and tensin homologue (PTEN), MCP-1, p120 Ras GTPase-activating protein 1(RASA1) (Jin *et al.*, 2012). These targets were validated by Jin *et al* in the rat VSMC and it was found that Ang II mediated increase in mirR-132/-212 resulted in decreased expression in PTEN and RASA1, leading to an increase in MCP-1 and phosphorylation of CREB, respectively (Jin *et al.*, 2012). PTEN is a lipid and protein phosphatase which primarily acts to dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PIP3), thereby inhibiting the actions of PI3K (Carracedo and Pandolfi, 2008). This second messenger is important for Akt activation, which promotes cell survival and growth, therefore PTEN activity reduces activation of this pathway leading to reduced cell proliferation (Huang and Kontos, 2002). Overexpression of PTEN has previously been reported to prevent proliferation and migration of VSMC (Huang and Kontos, 2002). MCP-1 is a pro-inflammatory cytokine with a key role in the progression and development of atherosclerosis, largely through promoting migration of sub-endothelial monocytes to the vessel wall and VSMC migration from the media to the neointima (Nelken *et al.*, 1991, Yu *et al.*, 1992, Sung *et al.*, 2001). MCP-1 has also been demonstrated to induce proliferation and migration of both human and rabbit VSMC (Viedt *et al.*, 2002, Ma *et al.*, 2007). RASA1 functions to increase the GTPase activity of Ras, which is active when guanosine triphosphate (GTP) is bound, resulting in reduced activity of Ras and inhibition of various downstream signal transduction pathways that have been reported to promote VSMC migration and proliferation, including MAPK signalling (Pamonsinlapatham *et al.*, 2009).

Furthermore, in Ang II-induced hypertension in the rat, miR-132 and miR-212 were upregulated in the heart, aorta and kidney in an AT_1R dependent manner (Eskildsen *et al.*, 2013). Additionally, miR-132 and miR-212 expression has been reported to be reduced in mammary arteries from patients who have undergone coronary artery bypass surgery and treated with AT_1R antagonists (Eskildsen *et al.*, 2013).

In comparison to Ang II, there is very little known about the counter-regulatory axis of the RAS and regulation of miRNAs, and a role for Ang-(1-9) in particular has yet to be identified. Ang-(1-7) has been demonstrated to exert protective effects in vascular endothelial cells through interaction with miR-146a (Wang *et*

al., 2013). The presence of plasma glycated albumin has been associated with vascular dysfunction in diabetes, partly through increased IL-6 expression on endothelial cells (Lu *et al*., 2009). In human coronary artery endothelial cells, Ang-(1-7) blocked glycated albumin-stimulated miR-146a down-regulation and the resultant increase in IL-6 expression, identifying a novel protective mechanism for Ang-(1-7) in diabetes (Wang *et al*., 2013).

3.2 Aims

The aims of this chapter were:

- To establish the optimal conditions for proliferation of HSVSMC and assess the effect of Ang II, Ang-(1-7) and Ang-(1-9), and the involvement of the AT_1R, AT_2R and Mas, in this setting.

- To assess the effect of Ang II, Ang-(1-7) and Ang-(1-9), and the involvement of the AT_1R, AT_2R and Mas, on HSVSMC migration.

- To investigate the mechanisms involved in Ang-(1-7) and Ang-(1-9)-mediated inhibition of Ang II-induced migration.

- To assess the involvement of Ang II mediated regulation of microRNA-132/-212 in HSVSMC migration.

3.3 Results

3.3.1 AT_1R, AT_2R and Mas expression in primary HSVSMC

Prior to investigating the effects of Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC, it was first confirmed that the AT_1R, AT_2R and Mas were expressed in HSVSMC via qRT-PCR (Figure 3.1 A). Results were expressed as the average delta Ct (dCt) ± S.E.M, relative to the endogenous housekeeping gene GAPDH, therefore a smaller dCt value was indicative of increased expression. The AT_1R was the most highly expressed in HSVSMC (dCt 8.6 ± 0.6), followed by Mas (dCt 9.15 ± 0.4) and then the AT_2R (dCt 10.6 ±0.4).

Next, it was established whether angiotensin peptides alter receptor expression as this may contribute any functional effect observed downstream. Stimulation of HSVSMC with Ang II, Ang-(1-7) or Ang-(1-9), alone or in combination, had no effect on AT_1R, AT_2R or Mas expression in comparison to unstimulated control HSVSMC (Figure 3.1 B-D), suggesting that the effects of the peptides in HSVSMC in this study were not due to alterations in receptor expression levels.

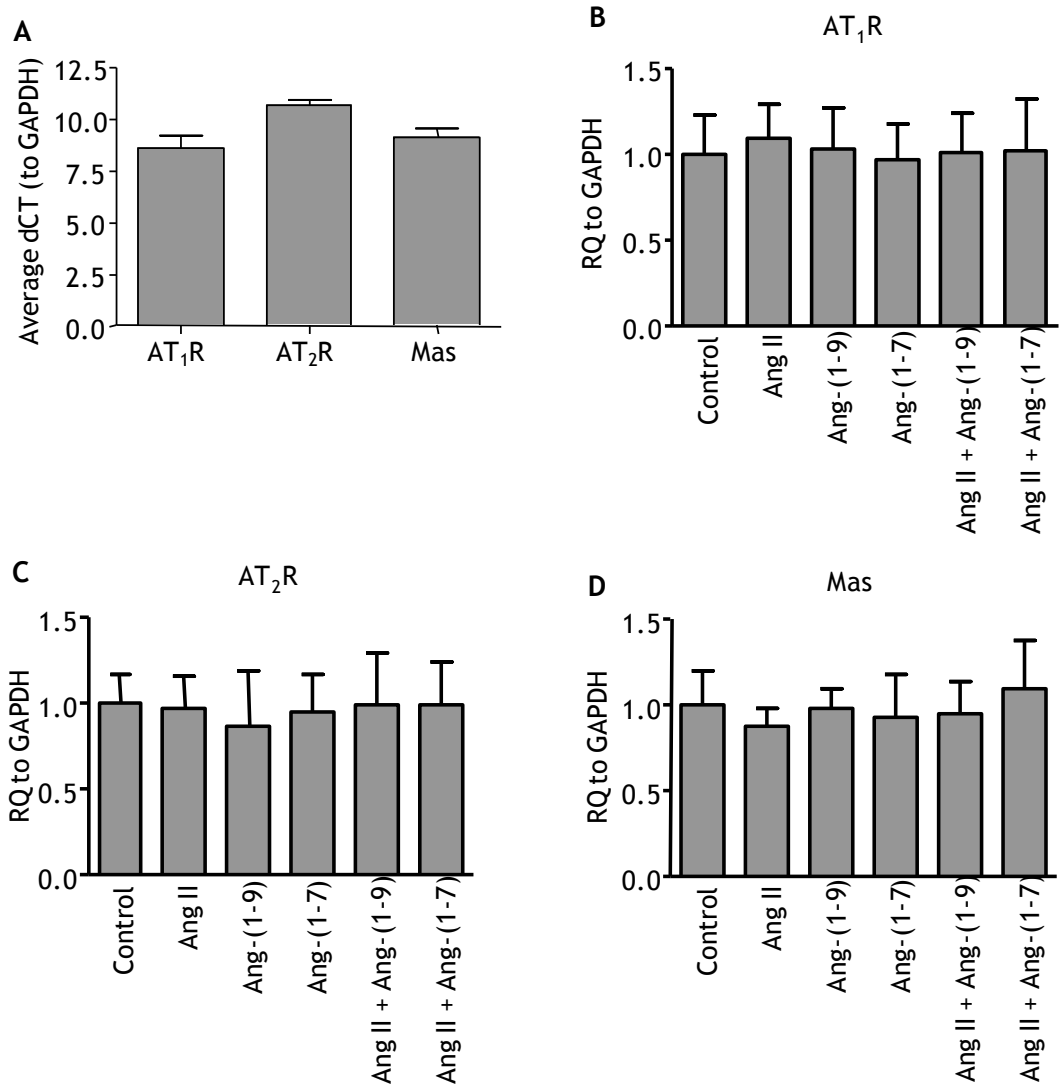

Figure 3.1 Expression of AT_1R, AT_2R and Mas in HSVSMC
Expression levels of AT_1R, AT_2R and Mas in HSVSMC was assessed via qRT-PCR using specific Taqman probes for each receptor. (A) Receptor expression was confirmed in unstimulated HSVSMC and results are expressed as average dCt ±S.E.M, relative to the endogenous housekeeping gene GAPDH. The effect of the RAS peptides on (B) AT_1R, (C), AT_2R and (D) Mas was also assessed and results are expressed as RQ to unstimulated control cells. N=3.

3.3.2 Assessing the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVSMC proliferation

To establish the optimal conditions for HSVSMC proliferation, cells were quiesced for 48 hours to arrest them in the resting phase of the cell cycle (Go) and then exposed to fresh media containing increasing concentrations of FCS (from 0 to 20 % v/v) for 48 hours. HSVSMC proliferation was then assessed using a MTS assay. FCS induced HSVSMC proliferation in a concentration dependent manner, with concentrations of 5% FCS and above producing a significant increase in proliferation in comparison to unstimulated, serum free control cells ($P<0.05$) (Figure 3.2 A). For subsequent proliferation experiments 5% FCS was used to induce cell proliferation as this concentration significantly increased HSVSMC growth in comparison to control cells without causing over-growth within the well.

To assess whether Ang-(1-7) or Ang-(1-9) blocked HSVSMC proliferation, quiescent cells were co-incubated with either Ang-(1-7) or Ang-(1-9) (both 200 nM) and 5% FCS for 48 hours. As expected, 5% FCS caused a significant increase in HSVSMC proliferation in comparison to control cells (Figure 3.2 B). Interestingly, Ang-(1-7) and Ang-(1-9) significantly blocked 5% FCS induced HSVSMC proliferation to similar levels as control, unstimulated cells ($P<0.05$ vs. 5% FCS).

To investigate if Ang II, Ang-(1-7) or Ang-(1-9) stimulation alone were able to induce quiescent HSVSMC to proliferate, each peptide was added individually to quiescent HSVSMC in serum free media for 48 hours and then cell proliferation assessed. While 5% FCS induced a significant increase in cell proliferation in comparison to control cells ($P<0.05$), no significant difference was observed between control cells and cells stimulated with either Ang II, Ang-(1-7) or Ang-(1-9) (Figure 3.2 C).

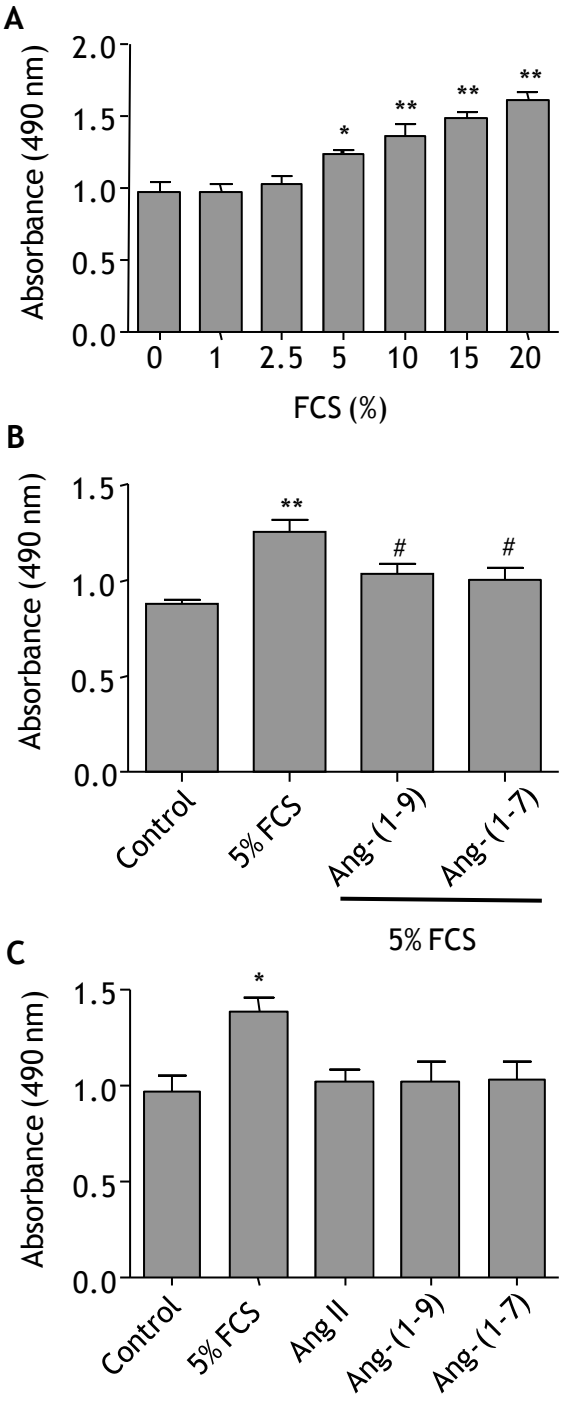

Figure 3.2 Assessment of the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVSMC proliferation

Proliferation of HSVSMC was assessed using the MTS assay. (A)To assess the concentration of FCS required to stimulated proliferation, HSVSMC were exposed to media containing increasing concentrations of FCS for 48 hours. (B) HSVSMC were incubated with Ang-(1-7) or Ang-(1-9) (200 nM) and stimulated with 5% FCS for 48 hours to assess the effect of these peptides on proliferation. (C) To assess if Ang II, Ang-(1-7) or Ang-(1-9) (200 nM) induce proliferation of HSVSMC in the absence of serum, cells were exposed to the peptides in serum free media for 48 hours; 5% FCS was used as a positive control. N=6. *P<0.05, **P<0.01 vs. control. $^{\#}$P<0.05 vs. 5% FCS.

As both Ang-(1-7) and Ang-(1-9) were found to inhibit serum induced proliferation, next the involvement of the AT_1R, AT_2R and Mas was assessed. Cells were incubated with the pharmacological antagonists losartan, PD123,319 or A779, which block the AT_1R, AT_2R and Mas, respectively, for 15 minutes prior to incubation with either Ang-(1-7) or Ang-(1-9). Cells were then stimulated with 5% FCS and proliferation assessed at 48 hours. As expected, 5% FCS caused a significant increase in HSVSMC proliferation in comparison to control non stimulated cells ($P<0.05$) (Figure 3.3). However, neither losartan, A779 nor PD123,319 had any effect on basal non-serum stimulated HSVSMC proliferation ($P>0.05$ vs. control) (Figure 3.3 A).

Next, the receptor through which Ang-(1-9) elicits its effects was assessed. As expected, Ang-(1-9) significantly reduced 5% FCS induced HSVSMC proliferation to similar levels as control (Figure 3.3 B). Importantly, this anti-proliferative effect was not altered by pre-incubation with losartan or A779 ($P>0.05$ vs. Ang-(1-9) + 5% FCS), but was blocked by PD123,319 ($P<0.05$ vs. Ang-(1-9) + 5% FCS) (Figure 3.3 B), suggesting that in this setting Ang-(1-9) acts via the AT_2R. Ang-(1-7) significantly reduced 5% FCS induced HSVSMC proliferation, resulting in a similar level of proliferation as control cells (Figure 3.3 C). This anti-proliferative effect was un-altered by pre-incubation with losartan or PD123,319 ($P>0.05$ vs. Ang-(1-7) + 5% FCS), but was inhibited by A779 ($P<0.05$ vs. Ang-(1-7) + 5% FCS), indicating that Ang-(1-7) acts via Mas (Figure 3.3 C).

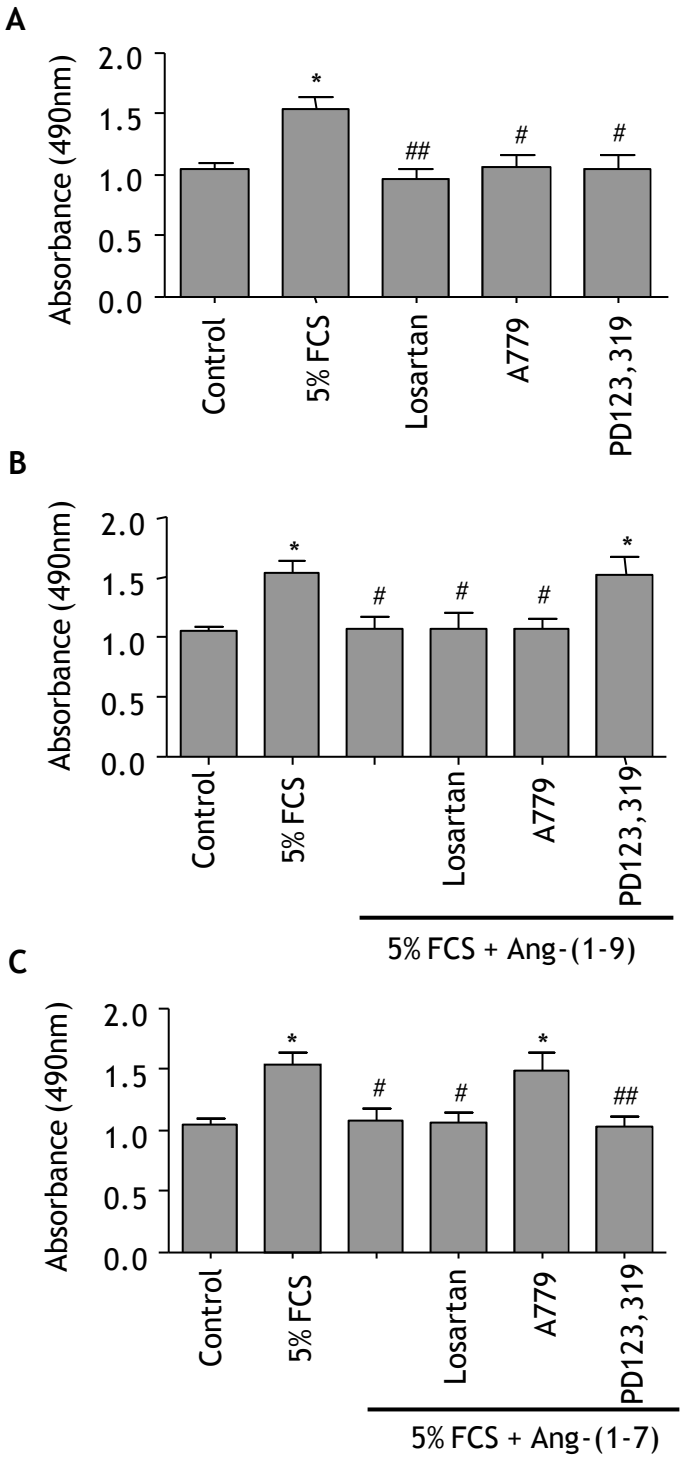

Figure 3.3 The role of AT_1R, AT_2R and Mas in the anti-proliferative effects of Ang-(1-9) and Ang-(1-7) in HSVSMC.

HSVSMC proliferation was assessed at 48 hours post stimulation with 5% FCS using the MTS assay. To assess the role of the RAS receptors in HSVSMC proliferation, cells were incubated with the pharmacological antagonists losartan (10μM), PD123,319 (500 nM) or A779 (100 μM) for 15 minutes prior to stimulation with Ang-(1-9) or Ang-(1-7) (200nM), and then 5% FCS . (A) It was first established if the antagonists influence HSVSMC proliferation. The effect of losartan, PD123,319 and A779 on the anti-proliferate effects of (B) Ang-(1-9) and (C) Ang-(1-7) was then assessed. All experimental conditions were included in replicate experiments and data has been separated into 3 panels due to the large number of groups; therefore, the values for control and 5% FCS are the same in all three panels. N=3. *P<0.05, **P<0.01 vs control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs 5% FCS.

3.3.3 Assessing the effects of Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC migration.

As VSMC migration is also largely involved in vascular remodelling, the effect of the RAS peptides on HSVSMC migration was assessed using a scratch assay. A confluent monolayer of cells was generated and then quiesced in serum free media for 48 hours. A wound in the cell monolayer was created with a pipette tip and cells were stimulated, in serum free media, with Ang II, Ang-(1-7) or Ang-(1-9) alone or in combination (200 nM each peptide). As a positive control for migration, cells were exposed to complete cell culture media containing 15% FCS for the duration of the experiment. Images of the scratches were taken at various time points, up to 30 hours so as to avoid induction of proliferation at later time points, and the reduction in scratch size was measured over time as a measure of cell migration (Figure 3.4 A).

Migration of HSVSMC over the full time course was quantified as a percentage reduction in scratch width at each time point relative to the 0 hours measurements (Figure 3.4 B). There was an increase in control cell migration over time resulting in a 53.2 ± 4.7% reduction in scratch size by 30 hours. In serum stimulated cells, 15% FCS induced a significant increase in HSVSMC migration in comparison to control cells at each time point measured and by 24 hours the wound had completely closed ($P<0.01$ vs. control) (Figure 3.4 B). Similarly, Ang II (200nM) induced a significant increase in VSMC migration from 12 hours post scratch in comparison to control cells and at each time point thereafter ($P<0.01$); by 24 hours the wound had closed (98.23 ± 1.2% reduction in scratch size) (Figure 3.4 B). Ang-(1-9) and Ang-(1-7) (200nM) resulted in an increase in migration of HSVSMC over time at a rate similar to control cells and resulted in a 52.3 ± 3.5% and 53.2 ± 5.3% reduction in scratch size by 30 hours, respectively (Figure 3.4 B). Ang-(1-9) and Ang-(1-7) both significantly inhibited Ang II-induced HSVSMC migration, producing similar levels of migration as control cells, resulting in a 51.4 ± 4.7% and 49.6 ± 5.5% reduction in scratch size, respectively ($P>0.05$ vs control) (Figure 3.4 B).

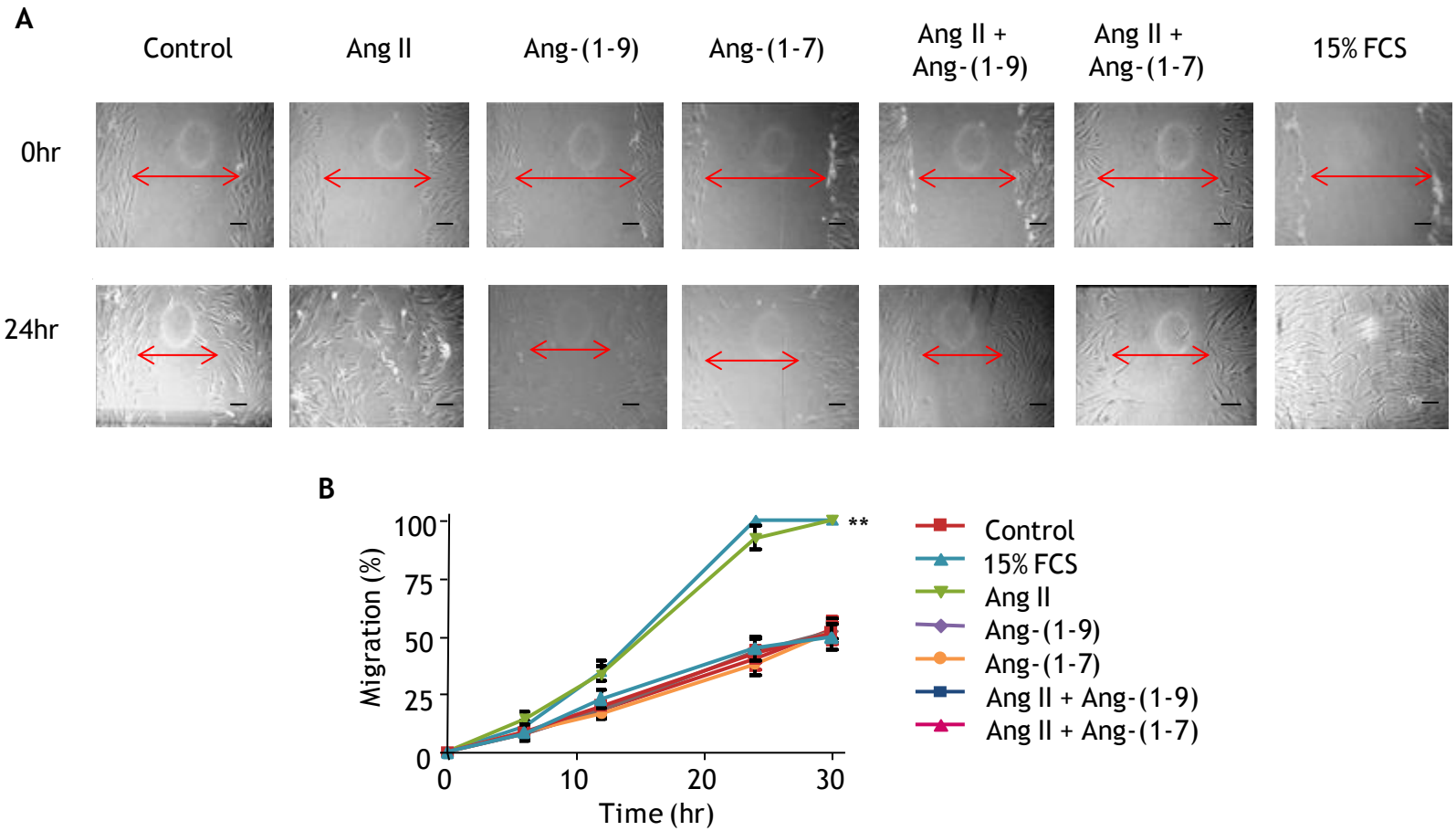

Figure 3.4 The effect of Ang II, Ang-(1-7) and Ang-(1-9) on HSVSMC migration.
HSVSMC migration was assessed using a scratch assay. Following a 48 hours quiescent period in serum free media, 3 scratches were induced in the cell monolayer in each well. Cells were stimulated with Ang II, Ang-(1-9) or Ang-(1-7) (200nM) alone or in combination in serum free media. Cells stimulated with 15% FCS were used as a positive control. Images of the scratch were taken at 0, 6, 12, 24 and 30 hours post scratch and the reduction in scratch size measured over time, as indicated by the red arrows. (A) Representative images of scratch at 0 hour and 24 hours. Scale bar = 40 µm. Magnification x10; (B) Migration was quantified as a reduction in scratch size (%) relative to 0 hour measurement. N=5. **P<0.01 vs. control.

Based on the finding that Ang-(1-7) and Ang-(1-9) block Ang II induced HSVSMC migration, a subsequent set of experiments was performed to investigate the involvement of the AT_1R, AT_2R and Mas. HSVSMC were incubated with losartan (10 μM), PD123,319 (500 nM) or A779 (100 μM) for 15 minutes prior to incubation with either Ang-(1-7) or Ang-(1-9). Migration was assessed at 24 hours post-stimulation (Figure 3.5)

First, it was established if the antagonists themselves had an effect on HSVSMC migration by incubating the cells with each antagonist in serum free media. While 15% FCS resulted in wound closure at 24 hours ($P<0.01$ vs control), it was found that neither losartan, A779 nor PD123,319 had any effect on HSVSMC migration in comparison to control cells ($P>0.05$ vs. control) (Figure 3.5 A).

Next the receptor through which Ang II induced migration occurs was assessed. Ang II caused a significant increase in HSVSMC migration at 24 hours in comparison to control cells (93.8 ± 8.0% vs. 52.8 ± 9.0% migration; $P<0.05$) which was significantly inhibited by losartan (53.3 ± 9.9% migration; $P<0.05$ vs. Ang II) but not PD123, 319 (92.4 ± 10.1% migration) nor A779 (93.2 ± 6.3% migration), indicating that Ang II induced migration is achieved via activation of the AT_1R (Figure 3.5 B).

As before, Ang–(1-9) significantly inhibited Ang II induced migration to similar levels as control cells (47.7 ± 8.9% migration; $P<0.05$ vs. Ang II) (Figure 3.5 C). While losartan and A779 did not alter the anti-migratory effect of Ang-(1-9) (52.7 ± 8.1% and 45.4 ± 12.7% migration respectively), PD123,319 blocked the inhibitory effects of Ang-(1-9), resulting in near complete wound closure (89.4 ± 9.3% migration), indicating that Ang-(1-9) mediated inhibition of Ang II-induced HSVSMC migration is via the AT_2R (Figure 3.5 C).

Similarly, Ang-(1-7) significantly inhibited Ang II-induced migration to an equivalent level to control cells (53.6 ± 10.1% migration; $P<0.05$ vs. Ang II) (Figure 3.5 D). However, the receptor through which Ang-(1-7) acts was found to be different to Ang-(1-9) as A779 blocked the anti-migratory effects of Ang-(1-7), resulting in near complete wound closure (92.6 ± 5.3% migration), while losartan and PD123,319 had no effect (43.4 ± 10.1% and 44.2 ± 9.6% migration

respectively), indicating that Ang-(1-7) mediated inhibition of Ang II induced HSVSMC migration is via the Mas receptor (Figure 3.5 D).

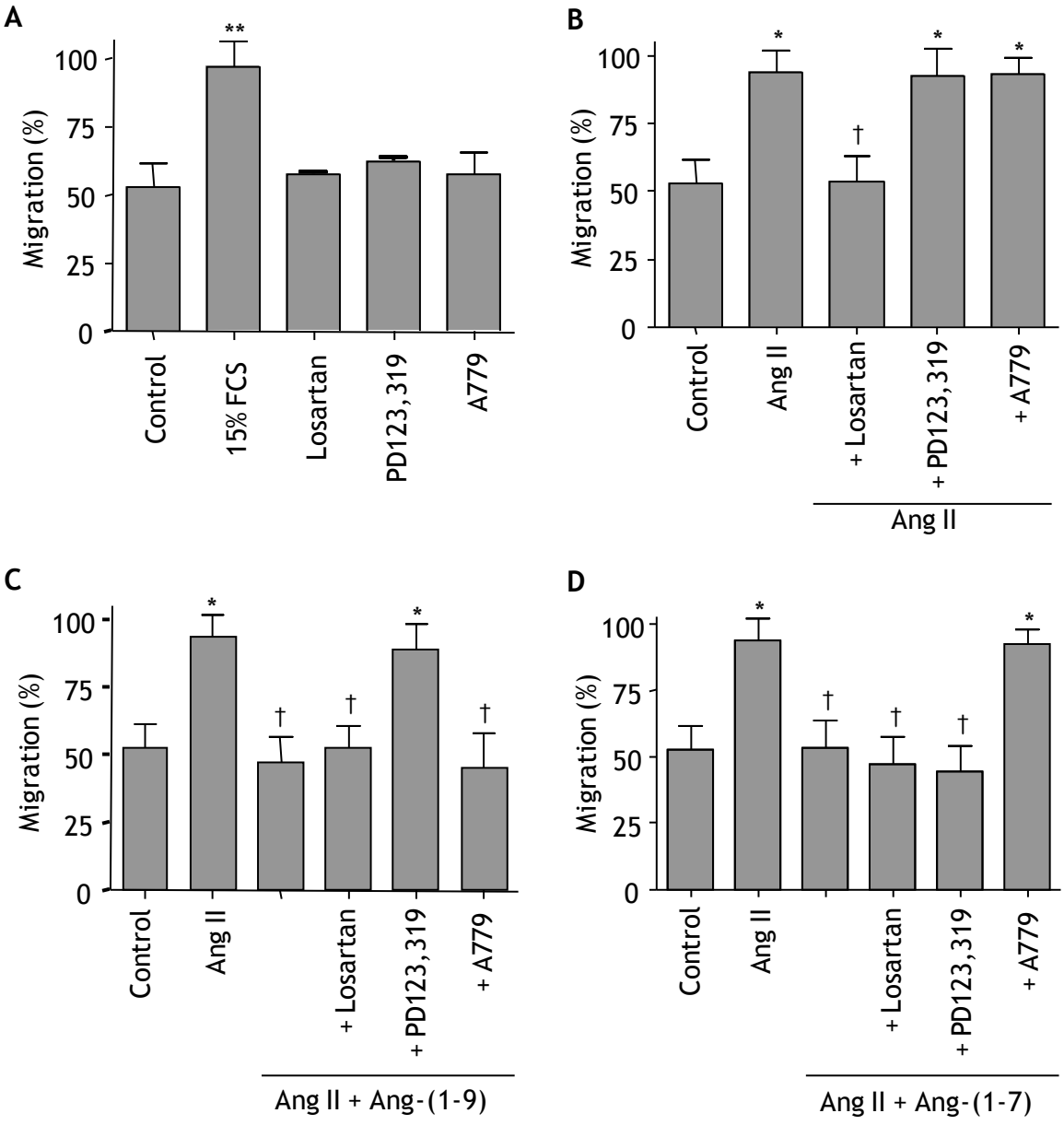

Figure 3.5 The role of the AT_1R, AT_2R and Mas in the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVSMC migration

HSVSMC migration was assessed using a scratch assay. To assess the role of the RAS receptors in HSVSMC migration, cells were incubated with the pharmacological antagonists losartan (10μM), PD123,319 (500 nM) or A779 (100 μM) for 15 minutes prior to stimulation with Ang-(1-9) or Ang-(1-7) (200nM). Cells stimulated with 15% FCS were used as a positive control. Images of the scratch were taken at 0 and 24 hours post stimulation and migration was quantified as a reduction in scratch size (%) relative to 0 hour measurement. (A) It was first established if the antagonists influence HSVSMC migration. The effect of losartan, PD123,319 and A779 on the effects mediated by (B) Ang II, (C) Ang-(1-7) and (D) Ang-(1-9) was then assessed. All experimental conditions were included in replicate experiments and data has been separated into 4 panels due to the large number of groups. N=3. *P<0.05, **P<0.01 vs. control; †P<0.05 vs. Ang II.

HSVSMC migration was also assessed using the xCELLigence system to allow for real time, automated analysis of migration, and corroboration of the results obtained using a traditional scratch assay. HSVSMC were seeded in specialised micro-titre plates (E-plates), allowed to adhere to the electrodes and establish a confluent monolayer overnight, and then quiesced for 48 hours. A scratch was induced in the cell monolayer causing a rapid drop in CI due to the reduced electrode coverage and cell migration measured as a change in CI due to movement of cells to the wounded area. Cells were stimulated with Ang II alone or in combination with Ang-(1-7) or Ang-(1-9) and cells in serum free media alone or complete growth medium (15% FCS) were used as control samples. CI was normalised at the time of stimulation with Ang II and is expressed relative to control cells; this time will hereafter be referred to as 0 hour.

CI of unstimulated control cells remained constant until 8 hours post stimulation and then gradually reduced at a steady rate; however, this reduction in CI is minimal and may represent a small degree of cell movement over the electrodes. Following stimulation with Ang II or 15% FCS there was an initial increase in CI followed by a small dip, then a gradual, steady increase in CI over time, indicative of increased cell migration (Figure 3.6 A). Cells exposed to Ang-(1-7) or Ang-(1-9) prior to stimulation with Ang II produced a similar level and pattern of migration to control cells, however, the reduction in CI from 8 hours post stimulation was less than that in control cells under these conditions (Figure 3.6 A).

The data was quantified as a fold change in normalised CI relative to control cells at 24 hours. A significant increase in CI in comparison to control cells was observed following stimulation with Ang II (1.9 ± 0.1 fold change in CI) or 15% FCS (2.0 ± 0.1 fold change in CI) ($P<0.001$ vs. control), indicative of enhanced HSVSMC migration (Figure 3.6 B). Ang II induced increased CI was significantly inhibited by Ang-(1-7) and Ang-(1-9), where a fold change in CI of 1.1 ± 0.1 and 1.0 ± 0.1 was observed, respectively (Figure 3.6 B).

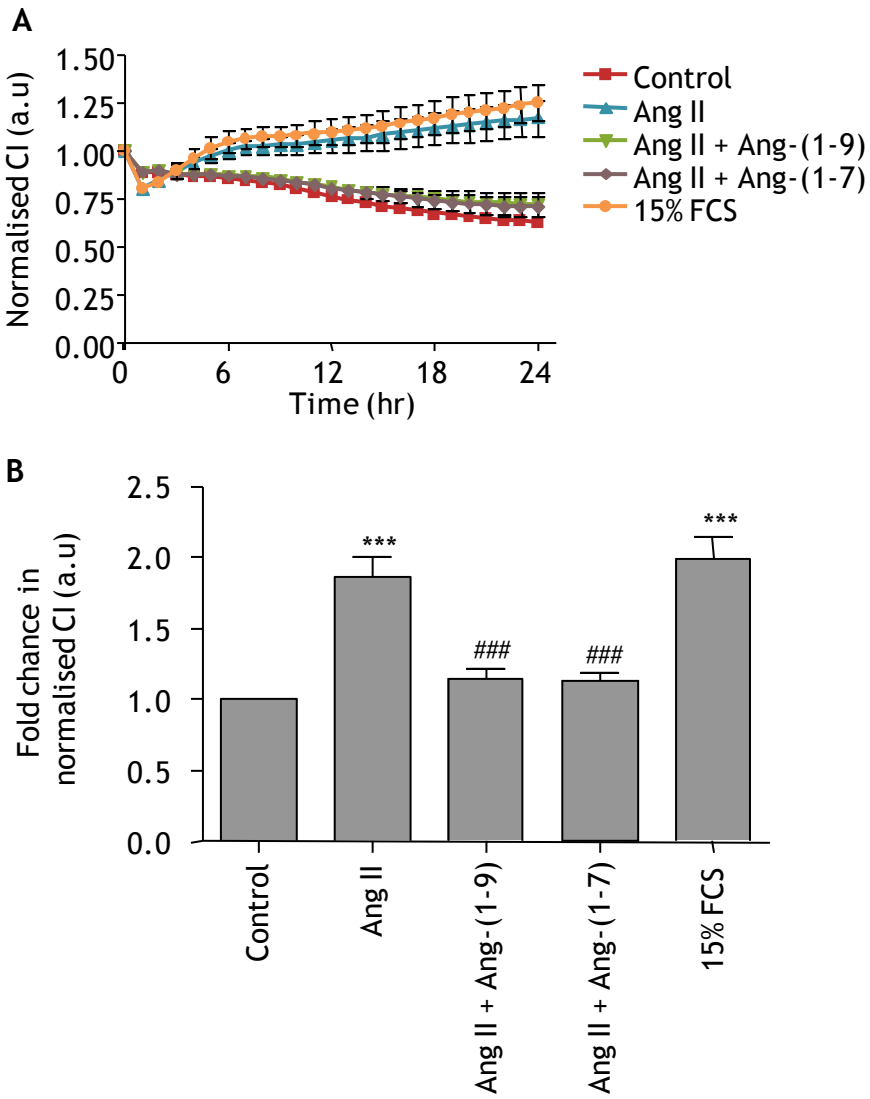

Figure 3.6 Analysis of the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVSMC migration using the xCELLigence system

HSVSMC migration was assessed in real time using the xCELLigence system. A scratch assay was performed as described in Section 2.5.1. Cells pretreated with Ang-(1-7) or Ang-(1-9) for 30 minutes prior to stimulation with Ang II (all peptides 200nM). (A) Representative CI trace. Cell index (CI) was normalised to control following addition of Ang II and changes in CI monitored over time. (B) Migration was quantified as a fold change in normalised CI at 24 hours relative to control. N=3. ***P<0.01 vs control, $^{###}$P<0.001 vs Ang II.

As in the traditional scratch assay, the involvement of the AT_1R, AT_2R and Mas in HSVSMC migration was also assessed using the xCELLigence system. The scratch assay was performed and analysed as described above, except cells were incubated with losartan (10 μM), PD123,319 (500 nM) or A779 (100 μM) for 15 minutes prior to incubation with either Ang-(1-7) or Ang-(1-9).

The findings from the scratch assay performed on the xCELLigence system were in line with the findings from the traditional scratch assay. While 15% FCS resulted in increased HSVSMC migration ($P<0.001$ vs. Control), stimulation of cells with losartan, PD123,319 or A779 resulted in a similar pattern and level of migration as control cells (losartan 1.0 ± 0.1; PD123,319 1.0 ± 0.1; A779 1.0 ± 0.1 fold change in CI; $P>0.05$ vs. control) (Figure 3.7 A).

Ang II caused a significant increase CI in comparison to control (2.0 ± 0.1 fold change in CI; $P<0.001$ vs. control) and this was significantly inhibited by Ang-(1-9) (1.30 ± 0.02 fold change in CI; $P<0.001$ vs. Ang II) (Figure 3.7 B). The effects of Ang-(1-9) were unaltered by A779 [1.2 ± 0.1 fold change in CI; $P>0.05$ vs. Ang II + Ang-(1-9)] (Figure 3.7 B). The effects of Ang-(1-9) were blocked by PD123,319, resulting in a similar pattern and level of migration as cells stimulated with Ang II (1.8 ± 0.1 fold change in CI; $P>0.05$ vs. Ang II) (Figure 3.7 B). Together, these findings confirm that Ang-(1-9) acts via the AT_2R and not Mas to prevent Ang II induced HSVSMC migration.

Conversely, while Ang-(1-7) significantly inhibited the Ang II induced increase in CI (2.0 ± 0.1 vs. 1.1 ± 0.1 fold change in CI; $P<0.001$ vs. Ang II) its effects were unaltered by PD123,319 [1.20 ± 0.1 fold change in CI; $P>0.05$ vs. Ang II + Ang-(1-7)] but effectively blocked by A779, resulting in a similar pattern and level of migration as cells stimulated with Ang II alone (1.9 ± 0.1 fold change in CI; $P>0.05$ vs Ang II) (Figure 3.7 C). Together these findings corroborated those from the traditional scratch assay and demonstrated that Ang-(1-7) acts via Mas to block Ang II-induced HSVSMC migration.

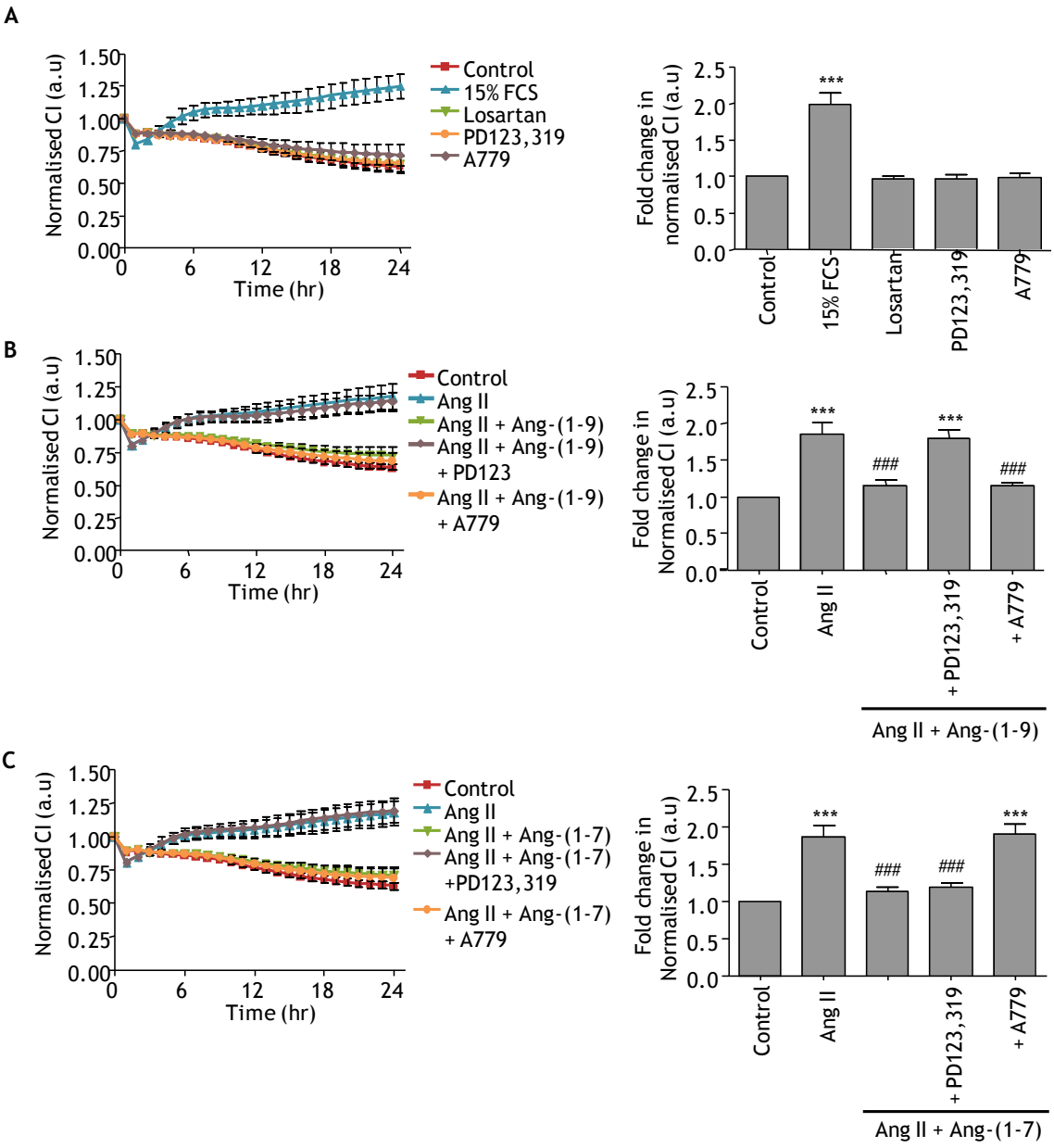

Figure 3.7 Analysis of the role of the AT_1R, AT_2R and Mas in the effects of Ang II, Ang-(1-9) and Ang-(1-7) on HSVSMC migration using the xCELLigence system.
HSVSMC migration was assessed in real time using the xCELLigence system. A scratch assay was performed as described in Section 2.5.1. To assess the role of the RAS receptors in HSVSMC migration cells were incubated with losartan (10 µM), PD123.319 (500 nM) or A779 (100 µM) for 15 minutes prior to stimulation with Ang-(1-7) or Ang-(1-9) (200 nM). Cells were then stimulated with Ang II 30 minutes later (200nM). Representative traces of CI measurements over time. Migration was quantified as a fold change in normalised CI at 24 hours relative to control. All experimental conditions were included in replicate experiments and data has been separated into 3 panels due to the large number of groups; therefore, the values for control and Ang II are the same in all panels. N=3. ***P<0.001 vs control; $^{###}$P<0.001 vs. Ang II.

3.3.4 Assessment of mechanisms involved in Ang-(1-7) and Ang-(1-9) inhibition of Ang II induced HSVSMC migration

In the current data set Ang-(1-7) and Ang-(1-9) have been demonstrated to prevent both HSVSMC proliferation and migration in response to FCS and Ang II, respectively. As one of the main aims of this thesis is to investigate the interaction of Ang II and the counter-regulatory peptides Ang-(1-7) and Ang-(1-9) in HSVSMC, it was decided that a more detailed assessment of the mechanisms involved in the effects of these peptides in HSVSMC migration would be performed, as there is clear modulation of the effects of Ang II by Ang-(1-7) and Ang-(1-9) in this setting.

3.3.4.1 Effect of Ang II, Ang-(1-7) and Ang-(1-9) on ERK phosphorylation in HSVSMC

Ang II via the AT_1R activates a number of intracellular signal transduction pathways that are linked to long term regulation of VSMC function, such as cell growth and migration (reviewed by [(Touyz and Schiffrin, 2000)]). These processes are initiated within minutes and involve phosphorylation of a number of protein kinases, including MAPK, such as ERK1/2, JNK and p38 MAPK (Xi *et al.*, 1999, Ohtsu *et al.*, 2005, Lee *et al.*, 2007). Ang II mediated phosphorylation of ERK1/2 in particular has been demonstrated to be an important mechanism involved in VSMC growth and migration (Xi *et al.*, 1999, Ohtsu *et al.*, 2005, Jiang *et al.*, 2008, Shen *et al.*, 2014) and importantly Ang-(1-7) has been demonstrated to prevent Ang II induced migration of rat VSMC via inhibition of Ang II-mediated phosphorylation of ERK1/2 (Zhang *et al.*, 2010b). However, as a role for Ang-(1-7) has yet to be defined in human VSMC migration and the effects of Ang-(1-9) on VSMC migration has yet to be explored it was first assessed if these peptides block Ang II induced HSVSMC migration through interaction with the ERK1/2 pathway.

To investigate ERK1/2 phosphorylation, quiescent HSVSMC were stimulated with Ang II, Ang-(1-7) or Ang-(1-9), alone or in combination (all peptides 200nM) for 5, 30 or 60 minutes; these time points were chosen as Ang II mediated ERK1/2 phosphorylation has been demonstrated to be transient, with a peak at 5 minutes (Eguchi *et al.*, 1996, Touyz *et al.*, 1999b). For ERK1/2, two bands were predicted at 42 and 44 kilodalton (kDa) and for GAPDH 1 band of 37kDa.

However, on the 30 minutes GAPDH representative blots 3 bands were present; this is due to residual detection of ERK1/2 following stripping of the membrane as opposed to non specific binding of the GAPDH antibody (Figure 3.8).

Stimulation of HSVSMC with Ang II for 5 minutes resulted in a significant increase in ERK1/2 phosphorylation in comparison to control cells ($P<0.05$ vs. control) (Figure 3.8). This effect of Ang II was transient as no differences in ERK1/2 phosphorylation were observed at the later time points (30 or 60 minutes). The transient Ang II mediated-increase in phosphorylation of ERK1/2 was blocked by Ang-(1-9) and Ang-(1-7), as in cells exposed to Ang II alongside either of these peptides ERK1/2 phosphorylation was similar to control levels (Figure 3.8). Ang-(1-7) and Ang-(1-9) alone had no effect on ERK1/2 phosphorylation at 5 minutes, indicating that the inhibitory effect on Ang II was not due to Ang-(1-7) or Ang-(1-9) directly reducing ERK1/2 phosphorylation but due to inhibition of Ang II signalling (Figure 3.8). At 30 minutes post stimulation, the RAS peptides, alone or in combination had no effect on phosphorylation of ERK1/2, as levels were comparable to control cells under all conditions, with the exception of the positive control 15% FCS, which was significantly increased in comparison to control ($P<0.01$) (Figure 3.8). In cells stimulated for 60 minutes with Ang II and Ang-(1-7), alone or in combination, no difference in ERK1/2 phosphorylation was observed in comparison to control (Figure 3.8). However, for Ang-(1-9), ERK1/2 phosphorylation was significantly elevated in cells stimulated with Ang-(1-9) alone and in combination with Ang II for 60 minutes in comparison to control cells ($P<0.05$ vs. control). As ERK1/2 was elevated in cells stimulated with Ang-(1-9) alone and alongside Ang II, but not in cells stimulated with Ang II alone this suggests that the increased ERK1/2 phosphorylation is induced by Ang-(1-9) (Figure 3.8). Cells were stimulated with FCS as a positive control and ERK1/2 phosphorylation was increased at 30 and 60 minutes, but not at 5 minutes, post stimulation with FCS ($P<0.01$ vs control cells) (Figure 3.8).

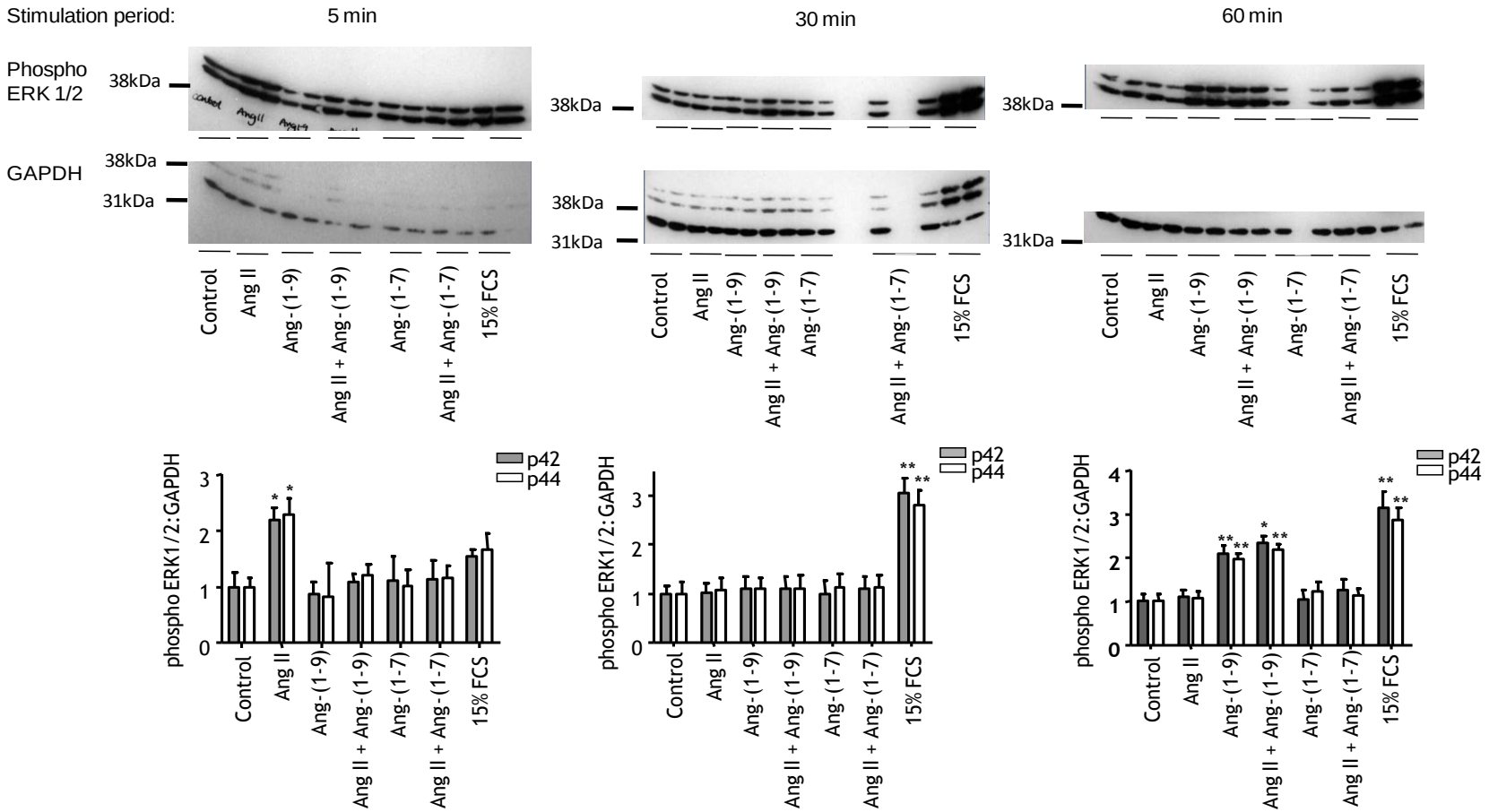

Figure 3.8 Effect of Ang II, Ang-(1-7) and Ang-(1-9) on ERK phosphorylation in HSVSMC.
Quiescent HSVSMC were stimulated with Ang II, Ang-(1-7) or Ang-(1-9), alone or in combination (all peptides 200nM) for 5, 30 or 60 minutes. Cells in serum free media alone or complete growth medium (15% FCS) were used as control samples. Cells were lysed for protein extraction and then Western immunoblotting performed. Expression of phosphorylated ERK1/2 was normalised to expression of the protein loading control GAPDH and quantified as a fold change relative to control at each time point. Representative blots shown. N=3. *P<0.05, **P<0.01 vs. control.

3.3.4.2 Effects of Ang II, Ang-(1-7) and Ang-(1-9) on matrix metalloproteinase expression in HSVSMC

It has been well documented that MMP2 and MMP9 are key endopeptidases involved in VSMC migration (Southgate *et al.*, 1996) therefore it was assessed if changes in MMP2 and MMP9 were involved. HSVSMC were subjected to a scratch assay and cells collected at 24 hours post-stimulation for RNA extraction and subsequent cDNA synthesis. Expression of MMP2 and MMP9 was assessed via qRT-PCR.

Ang II caused a significant reduction in MMP2 expression ($P<0.05$ vs. control), an effect significantly inhibited by Ang-(1-9) ($P<0.01$ vs. Ang II) but not Ang-(1-7) (Figure 3.9 A). The effect of Ang II was inhibited by losartan ($P<0.05$ vs. Ang II), resulting in similar expression levels to that observed in control cells, suggesting that Ang II acts via the AT_1R to down-regulate MMP2 expression. Co-incubation of PD123,319 partially attenuated the effect of Ang-(1-9) however, while MMP2 expression was lower than in both unstimulated cells and cells co-stimulated with Ang II and Ang-(1-9), this did not reach significance. Cells co-stimulated with Ang II and Ang-(1-7) and A779 had similar levels of MMP2 as cells stimulated with Ang II alone or alongside Ang-(1-7).

MMP9 was not detected in control, unstimulated cells and for this reason MMP9 expression is represented as average dCt and no statistical analysis has been performed (Figure 3.9 B). Stimulation of HSVSMC with Ang II results in induction of MMP9 expression, an effect blocked by Ang-(1-7) or Ang-(1-9). Similarly, Ang II-induced MMP9 expression was prevented by losartan, suggesting that this effect was dependent on interaction with the AT_1R. The effect of Ang-(1-9) and Ang-(1-7) on Ang II induced MMP9 expression was blocked by PD123,319 and A779, respectively, as under these conditions MMP9 was detected at similar levels to those produced in response to Ang II, suggesting that Ang-(1-9) acts via the AT_2R while Ang-(1-7) acts via Mas to prevent the effects of Ang II.

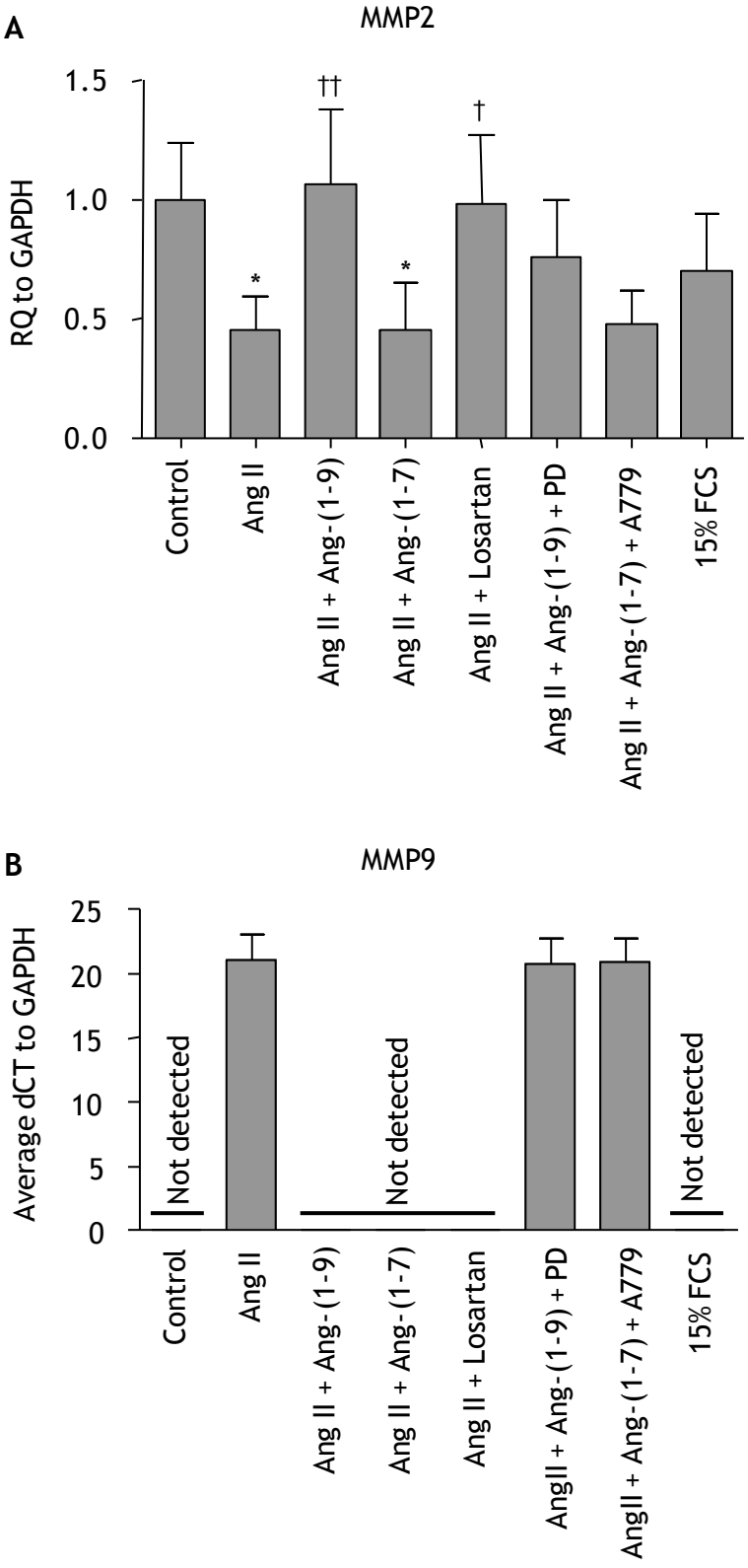

Figure 3.9 Differential regulation of MMP2 and MMP9 expression by Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC migration.

A scratch assay was performed in quiescent HSVSMC with 10 scratches induced per well to increase the population of migrating cells. Cells exposed to Ang II and Ang-(1-7) or Ang-(1-9) (200nM) for 24 hours and then cells were isolated for RNA extraction and subsequent cDNA synthesis. MMP2 and MMP9 expression was analysed by qRT-PCR using Taqman assays and normalised to GAPDH expression. (A) MMP2 expression is quantified as RQ to unstimulated cells. (B) MMP9 was not detected in control cells therefore expression is quantified as average dCt ± S.E.M. N=3. *P<0.05 vs. control; †P<0.05, ††P<0.01 vs. Ang II.

3.3.4.3 Effects of Ang II, Ang-(1-7) and Ang-(1-9) on miR-132/miR-212 expression and its downstream target genes in HSVSMC migration.

Ang II has previously been reported to regulate expression of the miR-132/-212 cluster in rat VSMC, resulting in down-regulation of various target genes that have been linked to VSMC proliferation and migration, including PTEN, RASA-1 and MCP-1 (Jin *et al.*, 2012). Therefore, regulation of the mir-132/212 cluster and its downstream targets was assessed. HSVSMC underwent the scratch assay protocol, except multiple scratches were induced in the cell monolayer to increase the proportion of migrating cells. HSVSMC were then stimulated with Ang II alone or in combination with Ang-(1-7) or Ang-(1-9). Unstimulated cells and cells stimulated with complete culture media (15% FCS) were used as a control. Cells were lysed for miRNA and RNA extraction at 24 hours post stimulation, and then miRNA and mRNA levels were analysed using q-RT PCR.

Both Ang II and 15% FCS induced a significant increase in miR-132 expression in comparison to control cells ($P<0.001$), suggesting miR-132 expression is increased during HSVSMC migration (Figure 3.10). Ang-(1-7) and Ang-(1-9) significantly reduced Ang II mediated increased miR-132 expression to similar levels as control cells (Ang II + Ang-(1-9) $P<0.01$; Ang II + Ang-(1-7) $P<0.001$ vs. Ang II) (Figure 3.10 A).

No changes in miR-212 expression were observed in cells stimulated with Ang II alone or alongside Ang-(1-7) or Ang-(1-9), suggesting that RAS peptides do not influence miR-212 expression under these conditions (Figure 3.10 B). While miR-212 expression in cells stimulated with 15% FCS appeared to be increased in comparison to control cells, this did not reach significance (Figure 3.10 B). This is unexpected as miR-132 and miR-212 have been reported to exist in a highly conserved cluster. However, the lack of regulation of miR-212 in comparison to miR-132 may be due to differences in basal expression of these miRNA's in HSVSMC, as in unstimulated cells miR-132 (average Ct 29.5 ± 0.5) is expressed at significantly higher levels than miR-212 (average Ct 33.6 ± 0.4).

Next, changes in miR-132 target gene expression; PTEN, RASA-1 and MCP1 was assessed (Figure 3.10 C-E). In line with increased miR-132 expression, HSVSMC stimulated with Ang II or 15% FCS displayed significantly lower expression of

PTEN in comparison to control cells (P<0.01 vs. control). Similarly, Ang-(1-7) and Ang-(1-9) blocked the effects of Ang II (P<0.01 vs Ang II) (Figure 3.11 C). Conversely, Ang II alone or in combination with Ang-(1-7) or Ang-(1-9) had no effect on either RASA-1 or MCP-1 expression in HSVSMC (Figure 3.10 D-E).

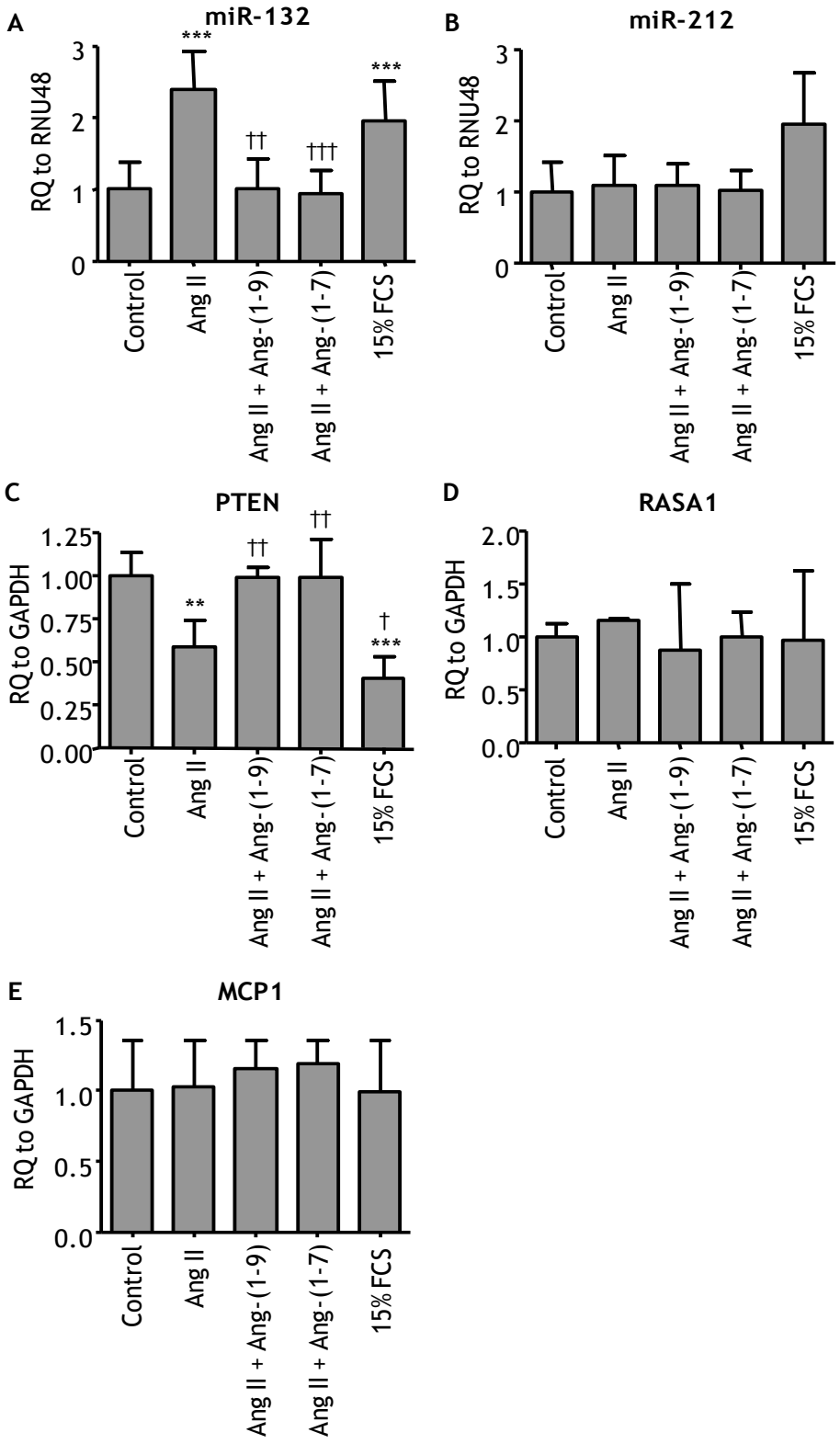

Figure 3.10 Effects of Ang II, Ang-(1-7) and Ang-(1-9) on miR-132/miR-212 expression and its downstream target genes during HSVSMC migration.

Quiescent HSVSMC were subjected to a scratch assay where multiple scratches were induced in the cell monolayer to increase the proportion of migrating cells. HSVSMC were then stimulated with Ang II alone or in combination with Ang-(1-7) or Ang-(1-9) (200nM). Unstimulated cells and cells stimulated with complete culture media (15% FCS) were used as a control. Cells were lysed for miRNA and RNA extraction at 24 hours post stimulation, and then miRNA and mRNA levels were analysed using q-RT PCR. Expression of (A) miR-132, (B) miR-212, (C) PTEN, (D) RASA1 and (E) MCP-1 was normalised endogenous housekeeper (RNU48 in A and B; GAPDH in C-E) and expression is expressed as RQ to control. N=6. *P<0.05, **P<0.01 ***P<0.001 vs control; †P<0.05, ††P<0.01, †††P<0.001 vs 5% Ang II.

The effect of Ang II on miR-132 expression at 24 hours post stimulation in comparison to control cells ($P<0.001$ vs control), was significantly inhibited by losartan, suggesting that Ang II mediated increase in miR-132 expression was via the AT_1R (Figure 3.11 A). The Ang II mediated increase in miR-132 expression was also blocked by Ang-(1-9) and Ang-(1-7) (Ang II + Ang-(1-9) $P<0.01$; Ang II + Ang-(1-7) $P<0.001$ vs Ang II) and similar levels of miR-132 were observed in cells stimulated with Ang II co-incubated with either Ang-(1-7) or Ang-(1-9) compared to control (Figure 3.12 A). The inhibitory effect of Ang-(1-9) was attenuated by PD123,319 ($P<0.05$ vs control, $P>0.05$ vs Ang II), and the effect of Ang-(1-7) was attenuated by A779 ($P<0.01$ vs control, $P>0.05$ vs Ang II), suggesting Ang-(1-9) may act via the AT_2R and Ang-(1-7) via Mas (Figure 3.11 A).

Ang II caused a significant reduction in PTEN expression in comparison to control cells, an effect blocked by losartan ($P<0.05$ vs Ang II) (Figure 3.11 B). Ang-(1-7) and Ang-(1-9) also prevented Ang II mediated reduction in PTEN expression, and cells stimulated with Ang II alongside either Ang-(1-7) or Ang-(1-9) displayed similar expression levels of PTEN as control (Figure 3.12 B). Again, the inhibitory effects of Ang-(1-7) and Ang-(1-9) were blocked by A779 and PD123,319, respectively ($P<0.05$ vs Ang II) (Figure 3.11 B).

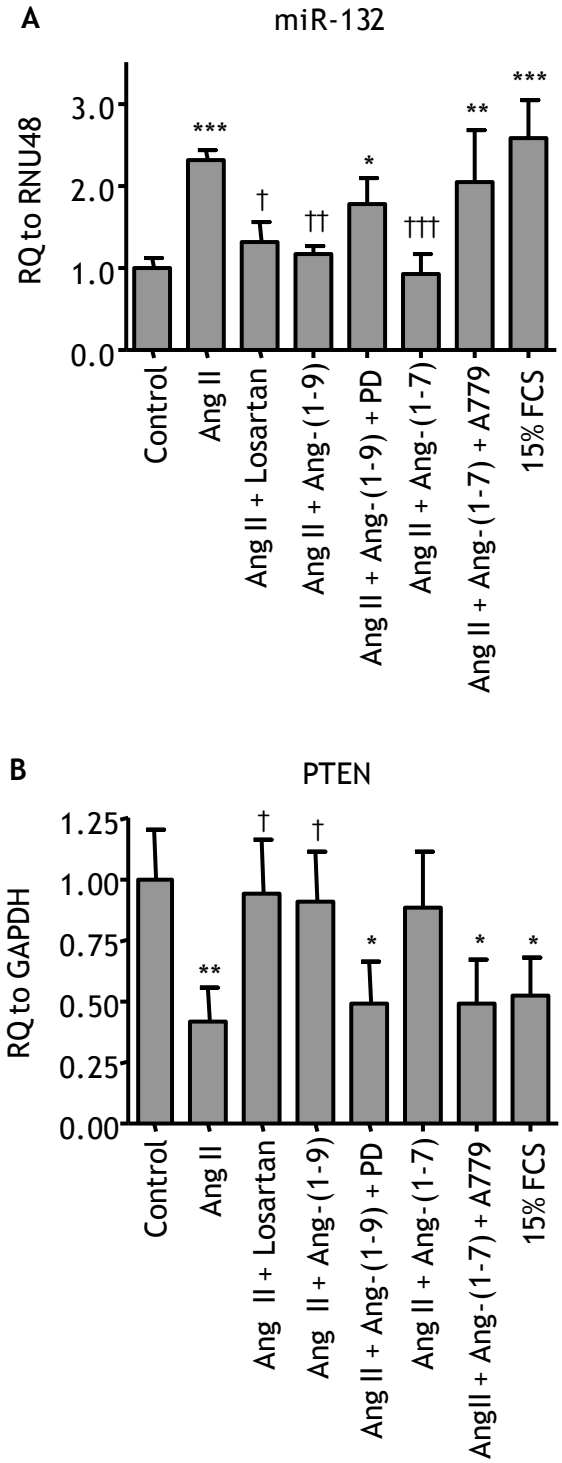

Figure 3.11 The role of the AT_1R, AT_2R and Mas in the effects of Ang II, Ang-(1-7) and Ang-(1-9) on miR-132 and PTEN expression in HSVSMC.

Quiescent HSVSMC were subjected to a scratch assay where multiple scratches were induced in the cell monolayer to increase the proportion of migrating cells. To assess the role of the RAS receptors in regulation of miR-132 and PTEN expression cells were incubated with losartan (10 µM), PD123.319 (PD) (500 nM) or A779 (100 µM) for 15 minutes prior to stimulation with Ang-(1-7) or Ang-(1-9) (200 nM). Cells were lysed for miRNA and RNA extraction at 24 hours post stimulation, and then miRNA and mRNA levels were analysed using q-RT PCR. Expression of (A) miR-132 and (B) PTEN was normalised endogenous housekeeper (RNU48 in A; GAPDH in B) and expression is expressed as RQ to control. N=3. *P<0.05, **P<0.01, ***P<0.001 vs control; †P<0.05, ††P<0.01, †††P<0.001 vs Ang II.

It was next assessed if Ang II induced reduction in PTEN protein levels and changes in downsteam targets including Akt (Huang and Kontos, 2002). Confluent HSVSMC were quiesced for 48 hours and then multiple scratches were induced in the monolayer followed by stimulation with Ang II alone or co-stimulated with Ang-(1-7) or Ang-(1-9) for 24 hours. Cells in serum free media or stimulated with 15% FCS were used as a control. At 24 hours post stimulation cells were lysed for protein extraction and Western immunoblotting performed using specific antibodies for PTEN and Akt. Protein expression was quantified as a ratio of the total protein (PTEN or Akt) to GAPDH, the loading control. No changes were observed in PTEN or Akt expression in HSVSMC stimulated with Ang II, alone or in combination with Ang-(1-7) or Ang-(1-9), or 15% FCS at 24 hours post stimulation in comparison to control cells ($P>0.05$ vs control) (Figure 3.12), indicating that the changes in PTEN expression observed at mRNA level were not apparent in protein levels at this time point.

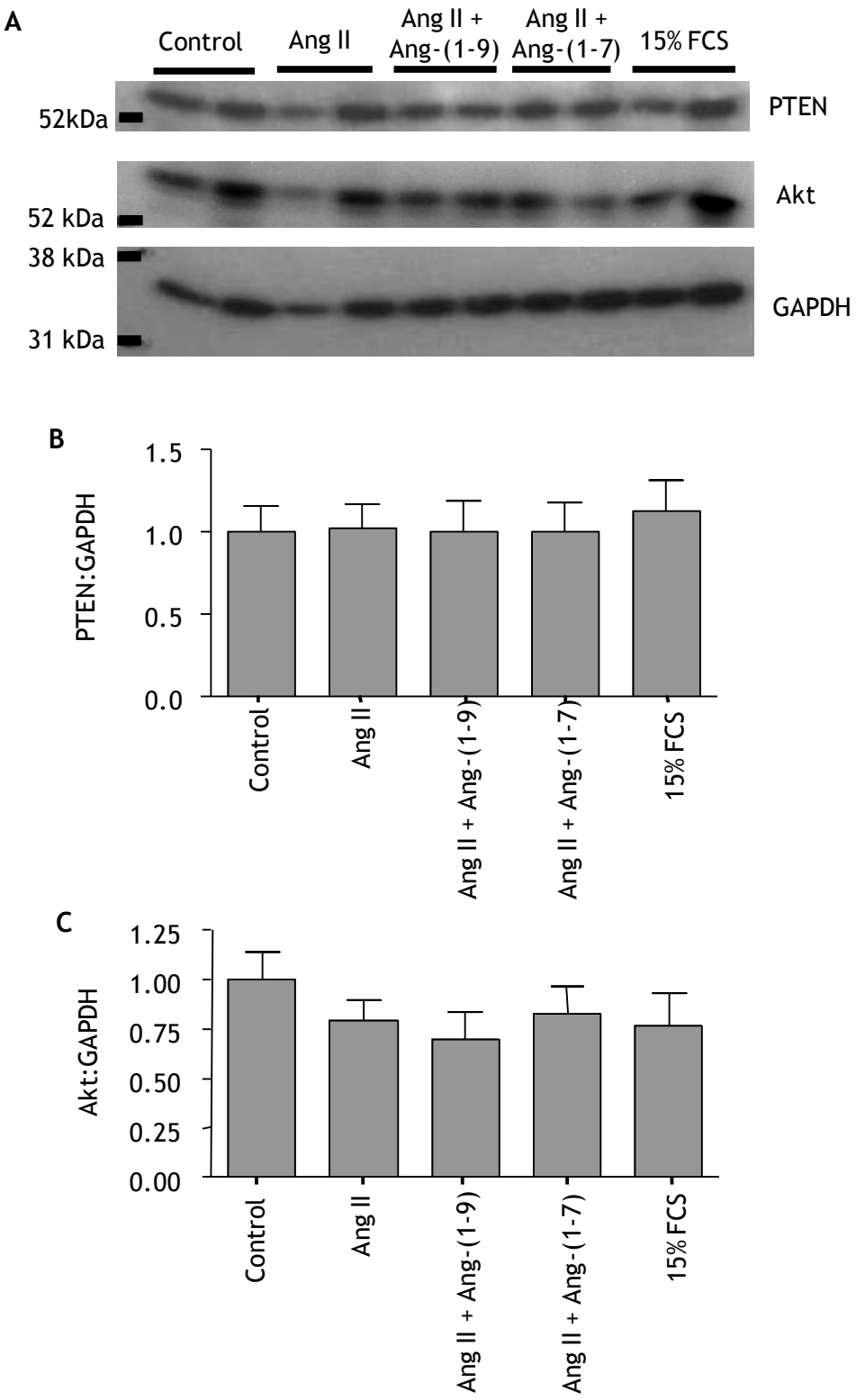

Figure 3.12 Effects of Ang II, Ang-(1-7) and Ang-(1-9) on PTEN and Akt protein expression in HSVSMC migration

Quiescent HSVSMC underwent a scratch assay with multiple scratches in the cell monolayer to increase the proportion of migrating HSVSMC. Cells were then stimulated with Ang II alone or alongside Ang-(1-7) or Ang-(1-9) for 24 hours. Cells in serum free media or stimulated with complete growth media (15% FCS) were used as a control. At 24 hours post stimulation cells were lysed for protein extraction and Western immunoblotting was then performed using speficic antibodies for PTEN and AKT. Protein expression was quantified as a ratio of the total protein expression of (B) PTEN or (C) Akt, to the loading control GAPDH. Representative immunoblots of PTEN, Akt and GAPDH expression. N=2.

To assess the role of miR-132 in Ang II mediated HSVSMC migration, a specific miR-132 inhibitor was used. miRNA inhibitors are small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit miRNA activity (Stenvang *et al.*, 2008, Weiler *et al.*, 2006). Therefore, these molecules are not designed to alter expression levels of the miRNA but prevent changes in target gene expression.

First the transfection efficiency of the miRNA inhibitor was assessed in HSVSMC using a Cy3 labelled negative control. As expected, no fluorescence was observed in nontransfected, control HSVSMC (NTC). No Cy3 positive cells were observed in HSVSMC transfected with 10nM or 30nM of the labelled non targeting molecule and only a small proportion of cells transfected at 60nM were positive for Cy3, suggesting a low transfection efficiency (Figure 3.13 A). As cells transfected with 60nM of the Cy3 control displayed the highest positive cell number, this concentration was used for all subsequent experiments.

Expression of PTEN was used to assess efficiency of the miR-132 inhibitor. In nontransfected cells, Ang II and 15% FCS induced a significant reduction in PTEN expression in comparison to control (Ang II $P<0.01$; 15% FCS $P<0.05$ vs control) (Figure 3.13 B). In cells transfected with the non targeting negative control (scramble), stimulation with either Ang II resulted in a significant reduction in PTEN expression ($P<0.05$), and while 15% FCS also reduced this was not significantly different to control (Figure 3.13 B). A similar effect was observed in cells transfected with the miR-132 inhibitor, with both Ang II and 15% FCS significantly reduced PTEN expression in comparison to control, suggesting that the transfection efficiency attained was possibly not high enough. (Figure 3.13 B).

Conversely, in nontransfected cells, Ang II and 15% FCS induced a significant increase in miR-132 expression in comparison to control ($P<0.05$) (Figure 3.13 C). In cells transfected with the non targeting negative control (scramble), stimulation with either Ang II resulted in a significant increase in miR-132 expression ($P<0.01$), and while 15% FCS also increased miR-131 expression this was not significantly different to control (Figure 3.13 C). A similar pattern in response to Ang II and FCS was observed in cells transfected with the miR-132 inhibitor, however, these changes were not significantly different to

unstimulated cells and miR-132 expression was lower under all stimulation conditions compared to non transfected cells and cells transfected with the non tragetting miRNA inhibitor (Figure 3.13 C).

HSVSMC migration was quantified at 24 hours post stimulation, in untransfected cells and cells transfected with either the non targeting molecule or miR-132 inhibitor. Under all conditions, both Ang II and 15% FCS caused a significant increase in HSVSMC migration at 24 hours post stimulation in comparison to control ($P<0.001$) (Figure 3.13 D). However, in unstimulated cells transfected with the miR-132 inhibitor, there was a significant increase in HSVSMC migration in comparison to unstimulated, untransfected cells ($P<0.01$). In both untransfected cells and cells transfected with scramble miR inhibitor basal HSVSMC migration at 24 hours was comparable at 52.8 ± 7.4 % and 52.2 ± 7.8 % migration, respectively (Figure 3.13 D). However, in unstimulated cells transfected with the miR-132 inhibitor, basal HSVSMC migration at 24 hours was significantly higher at 71.1 ± 12.1 % migration, suggesting that the miR-132 inhibitor influenced basal HSVSMC migration (Figure 3.13 D).

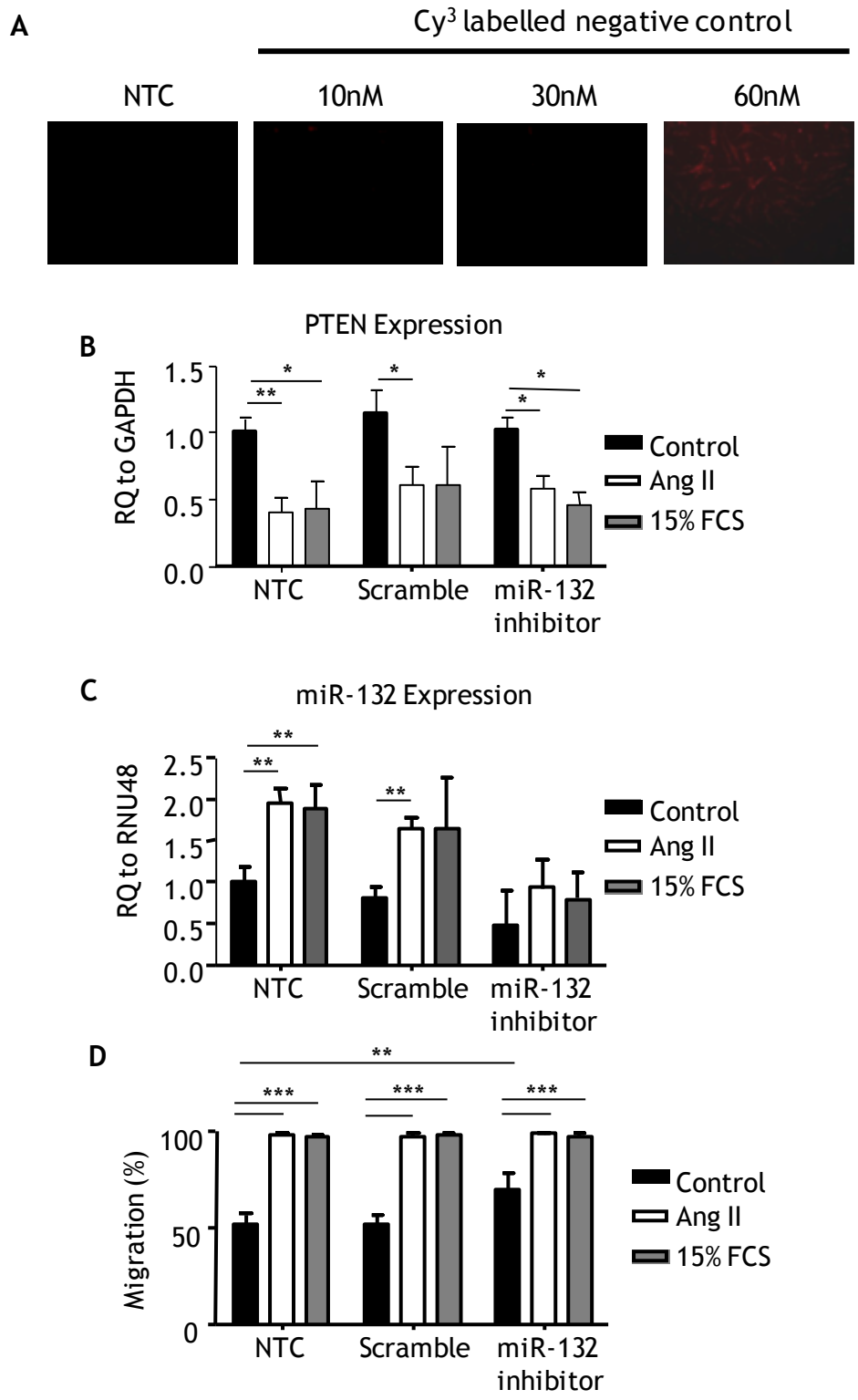

Figure 3.13 Effect of mir-132 inhibition on PTEN expression and HSVSMC migration.
HSVSMC were transfected with either a Cy3 labelled non targeting miRNA inhibitor or a miR-132 inhibitor as described in section 2.7. (A) Transfection efficiency was assessed by visualisation of fluorescent cells. (B) Cells were transfected with either a scramble miR-inhibitor or the miR-132 inhibitor at 60 nM described, then stimulated with Ang II or 15% FCS for 24 hours. Cells were then lysed for RNA extraction and subsequent synthesis to cDNA. (B) PTEN and (C) miR-132 expression was assessed using qRT-PCR and expressed as RQ to control. (D) HSVSMC migration was quantified as a reduction in scratch width (%) at 24 hours relative to the 0 hour measurement. N=3. *P<0.05, **P<0.01, ***P<0.001 vs control.

3.3.4.4 The role of miRNA in Ang II induced HSVSMC migration

Although Ang II increased miR-132 expression in HSVSMC, it was not essential for HSVSMC migration. Therefore, an alternative approach was taken to determine whether miRNA regulation essential for Ang II-mediated HSVSMC migration. To do this an experiment to inhibit DICER, the key enzyme involved in miRNA generation was undertaken. This was achieved through the use of a specific siRNA to knock down DICER expression within VSMC stimulated with Ang II.

To assess the optimal concentration of siRNA required for maximal transfection of HSVSMC, cells were transfected with increasing concentrations (10-60 nM) of a Cy3 labelled control siRNA (Figure 3.14 A). In HSVSMC transfected with the Cy3 labelled control siRNA, the number of Cy3 positive cells increased in a concentration-dependent manner, as indicated by increased red fluorescence (Figure 3.14 A). The majority of cells transfected at 60 nM were positive for Cy3. Next, to assess the optimal concentration of siRNA required for efficient knock down of gene expression in HSVSMC, cells were transfected with increasing concentrations of a siRNA against GAPDH. In HSVSMC transfected with the GAPDH siRNA, there was a reduction in GAPDH expression in a concentration dependent manner (Figure 3.14 B). GAPDH expression was significantly reduced in comparison to cells transfected with the scrambled siRNA at concentrations of 30nM and above, however, maximal knockdown of GAPDH was observed in cells transfected with 60 nM siRNA (Figure 3.14 B). Cy3 labelled control siRNA transfection (scramble siRNA) did not lead to alterations in GAPDH expression (Figure 3.14 B).

Transfection with the DICER targeting siRNA at 60 nM significantly reduced DICER expression in HSVSMC in comparison to untransfected cells or cells transfected with the non targeting siRNA (scramble) ($P<0.001$) (Figure 3.14 C).

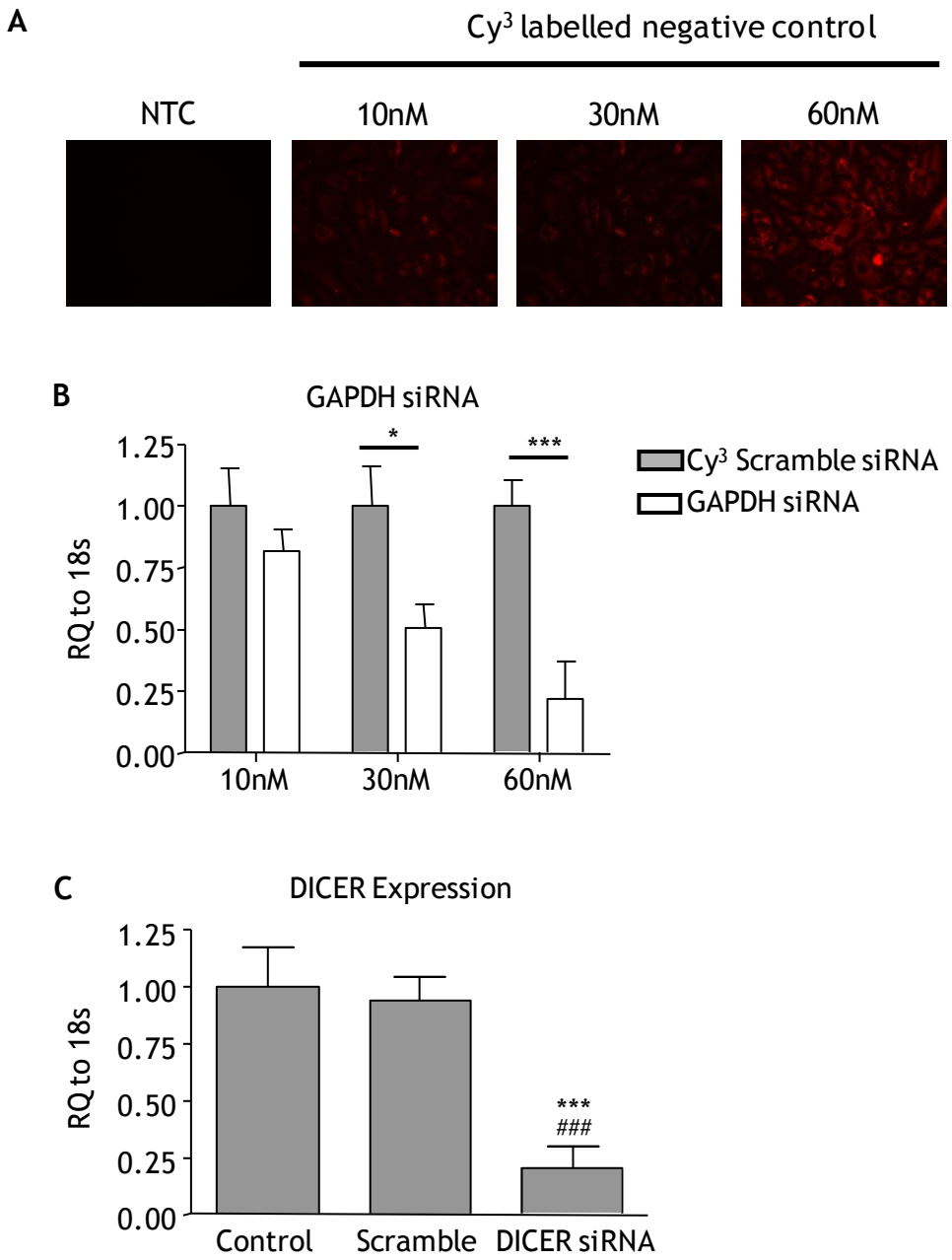

Figure 3.14 Transfection efficiency of siRNA in HSVSMC
The transfection efficiency of siRNAs in HSVSMC was assessed as described in section 2.7. (A) Cells were transfected with increasing concentration of a Cy3 labelled non targeting siRNA (10, 30, 60 nM) and transfection efficiency assessed by visualisation of fluorescent cells. (B) To assess the optimal concentration for efficient gene knock down, cells were transfected with a siRNA targeting GAPDH (10, 30, 60 nM). GAPDH expression was normalised to 18s and expressed as RQ to the comparable concentration of the scrambled siRNA. (C) DICER expression following transfection of HSVSMC with 60 nM DICER siRNA. N=3. *P<0.05, ***P<0.001 vs control; $^{\#\#}$P<0.01 vs scramble siRNA.

Although it was previously demonstrated that Ang II mediated miR-132 expression was not essential for HSVSMC migration, expression levels of this miRNA and its target PTEN were assessed to confirm that siRNA-mediated knockdown of DICER had functional biological effects on miRNA and mRNA expression. HSVSMC underwent a scratch assay following transfection and then stimulated with Ang II or 15% FCS to induce migration. Cells were lysed for RNA extraction at 24 hours post stimulation and miR-132 and PTEN expression assessed. In untransfected cells or cells transfected with a non targeting siRNA (scramble), there was a significant increase in miR-132 expression in HSVSMC stimulated with Ang II or 15% FCS in comparison to control ($P<0.05$ vs. control) (Figure 3.15 A). In cells transfected with the DICER siRNA, miR-132 expression was unaltered by Ang II in comparison to control, suggesting that knockdown of DICER blocked the Ang II mediated increase in miR-132 expression (Figure 3.15 A). miR-132 expression was elevated in cells transfected with DICER siRNA when stimulated with 15% FCS in comparison unstimulated cells, however this change did not reach significance (Figure 3.15 A).

Furthermore, in untransfected cells or cells transfected with the scramble siRNA there was a significant reduction in PTEN expression in HSVSMC stimulated with Ang II or 15% FCS in comparison to control ($P<0.05$ vs. control) (Figure 3.15 B). In cells transfected with the DICER siRNA, PTEN expression was unaltered in response to Ang II or 15% FCS, suggesting that Ang II mediated reduction in PTEN occurs via a mechanism dependent on DICER expression (Figure 3.15 B).

In addition to assessing the effects of DICER siRNA on Ang II induced changes in miR-132 and PTEN expression, it was also established if knockdown of DICER had functional consequences on Ang II induced HSVSMC migration. In untransfected control cells, and cells transfected with a scramble siRNA or DICER siRNA, there was a significant increase in HSVSMC migration in response to Ang II and 15% FCS at 24 hours post stimulation in comparison to unstimulated cells (Figure 3.15 C). Under all transfection conditions, stimulation of cells with Ang II or 15% FCS resulted in wound closure by 24 hours. However, in cells transfected with DICER siRNA, there was a significant increase in basal migration of HSVSMC in comparison to untransfected cells and cells transfected with the scramble siRNA ($P<0.01$) (Figure 3.15 C). In both untransfected cells and cells transfected with scramble siRNA basal HSVSMC migration at 24 hours was comparable at 50.36 ±

5.3 % and 51.1 ± 4.0 % migration, respectively (Figure 3.16 C). However, in unstimulated cells transfected with DICER siRNA, basal HSVSMC migration at 24 hours was significantly higher at 70.4 ± 14.4 % migration, suggesting that alterations in miRNA expression through reduced DICER expression promote HSVSMC migration in the absence of other stimuli (Figure 3.15 C).

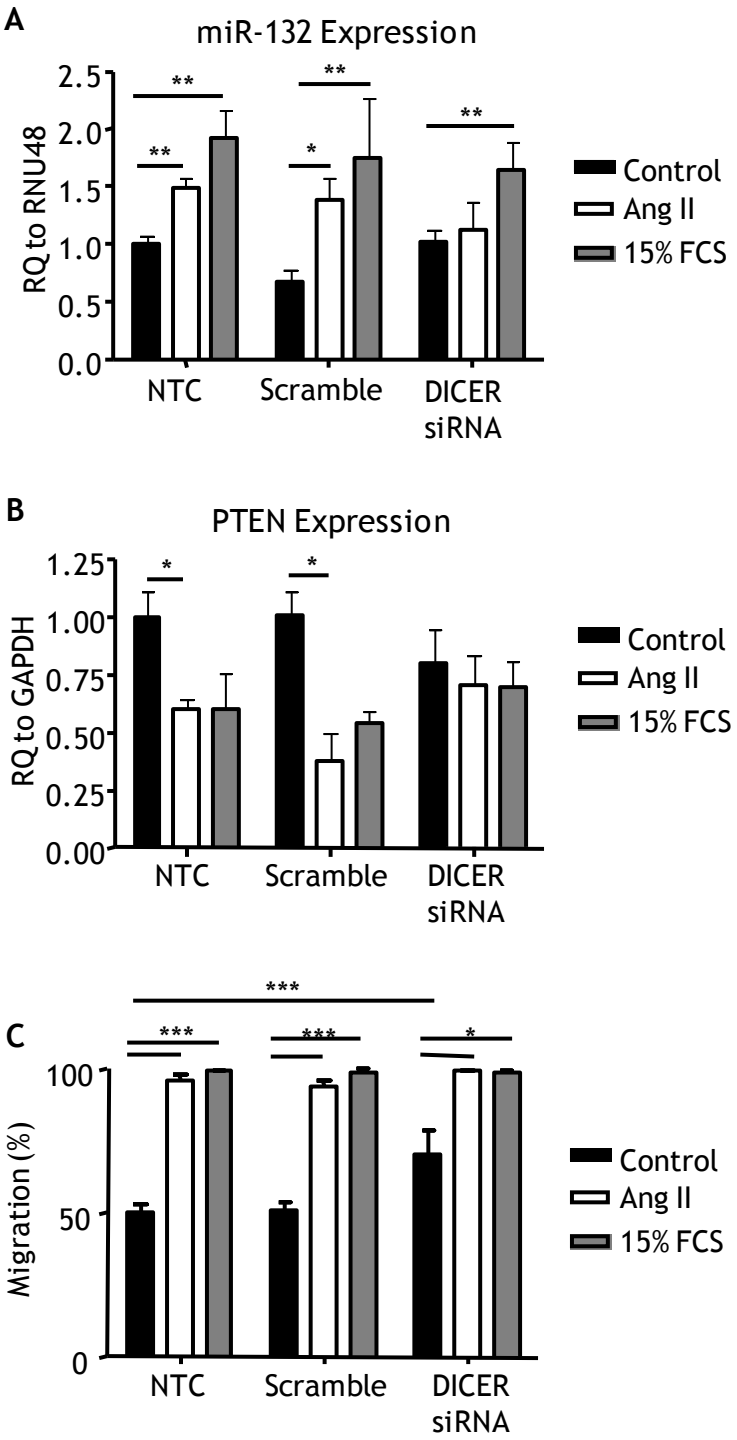

Figure 3.15 Effect of DICER knockdown on Ang II-mediated regulation of miR-132 and PTEN expression, and HSVSMC migration.

HSVSMC were transfected with 60nM scramble or DICER siRNA and then underwent the scratch assay protocol as described in section 2.7. Cells were stimulated with Ang II or 15% FCS for 24 hours and then lysed for miRNA and RNA extraction. (A) miR-132 and (B) PTEN expression was assessed via qRT-PCR and expressed as RQ to control. (C) HSVSMC migration was quantified as a reduction in scratch width (%) at 24 hours relative to the 0 hour measurement. N=3. *P<0.05, **P<0.01, ***P<0.001 vs control.

3.4 Discussion

This study is the first to report a role for Ang-(1-9) in human VSMC proliferation and migration, and to provide a direct comparison between Ang-(1-7) and Ang-(1-9) in these processes. The data demonstrate that Ang-(1-7) and Ang-(1-9) inhibit serum induced proliferation of HSVSMC via Mas and AT_2R, respectively. While Ang II was unable to induce proliferation of HSVSMC in this study, it was demonstrated to have potent pro-migratory effects. Ang II-induced HSVSMC migration via the AT_1R in agreement with previous reports (Schieffer *et al.*, 1996, Epstein *et al.*, 1997, Touyz *et al.*, 1999a, Xu *et al.*, 1996, Lee *et al.*, 2007, Kyaw *et al.*, 2004, Yang *et al.*, 2005, Mugabe *et al.*, 2010), and its effect was inhibited by both Ang-(1-7) and Ang-(1-9) via Mas and the AT_2R, respectively. The effect of Ang-(1-7) via Mas agrees with previous reports in rat VSMC (Zhang *et al.*, 2010b). Further investigation into the functional interplay of the RAS peptides in HSVSMC migration identified alterations in ERK1/2 activity and, MMP2 and MMP9 expression as potential mechanisms contributing to the observed results.

Additionally, as Ang II has recently been demonstrated to regulate expression of the miR-132/-212 cluster in rat aortic VSMC, thereby regulating a number of target genes involved in VSMC migration including PTEN, MCP-1 and RASA-1 (Jin *et al.*, 2012), this pathway was also assessed. Ang II-mediated HSVSMC migration was associated with an increase in miR-132 but not miR-212 expression, and a decrease in PTEN expression at the mRNA level. These changes were mediated via the AT_1R and were inhibited by Ang-(1-7) and Ang-(1-9); the effects of Ang-(1-7) and Ang-(1-9) were partially attenuated by antagonism of Mas and the AT_2R, respectively, suggesting a role for these receptors in this setting. However, PTEN protein levels were unchanged. Similarly, there was no change in MCP-1 expression at mRNA or Akt at protein levels, both of which are key proteins involved in the downstream signalling pathways of PTEN, suggesting the changes in PTEN gene expression were not converted to changes in protein levels under these experimental conditions. Additionally, in contrast to the findings in rat aortic VSMC, no changes in RASA-1 were observed. While highlighting a potential mechanism involved in HSVSMC migration, these results also demonstrate key differences in the miRNA response to Ang II between rat and human VSMC (Jin *et al.*, 2012). The role of miRNA-132 in Ang II induced HSVSMC

migration was further investigated through the use of a miR-132 inhibitor, which blocks miR-132 activity and through siRNA-mediated downregulation of DICER, a key enzyme involved in miRNA biogenesis. It was found that Ang II mediated HSVSMC migration was not dependent on miR-132 or synthesis of an alternative miRNA. However, reduced miR-132 activity and DICER expression increased basal migration of unstimulated HSVSMC.

Ang II is a potent mitogen and widely accepted to promote VSMC proliferation via stimulation of the AT_1R [extensively reviewed by (Touyz and Schiffrin, 2000)]. However, in the majority of the studies demonstrating a proliferative role for Ang II the cells used have been isolated from arteries and evidence for Ang II induced proliferation in human VSMC isolated from saphenous veins is conflicting (Mii *et al.*, 1994, Patel *et al.*, 1996). In the present study, Ang II had no effect on proliferation of quiescent HSVSMC following a 48 hours stimulation period. This is in line with previous findings from Mii and colleagues who demonstrated that Ang II (1-1000 nM) had no effect on HSVMC proliferation when stimulated in the presence of either 0.5 % or 10% FCS (Mii *et al.*, 1994). In a later study by Patel *et al*, it was demonstrated that Ang II caused increased HSVSMC proliferation at 1 nM but not at higher concentrations (10 nM or 1 μM) and that this increase in proliferation was via the AT_1R and associated with increased expression of c-fos (Patel *et al.*, 1996).

One possible explanation for the difference in the response to Ang II in cells isolated from arteries and veins may lie in their basal phenotypic differences. Primary VSMC cultures isolated from veins and arteries share many common features, including similarities in morphology and responses to mitogens and chemoattractants (Deng *et al.*, 2006, Yang *et al.*, 1998, Liu *et al.*, 2004a). However, it has been demonstrated that although some responses are similar, VSMC from arteries and veins in culture maintain distinct cell lineage gene expression programmes and responses (Deng *et al.*, 2006). For example, stimulation of human coronary artery smooth muscle cells (HCASMC) in culture with oxidised LDL led to reduced proliferation and migration, and increased expression of growth-inhibitory genes involved in cell cycle control such as cyclin-dependent inhibitors cyclin-dependent kinase inhibitor 1 (CDKN1a) and cyclin-dependent kinase 4 inhibitor C (CKDN2C) (Deng *et al.*, 2006). Conversely, stimulation of HSVSMC under the same conditions resulted in increased

proliferation, partially via increase IGF-1 signalling and activation of NFkB (Deng *et al.*, 2006). While this has yet to be demonstrated to be the case for Ang II, it may explain the differences observed in the proliferative response to Ang II documented in the literature.

While Ang II had no effect on HSVSMC proliferation, stimulation with increasing concentrations of serum resulted in a concentration-dependent increase in HSVSMC proliferation. As Ang-(1-7) has previously been shown to inhibit serum induced proliferation (Tallant and Clark, 2003), FCS was used as the mitogen to assess the effects of Ang-(1-7) and Ang-(1-9) on HSVSMC proliferation. A serum concentration of 5% FCS was chosen as this was sufficient to induce a significant increase in proliferation in comparison to control, unstimulated cells but low enough to avoid overgrowth of the cells within the well.

This study demonstrates for the first time an anti-proliferative role for Ang-(1-7) in human VSMC. In line with previously published data, Ang-(1-7) inhibits HSVSMC proliferation via Mas (Tallant and Clark, 2003, Tallant *et al.*, 1999, Freeman *et al.*, 1996, Zhang *et al.*, 2010b). While in this current study the mechanisms involved in the anti-proliferative effects of Ang-(1-7) have not been defined, previous studies have identified inhibition of MAPK signalling pathways and release of prostacyclin as key in the anti-proliferative effects of Ang-(1-7) (Tallant and Clark, 2003, Zhang *et al.*, 2010b). Ang-(1-7) blocked Ang II- and PDGF-induced activation of ERK1/2 signalling in rat aortic SMC and Ang II-induced activation of ERK1/2 in mouse aortic VSMC (Tallant and Clark, 2003). However, the upstream mechanisms of Ang-(1-7) mediated inhibition of ERK1/2 have still to be established. Additionally, Ang-(1-7) has been demonstrated to inhibit VSMC proliferation through stimulation of COX-mediated PGI2 release (Tallant and Clark, 2003, Jaiswal *et al.*, 1993b, Jaiswal *et al.*, 1993a, Muthalif *et al.*, 1998).

This study also demonstrates for the first time an anti-proliferative role for Ang-(1-9) in human VSMC. In fact, these findings are the first to identify a direct effect of Ang-(1-9) in VSMC via the AT_2R, and although signalling via this receptor is poorly defined, the AT_2R has been linked to reduced VSMC proliferation *in vitro*. For example, adenoviral-mediated over expression of the AT_2R in rat VSMC resulted in a reduction in Ang II mediated VSMC proliferation

via the AT_2R, an effect which was linked to reduced MAPK activity (Nakajima *et al.*, 1995, Stoll *et al.*, 1995). While the mechanisms involved in the anti-proliferative effects of Ang-(1-9) in HSVSMC proliferation have yet to be established, inhibition of MAPK signalling may be involved as data from this present study suggests that Ang-(1-9) modulates ERK1/2 phosphorylation, as discussed below.

In addition to VSMC proliferation, Ang II is known to promote VSMC migration, another key process in vascular remodelling. The data from this study demonstrate that Ang II promotes HSVSMC migration via the AT_1R. This finding is in line with previously published studies (Schieffer *et al.*, 1996, Epstein *et al.*, 1997, Touyz *et al.*, 1999a, Xu *et al.*, 1996, Lee *et al.*, 2007, Kyaw *et al.*, 2004, Yang *et al.*, 2005, Mugabe *et al.*, 2010). The findings from this current study so far demonstrate that Ang-(1-7) and Ang-(1-9) prevent both HSVSMC proliferation and migration in response to FCS and Ang II, respectively. However, as one of the main aims of this thesis is to investigate the interaction of Ang II and the counter-regulatory peptides Ang-(1-7) and Ang-(1-9) in HSVSMC, it was decided that a more detailed assessment of the mechanisms involved in the effects of these peptides in HSVSMC migration would be performed, as there is clear modulation of the effects of Ang II by Ang-(1-7) and Ang-(1-9) in this setting.

Ang II via the AT_1R activates a number of intracellular signal transduction pathways that are linked to long term regulation of VSMC function, such as cell growth and migration. These processes are initiated within minutes and involve phosphorylation of a number of protein kinases, including MAPK, such as ERK1/2, JNK and p38 MAPK (Xi *et al.*, 1999, Ohtsu *et al.*, 2005, Lee *et al.*, 2007). Ang II mediated phosphorylation of ERK1/2 in particular has been demonstrated to be an important mechanism involved in VSMC growth and migration (Xi, Graf *et al.* 1999; Ohtsu, Mifune *et al.* 2005; Jiang, Bujo *et al.* 2008; Shen, Zhu *et al.* 2014) and importantly Ang-(1-7) has been demonstrated to prevent Ang II induced migration of rat VSMC via inhibition of Ang II-mediated phosphorylation of ERK1/2 (Zhang *et al.*, 2010b). In this present study it was found that Ang II cause a transient increase in ERK1/2 phosphorylation at 5 minutes post-stimulation and ERK1/2 levels returned to similar levels as unstimulated cells by 30 minutes. This increase in ERK1/2 phosphorylation in the minutes following stimulation with Ang II is consistent with previous studies in rat VSMC where it was found that Ang

II induced ERK1/2 phosphorylation was maximal 3 to 10 minutes post stimulation (Eguchi *et al.*, 1996, Touyz *et al.*, 1999d, Kyaw *et al.*, 2004, Mugabe *et al.*, 2010). However, in contrast with the findings from this present study, others have found that Ang II-induced phosphorylation of ERK1/2 remained elevated at both 30 at 60 minutes post stimulation (Zhang *et al.*, 2010b, Eguchi *et al.*, 1996, Touyz *et al.*, 1999d), however, this may be due to variation between cells from different vascular beds and species.

Interestingly, both Ang-(1-7) and Ang-(1-9) blocked Ang II-induced ERK1/2 phosphorylation in HSVSMC at 5 minutes post stimulation. For Ang-(1-7) this is consistent with a previous study in rat VSMC using a scratch assay, where Ang-(1-7) inhibited Ang II-induced VSMC migration via inhibition of ERK1/2 signalling (Zhang *et al.*, 2010b). While the signalling mechanisms involved have yet to be defined, it is likely that Ang-(1-7) can activate a signalling pathway that leads to blockage of ERK1/2 activation or its downstream signalling and therefore can inhibit Ang II-induced ERK1/2 activation and Ang II-stimulated HSVSMC migration. For example, Ang II mediated activation of c-Src, a vital component in the regulation of focal contact formation, is blocked by Ang-(1-7) in human endothelial cells, and c-Src is upstream of ERK1/2 signalling (Sampaio *et al.*, 2007b). Therefore, a similar effect may occur in HSVSMC; however, further investigation is required to confirm this.

Ang-(1-9) was shown to inhibit Ang II-mediated HSVSMC migration via the AT_2R, therefore it is plausible that Ang-(1-9) also inhibits Ang II-induced ERK1/2 phosphorylation via the AT_2R. While signalling via the AT_2R is poorly characterised, activation of various pathways that involve tyrosine or serine/threonine phosphatases has been suggested as potential signalling pathways (Horiuchi *et al.*, 1999). Phosphatases suggested to be involved in AT_2R signalling include mitogen-activated protein kinase phosphatase 1 (MKP-1), SH2 domain containing phosphatase (SHP-1) and protein phosphatase 2A (PP2A) (Nouet and Nahmias, 2000). Interestingly, these phosphatases have been shown to interact with the ERK1/2 pathway, identifying them as potential regulators of Ang-(1-9)-mediated inhibition of ERK1/2 activation (Calo *et al.*, 2010b). At 60 minutes post-stimulation there was a significant increase in ERK1/2 phosphorylation in cells stimulated with Ang-(1-9) alone or in combination with Ang II. As no changes were observed in ERK1/2 phosphorylation at this time point

in cells stimulated with Ang II alone, this increased phosphorylation is likely due to a direct action of Ang-(1-9). This is the first evidence of a direct effect of Ang-(1-9) in VSMC. ERK1/2 activation is most often associated with pro-proliferative and pro-migratory effects in VSMC [reviewed by (Touyz and Schiffrin, 2000)], while Ang-(1-9) has been demonstrated to have no effect on HSVSMC migration alone and have anti-migratory and anti-proliferative effects in response to Ang II and serum, respectively. Therefore, it is unclear what the functional consequences of Ang-(1-9) mediated ERK1/2 phosphorylation are and further investigation is required.

It has been well documented that MMP2 and MMP9 are involved in VSMC migration by degradation of the ECM and are upregulated in various pathologies involving VSMC migration (Southgate *et al.*, 1996). The data in this current study suggest Ang II reduces MMP2 expression, an effect prevented by Ang-(1-9) but not Ang-(1-7), and AngII causes an increase in MMP9 expression, which is prevented by Ang-(1-9) and Ang-(1-7). While Ang II has previously been shown to upregulate MMP2 and MMP9 expression in cardiovascular pathology (Jung *et al.*, 2010), it has been shown that in the same cell type Ang II can cause a reduction in MMP2 expression and this effect is unaltered by Ang-(1-7), while MMP9 is upregulated by Ang II and this upregulation is inhibited by Ang-(1-7) (Pan *et al.*, 2008). This is consistent with the findings from this study. However, it is important to note that these changes have only been demonstrated at mRNA level and a further assessment of protein expression and activity would be required. These differential effects of Ang-(1-7) and Ang-(1-9) on MMP expression indicate that they may have differential mechanisms of actions, consistent with the use of different receptors.

Ang II has recently been demonstrated to regulate expression of the miR-132/-212 cluster in rat aortic VSMC, leading to alterations in various target genes relevant to VSMC migration, such as PTEN, MCP-1 and RASA-1 (Jin *et al.*, 2012). Therefore, this was also assessed in the present study to investigate a role for Ang II-mediate miR-132/-212 regulation in HSVSMC migration. The data demonstrate that Ang II-mediated HSVSMC migration was associated with an increase in miR-132 but not miR-212 expression. As both miRNA's exist in a cluster it would be expected that as one miRNA increases in expression the other would increase (van Rooij and Olson, 2007). However, this differential

expression of miR-132 and miR-212 in response to Ang II has also been observed in adult rat cardiac fibroblasts (Jiang *et al.*, 2013). In these cells miR-132 was found to be increased in response to Ang II at 24 hours post stimulation, however, miR-212 was not found to be regulated (Jiang *et al.*, 2013). It is possible that the miR-132 and miR-212 responses to Ang II differ between species and cell type. Based on the findings that Ang II regulated miR-132 in HSVSMC, expression of its validated targets PTEN and RASA-1 was assessed. While no changes were observed in RASA-1 expression, Ang II significantly reduced PTEN expression. Additionally, MMP9 and the AT_1R have both been identified as potential targets of miR-132 in cardiac fibroblasts and rat VSMC, respectively (Jiang *et al.*, 2013, Elton, 2008), however, these findings were not replicated in this current data set as MMP9 expression was increased in response to Ang II while AT_1R expression was unchanged.

Increased PTEN levels are associated with reduced VSMC migration, due to inhibition of PI3K signalling (Huang and Kontos, 2002). Overexpression of PTEN via an adenoviral vector (AdPTEN) inhibited Ang II and PDGF mediated proliferation and migration of VSMCs (Huang and Kontos, 2002, Dong *et al.*, 2013). Both these studies reported a reduction in phosphorylation of Akt and FAK as a result of PTEN expression. Interestingly, phosphorylation of Akt and FAK have previously been demonstrated to increase in a time dependent manner in response to Ang II stimulation of VSMC, highlighting the importance of Ang II induced reduction of PTEN expression in VSMC migration (Dong *et al.*, 2013). Inhibition of Akt in VSMCs has been demonstrated to inhibit migration which is thought to occur due to prevention of cytoskeleton remodelling (Galaria *et al.*, 2005). FAK however is important for the generation of focal contacts in migration (Gerthoffer, 2007), therefore suggesting PTEN overexpression may also reduce focal contact formation. Furthermore, increased expression of PTEN has been linked to reduce expression of the pro-inflammatory cytokine MCP-1 following cuff injury in rats (Koide *et al.*, 2007). This was suggested to be linked to inactivation of NF-κB due to the reduced phosphorylation of inhibitor of kappa b-alpha (IκB-α), an inhibitory protein that dissociates from NF-κB when phosphorylated allowing activation, in AdPTEN transduced mice (Koide *et al.*, 2007). This pathway was further supported in a study using rat arterial VSMCs

which were deficient in PTEN, where increased cytokine production was inhibited by PI3K/Akt or NF-κB suppression (Furgeson *et al.*, 2010).

Due to the fact that Ang II stimulation of HSVSMC led to changes in PTEN at the mRNA level the PTEN pathway was investigated further, by assessing protein levels of PTEN and changes in expression levels of molecules downstream from PTEN in the signalling cascade. In this study, PTEN protein levels were unchanged at 24 hours following stimulation with Ang II. In a recent study, Ang II was found to reduce PTEN expression at the protein level in rat aortic VSMC at 12 hours following stimulation. Along with the different origins of these cells, inconsistent PTEN regulation may be due to a greater reduction in PTEN mRNA levels in rat arterial VSMCs of approximately 0.4 fold after 12 hours in contrast to around 0.6 fold in the present study following 24 hours Ang II stimulation. The lack of change in PTEN protein does not however rule out the regulation of PTEN post-translation by Ang II. Dong *et al* demonstrated that Ang II increased ROS production in rat VSMC leading to rapid phosphorylation or oxidation of PTEN, resulting in its de-activation (Dong *et al.*, 2013). However this regulation would be independent of miR-132 due to the temporal aspects of the reported de-activation of PTEN; 30 minutes and 120 minutes for phosphorylation and oxidation respectively (Dong *et al.*, 2013). Similarly, there was no change in MCP-1 expression at mRNA or Akt at protein levels observed in the current study, both of which are key proteins involved in the downstream signalling pathways of PTEN, suggesting these changes in gene expression were not converted to protein within these experimental conditions. MCP-1 is known to promote VSMC migration (Ma *et al.*, 2007) and mRNA levels of MCP-1 have been reported to be increased in a concentration-and time dependent manner in response to Ang II in rat arterial VSMC (Chen *et al.*, 1998), which is inconsistent with the results from the present study where there was no change in MCP-1 mRNA expression following Ang II stimulation for 24 hours. This discrepancy may be due to the HSVSMC used in this study in comparison to rodent arterial VSMC used in other studies (Ma *et al.*, 2007, Jin *et al.*, 2012).

The role of miRNA-132 in Ang II-induced HSVSMC migration was further investigated through the use of a miR-132 inhibitor, which blocks miR-132 activity and through siRNA-mediated downregulation of DICER, a key enzyme involved in miRNA biogenesis (van Rooij and Olson, 2007, Bartel, 2009). It was

found that Ang II-mediated HSVSMC migration was not dependent on miR-132 or through synthesis of an alternative miRNA via DICER. However, this is not entirely unexpected as Ang II is a powerful mitogen and has been demonstrated to promote VSMC proliferation and migration through a cascade of various cell signalling pathways [reviewed by (Touyz and Schiffrin, 2000)]. Therefore, it is highly possible that Ang II mediate HSVSMC migration is via an alternative pathway independent of regulation of miRNA. However, reduced miR-132 activity and DICER expression increased basal migration of unstimulated HSVSMC. There is no published evidence to support a role for alterations in miR-132 or -212 in the absence of stimuli. However, a number of miRNAs have been demonstrated to be active during the phenotypic switching of VSMC from the synthetic to the quiescent state, and would therefore be active during serum starvation (Leeper *et al.*, 2011). As HSVSMC were transfected with the DICER siRNA prior to being quiesced it is possible that during the 48 hours quiescent period, DICER levels were inhibited and as a result miRNAs involved in maintaining the cells in a quiescent state were not activated. However, further work is required to establish if this is the case and an assessment of miRNA levels in comparison to non-quiescent HSVSMC would be required.

3.5 Conclusion

In summary, the data demonstrate that Ang-(1-7) or Ang-(1-9),via Mas and the AT_2R respectively, block FCS induced proliferation and Ang II-induced migration of HSVSMC. Investigation into the functional interplay of Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC identified regulation of ERK1/2 activity, and MMP2 and MMP9 as potential mechanisms contributing to the observed results. While further work is required to fully elucidate the signal transduction pathways of Ang-(1-7) and Ang-(1-9) in HSVSMC, this data demonstrates a functional role for of Ang-(1-9) in VSMC and is the first to directly compare the effects of Ang-(!-7) and Ang-(1-9) in the vasculature, identifying both peptides as potential therapeutic targets in acute vascular injury.

Additionally, Ang II-mediated HSVSMC migration was demonstrated to be associated with an increase in miR-132 expression and downregulation of its target PTEN at the mRNA level, consistent with previous reports in rat VSMC (Jin *et al.*, 2012). These changes were found to be via the AT_1R and inhibited by Ang-

(1-7) and Ang-(1-9). However, PTEN protein levels were unchanged and no changes were observed in key proteins involved in the downstream signalling pathways of PTEN, including Akt and MCP-1. The role of miR-132 in Ang II induced HSVSMC migration was further investigated through the use of a miR-132 inhibitor and downregulation of DICER. While it was found that Ang-II mediated HSVSMC migration was not dependent on miR-132 or regulation of an alternative miRNA via DICER, it was found that inhibition of miR-132 or DICER expression increased basal HSVSMC migration. Together these findings demonstrate key differences in the miRNA response to Ang II between rat and human VSMC, and identify a further potential mechanism of HSVSMC migration independent of Ang II.

Chapter 4

The effects of Ang-(1-7) and Ang-(1-9) on vascular endothelial cell function

4.1 Introduction

In the vasculature the endothelium exists as a single cell monolayer surrounding the lumenal surface of the vessel wall. The endothelium is able to respond to a large number of physical and chemical signals leading to the production of a vast number of factors that regulate vascular tone, cell adhesion, thrombogenicity, inflammation and VSMC proliferation (White *et al.*, 1994, Furchgott and Zawadzki, 1980, Saye *et al.*, 1984, Kinlay *et al.*, 2001, Ghosh and Karin, 2002, Gryglewski *et al.*, 1986, Darley-Usmar *et al.*, 1992). With the endothelium playing such an important role in vascular homeostasis, it is no surprise that damage to the endothelial layer leads to the development and progression of various vascular pathologies. Reduced NO bioavailability occurs as a result of increased NO degradation or reduced NO production, both of which are largely triggered by oxidative stress and the production of ROS (White *et al.*, 1994, Darley-Usmar *et al.*, 1992, Gryglewski *et al.*, 1986). ROS production is elevated by a number of CVD risk factors including hypertension, hypercholesterolaemia and smoking. This alteration in ROS levels leads to reduced endothelial function, increased VSMC growth and increased vascular inflammation, all processes which underlie the pathology of vascular remodelling and disease (Cai and Harrison, 2000).

Prolonged exposure to CVD risk factors, or physical endothelial denudation (as occurs during stent deployment in angioplasty procedures or surgical preparation of bypass grafts prior to CABG procedures), leads to reduced endothelial function and integrity, and untimately to remodelling of the vasculature and failure of revascularisation attempts (Kipshidze *et al.*, 2004, Inoue *et al.*, 2011, Van Belle *et al.*, 1998). Importantly, the functioning and integrity of the endothelium not only depends on the extent of the injury but also on the endogenous capacity for repair (Deanfield *et al.*, 2007). One mechanism through which the endothelial layer is repaired is by proliferation of adjacent mature endothelial cells, which can then migrate to the denuded area to replace the lost and damaged cells (Deanfield *et al.*, 2007). Following vascular injury both endothelial cells and VSMC become activated, leading to the production and release of various growth factors, including bFGF and vascular endothelial growth factor (VEGF). These growth factors have been demonstrated to

contribute to enhanced endothelial cell growth (Schweigerer *et al.*, 1987, Tsurumi *et al.*, 1997, Lindner *et al.*, 1990).

Re-growth of the endothelial layer is essential in acute vascular injury and has been demonstrated to repress neointimal thickening and the occurrence of thrombosis. However, the new endothelial layer may also be dysfunctional, resulting in decreased vascular integrity, increased permeability and impaired vasodilation (Weidinger *et al.*, 1990, Hamon *et al.*, 1995, Hamon *et al.*, 1996, Kipshidze *et al.*, 2004). This impaired functioning of the neo-endothelial layer has been shown to be maintained for some time following the completion of re-endothelialisation (Shimokawa *et al.*, 1987, Weidinger *et al.*, 1990). The dysfunctional neo-endothelial layer has been linked to impaired vasomotion and responses to both vasodilator and vasoconstrictor stimuli (Shimokawa *et al.*, 1989, Shimokawa *et al.*, 1987, Mc Fadden *et al.*, 1993, Hamon *et al.*, 1996). For example, enhanced constrictor responses have been observed in human arterial segments previously subjected to angioplasty (Mc Fadden *et al.*, 1993, Hamon *et al.*, 1996). Furthermore, the neo-endothelial layer has also been demonstrated to have a reduced ability to generate NO, linking this dysfunctional re-growth to increased vascular remodelling, inflammation and thrombus formation (Weidinger *et al.*, 1990, Saroyan *et al.*, 1992, Hamon *et al.*, 1995). Therefore, the optimal treatment strategy in acute vascular injury would not only allow complete re-endothelialisation but would contribute to improved functioning of the neo-endothelium.

A role for Ang-(1-7) in improved endothelial function has been well established. Ang-(1-7) has been shown to increase NO release via two main mechanisms. First, directly through interaction with Mas, Ang 1-7 stimulates eNOS activation via reciprocal phosphorylation/dephosphorylation at $Serine^{1177}$/$Threonine^{495}$ further leading to sustained Akt phosphorylation (Sampaio *et al.*, 2007b). Second, Ang-(1-7) indirectly increases NO release via production of bradykinin and receptor cross talk with the bradykinin BK_2R (Jackman *et al.*, 2002, Sampaio *et al.*, 2007a). Ang-(1-7)-mediated NO release has been linked to improved vascular endothelial function, vasodilation, and reduced ROS production, inflammation and thrombosis (Brosnihan *et al.*, 1996, Faria-Silva *et al.*, 2005, Stegbauer *et al.*, 2011). Combined, these effects of Ang-(1-7) have been linked

to reduced atherosclerosis and improved endothelial function following vein grafting (Langeveld *et al.*, 2008, Stegbauer *et al.*, 2011).

However, there is much less evidence from the literature in support of a direct effect of Ang-(1-9) in the endothelium. Ang-(1-9) has been shown to potentiate NO release indirectly via interaction with bradykinin signalling. For example, in human pulmonary artery endothelial cells Ang-(1-9) has been shown to stimulate bradykinin release and to enhance the effects of bradykinin in EC by augmenting NO and arachidonic acid release (Erdos *et al.*, 2002, Jackman *et al.*, 2002). Importantly, it was shown that not only was Ang-(1-9) an active peptide but that it is more potent than Ang-(1-7) in achieving these results (Jackman *et al.*, 2002). Ang-(1-9) has been demonstrated to have a direct beneficial effect in the vasculature by increasing NO bioavailability (Flores-Munoz *et al.*, 2012). Continuous infusion of Ang-(1-9) improved aortic vasorelaxation in the SHRSP through increased NO bioavailability, an effect which was blocked by PD123,319, suggesting that these effects are due to Ang-(1-9) acting via the AT_2R (Flores-Munoz *et al.*, 2012). More recently, Ang-(1-9) was found to induce relaxation of rat aortic rings in a concentration- and endothelium-dependent manner; this effect was inhibited by PD123,319 but not A779 or losartan, suggesting that in this setting Ang-(1-9) mediated vasdodilation of aortic rings via the AT_2R (Ocaranza *et al.*, 2014). However, the mechanisms through which Ang-(1-9) acts to achieve this is currently unknown and a direct effect of Ang-(1-9) on NO release in human endothelial cells has yet to be demonstrated.

4.1.1 Aims

The aims of this chapter were to:

- To establish the optimal conditions for proliferation of HSVEC and assess the effect of Ang-II, Ang-(1-7) and Ang-(1-9).

- To assess the effect of Ang II, Ang-(1-7) and Ang-(1-9) on HSVEC migration.

- To determine the effects of Ang-(1-9) on release of NO from HSVEC.

- To investigate the effect of Ang-(1-9) on vascular tone in the aorta and mesenteric arteries of $AT_2R^{-/-}$ mice.

4.2 Results

4.2.1 AT_1R, AT_2R and Mas expression in primary HSVEC

Prior to investigating the effects of Ang-(1-7) and Ang-(1-9) in HSVEC, it was first confirmed that the AT_1R, AT_2R and Mas were expressed in HSVEC via qRT-PCR (Figure 4.1). The AT_1R (dCt 12.1 ± 2.3) was the most highly expressed of the RAS receptors, followed by Mas (dCt 14.2 ± 0.1) and then the AT_2R (dCt 16.3 ± 1.9) (Figure 4.1).

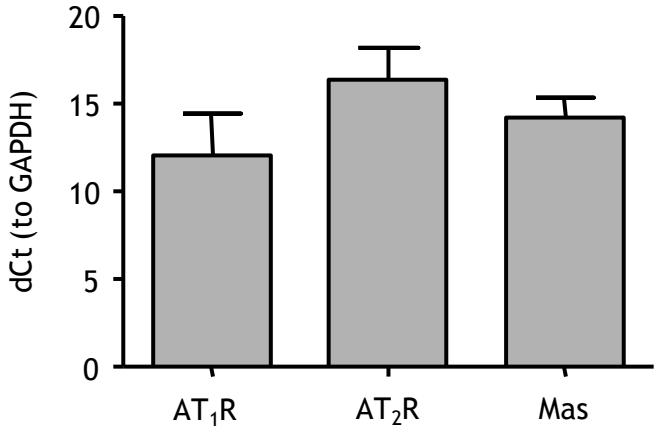

Figure 4.1 Expression of AT_1R, AT_2R and Mas in HSVEC.
Expression levels of AT_1R, AT_2R and Mas in HSVEC was assessed via qRT-PCR using specific Taqman probes for each receptor. Results are expressed as average dCt ± S.E.M, relative to the endogenous housekeeping gene GAPDH. N=3.

4.2.2 Assessing the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVEC proliferation

To establish the optimal conditions for HSVEC proliferation, cells were quiesced for 24 hours and then exposed to fresh media containing increasing concentrations of FCS (0-20% v/v) for 24 hours and HSVEC proliferation assessed via the MTS assay. It was found that FCS induced HSVEC proliferation in a concentration-dependent manner, with concentrations of 5% FCS and above producing significantly increased proliferation in comparison to unstimulated, serum free control cells ($P<0.05$) (Figure 4.2 A). Therefore, for subsequent experiments 5% FCS was used to induce HSVEC proliferation.

To assess whether Ang-(1-7) or Ang-(1-9) blocked HSVEC proliferation quiescent cells were incubated with either Ang-(1-7) or Ang-(1-9) (200 nM) and stimulated with 5% FCS for 24 hours. As expected, 5% FCS caused a significant increase in HSVEC proliferation in comparison to control cells (Figure 4.3 B). However, proliferation was unaffected by Ang-(1-9) and Ang-(1-7) and proliferation remained significantly increased in comparison to control cells ($P<0.05$ vs control cells) (Figure 4.2 B).

To investigate if Ang II, Ang-(1-7) or Ang-(1-9) themselves were able to induce HSVEC proliferation, quiescent cells were stimulated with each peptide in serum free media for 24 hours and then cell proliferation was assessed. While 5% FCS induced a significant increase in cell proliferation in comparison to control cells ($P<0.05$), no significant difference was observed between control cells and cells stimulated with either Ang II, Ang-(1-7) or Ang-(1-9) (Figure 4.2 C).

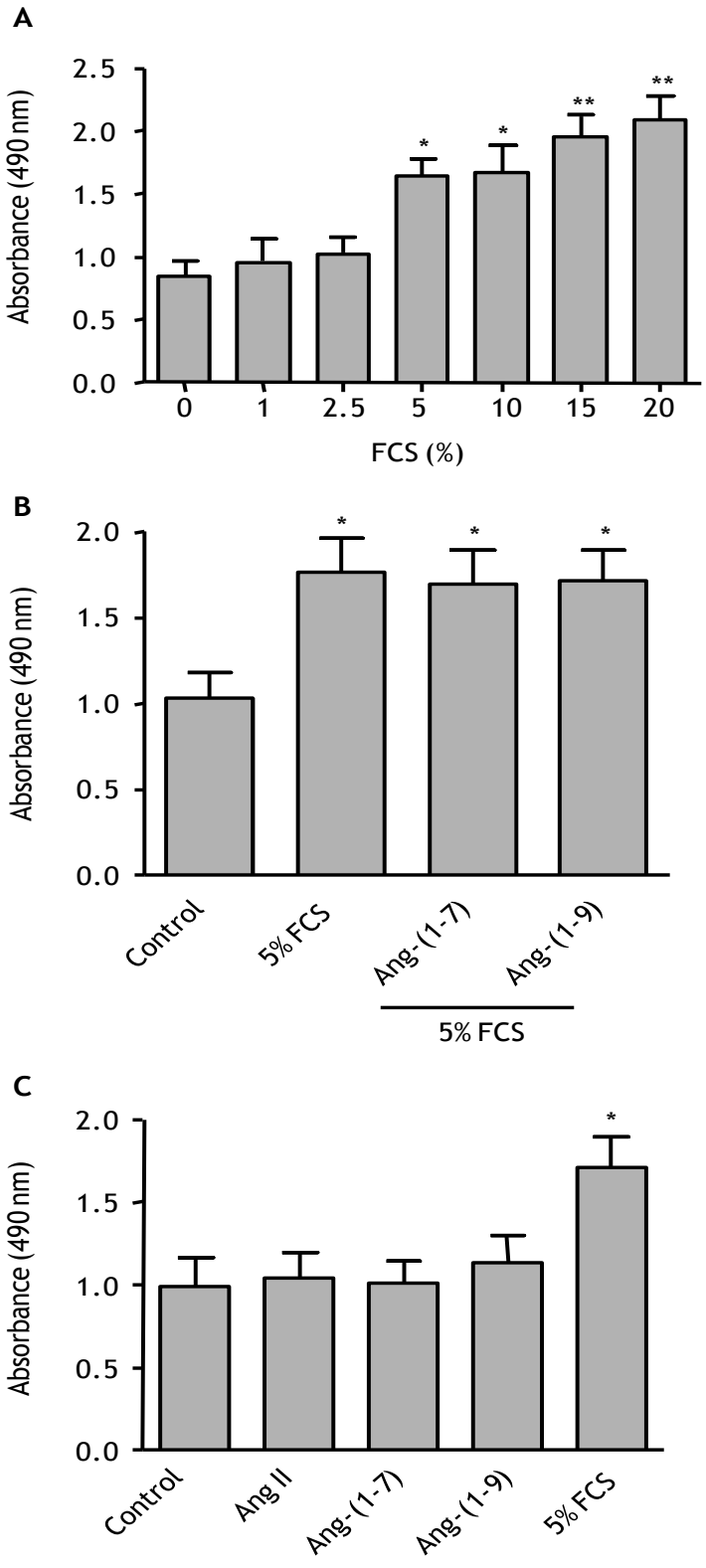

Figure 4.2 Assessment of the effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVEC proliferation

Proliferation of HSVEC was assessed using the MTS assay. (A) To assess the concentration of FCS required to stimulate proliferation, HSVEC were exposed to media containing increasing concentrations of FCS for 24 hours. (B) Cells were incubated with Ang-(1-7) or Ang-(1-9) for 24 hours, and stimulated with 5% FCS to assess the effect of the peptides. (C) To assess if Ang II, Ang-(1-7) or Ang-(1-9) induced proliferation of HSVEC cells were exposed to the peptides in the absence of serum for 24 hours; 5% FCS was used as a positive control. N=3-4. *P<0.05, **P<0.01 vs. control.

4.2.3 Effect of Ang II, Ang-(1-7) and Ang-(1-9) on HSVEC migration

As migration of resident endothelial cells is an important process in re-endothelialisation following vascular injury (Deanfield *et al.*, 2007), the effects of Ang II, Ang-(1-7) or Ang-(1-9), alone or in combination, on HSVEC migration was assessed. This was achieved using a scratch assay where scratches were induced in the monolayer of quiescent HSVEC prior to stimulation with RAS peptides. HSVEC migration was quantified as a percentage reduction in scratch width over time. Cells in serum free media alone were used as a control and basal HSVEC migration over time resulted in a 43.7 ± 4.5 % reduction in scratch size by 30 hours (Figure 4.3). Ang II, Ang-(1-9) and Ang-(1-7), alone or in combination had no effect on HSVEC migration in comparison to control cells at any time point ($P>0.05$), suggesting they do not induce HSVEC migration (Figure 4.3). HSVEC stimulated with complete HSVEC growth media containing 20% (v/v) FCS were included as a positive control and it was found that 20% FCS resulted in a significant increase in HSVEC migration in comparison to control cells at each time point ($P<0.05$) and by 24-30 hours the wound had completely closed (Figure 4.3).

To assess if Ang II, Ang-(1-7) or Ang-(1-9), alone or in combination, inhibited serum stimulated HSVEC migration, HSVEC were stimulated with 20% FCS and each peptide alone or in combination. The peptides alone or in combination did not affect FCS induced HSVEC migration, as there was an equivalent increase in HSVEC migration over time as 20% FCS alone and complete wound closure by 24 hours for all combinations of peptides (Figure 4.4).

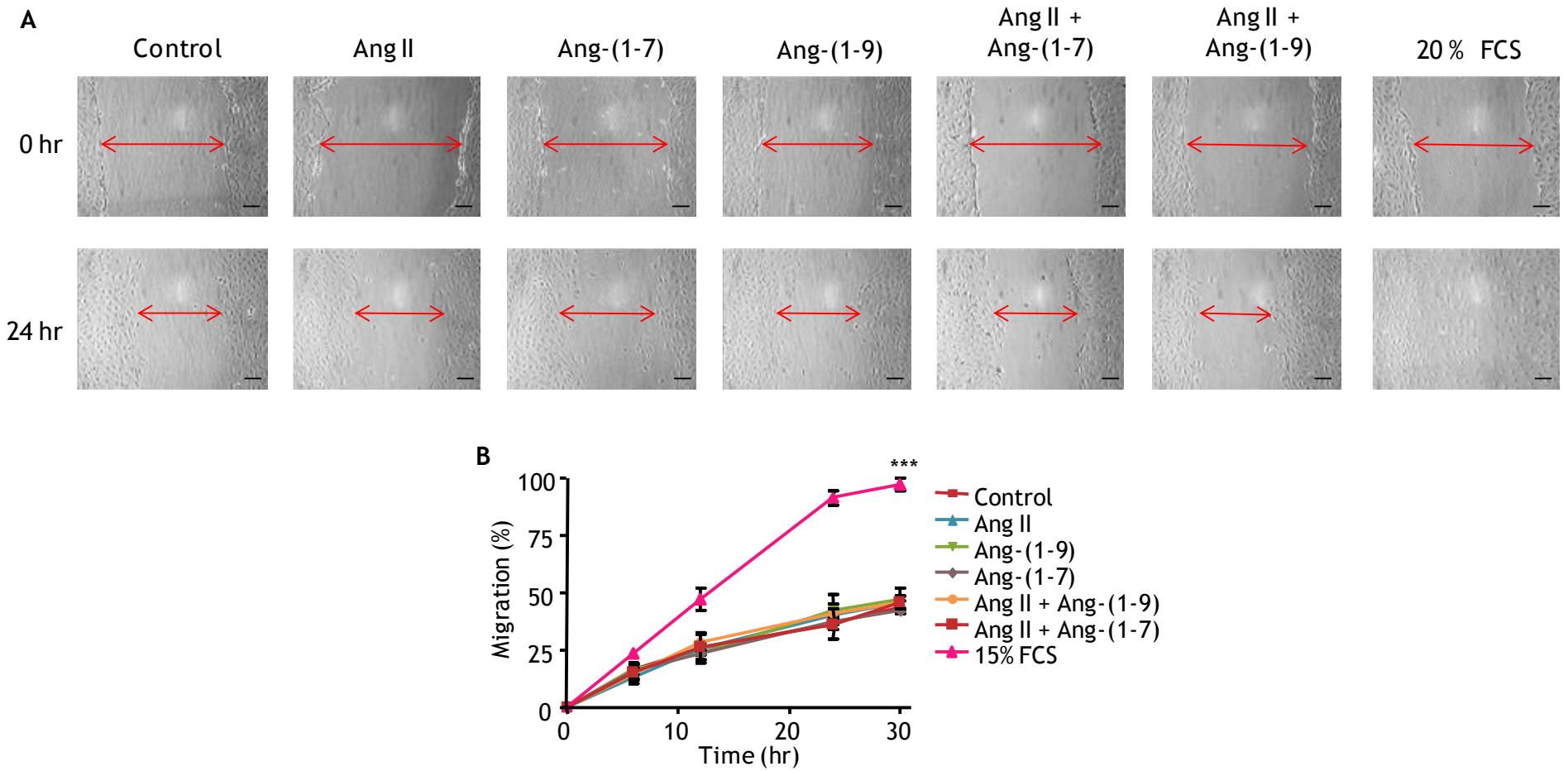

Figure 4.3 The effects of Ang II, Ang-(1-7) and Ang-(1-9) on HSVEC migration
HSVEC migration was assessed using a scratch assay. Following starvation in serum free media for 24 hours, 3 scratches were induced in the cell monolayer of each well. Cells were then stimulated with Ang II, Ang-(1-9) or Ang-(1-7), alone or in combination (200nM each), in serum free media. Images of the scratch were taken at 0, 6, 12, 24 and 30 hours post scratch. (A) Representative images of scratch at 0 hours and 24 hours. Scale bar = 40 µm. Magnification 10x.(B) Migration was quantified as a reduction in scratch size (%) relative to the 0 hour measurement (B). N=3. ***P<0.001 vs. control.

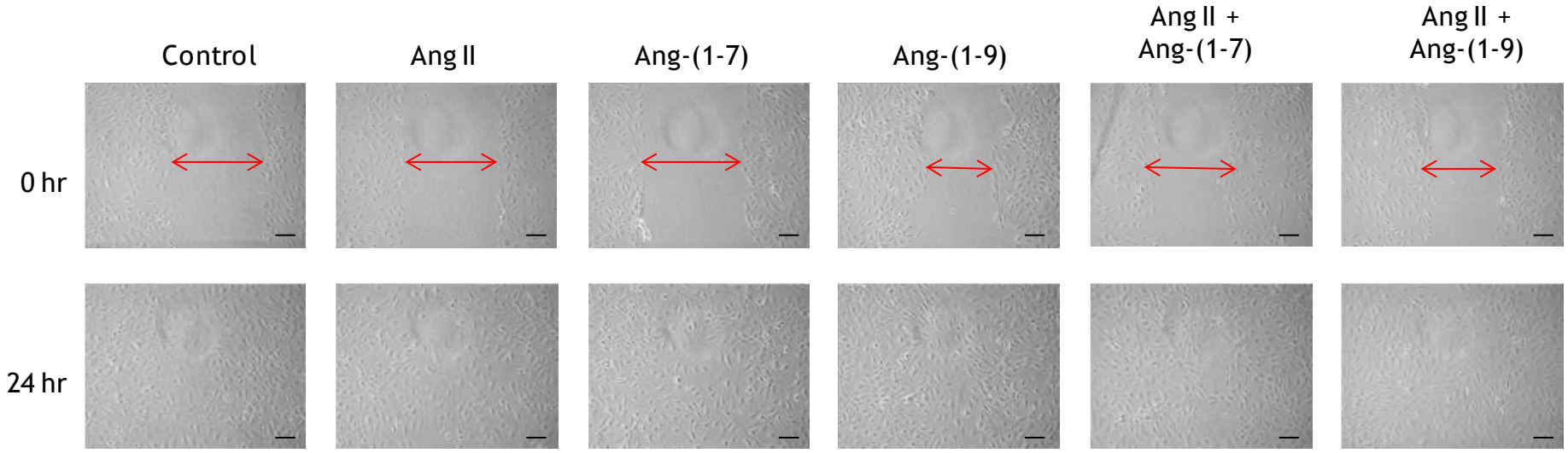

Figure 4.4 The effect of Ang II, Ang-(1-7) and Ang-(1-9) on serum induced migration of HSVEC
HSVEC migration was assessed using a scratch assay. Following starvation in serum free media for 24 hours, 3 scratches were induced in the cell monolayer of each well. Cells were then stimulated with Ang II, Ang-(1-9) or Ang-(1-7), alone or in combination (200nM each), in the presence of 20% FCS. Images of the scratch were taken at 0 and 24 hours. Representative images of each condition from an individual experiment which was performed on 3 separate occasions. Scale bar = 40 µm. Magnification 10x.

4.2.4 Effects of Ang-(1-9) on nitric oxide release

In order to investigate the effect of Ang-(1-9) on NO release the cell-permeable, fluorescent indicator of NO, DAF-FM, was used. All cells were serum starved for 2 hours in phenol free media to minimise basal NO release, however, some NO activity was maintained in control, unstimulated HSVEC as evidenced by the observed fluorescence signal when cells were loaded with DAF-FM (Figure 4.5). In control cells the fluorescence signal was most intense in the centre of the cells, however, further cellular staining would be required to accurately assess NO localisation within the cell. When stimulated with Ang-(1-9) at 200 nM or 1 µm, there was an increase in the intensity of the fluorescence signal within discrete central regions of the cell and there was a change in cell morphology, with cells becoming smaller and losing their characteristic cobblestone appearance, in comparison to unstimulated control cells at 15 minutes post-stimulation (Figure 4.5). The Ang-(1-9)-induced increase in fluorescence and change in cell morphology was blocked by co-incubation with PD123,319, with cells exposed to both peptide and antagonist being similar to control cells (Figure 4.5). When HSVEC were stimulated with PD123,319 alone no changes in fluorescence or cell morphology in comparison to unstimulated cells were observed (Figure 4.5).

To further assess the involvement of the AT_2R in Ang-(1-9) mediated NO release, CHO cells were transfected with a plasmid engineered to express the human AT_2R and then loaded with DAF-FM before being stimulated with Ang-(1-9) (Figure 4.6). Untransfected cells and cells transfected with the AT_2R displayed no basal NO release, as evidenced by the lack of fluorescence signal observed (Figure 4.6) While Ang-(1-9) had no effect on untransfected CHO cells, in CHO cells expressing the AT_2R stimulation with Ang-(1-9) led to generation or release of NO, evidenced by increased fluorescence when treated with Ang-(1-9) (Figure 4.6). In cells expressing the AT_2R, PD123,319 blocked the response to Ang-(1-9) but had no effect when added alone, providing further evidence to suggest that Ang-(1-9) acts via the AT_2R in this setting (Figure 4.6).

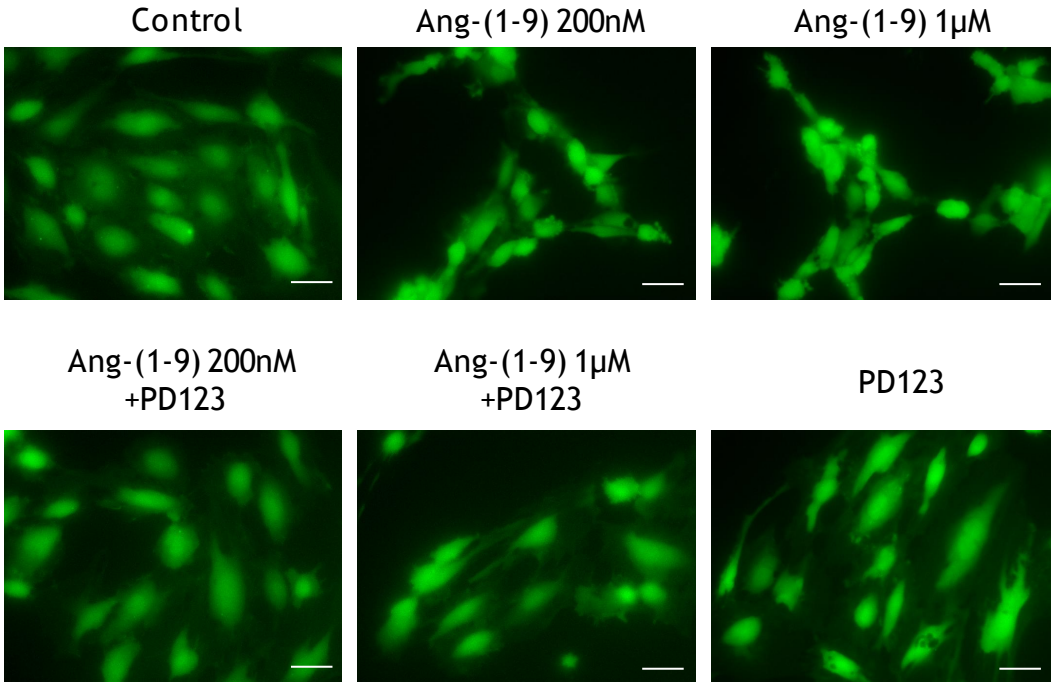

Figure 4.5 Effect of Ang-(1-9) on NO release in HSVEC
Ang-(1-9) mediated NO release from HSVEC was assessed using DAF-FM. Cells were serum starved in phenol free, serum free media for 2 hours to minimise basal NO signalling and then loaded with 5 µM DAF-FM for 30 minutes. Cells were stimulated with Ang-(1-9) (200 nM or 1 µM) alone or in the presence of PD123,319 (PD123) (500nM); cells were also exposed to PD123,319 alone. Images of the cells were taken at 15 minutes post-stimulation with peptide or antagonist of 5 individual areas per condition. Representative images of an experiment performed on 2 separate occasions. Scale bar = 25 µm.

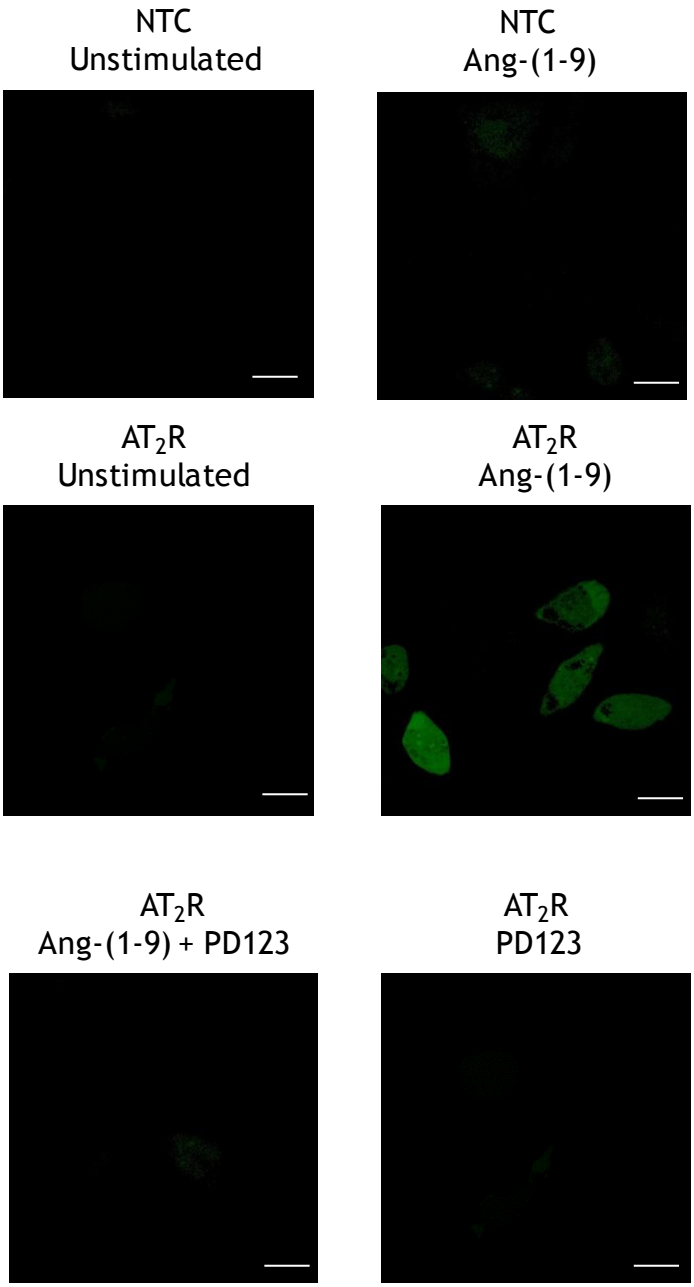

Figure 4.6 Role of the AT_2R in Ang-(1-9) induced NO release.
CHO cells were transfected with a plasmid engineered to express the human AT_2R. The following day cells were loaded with DAF-FM (5 µM) for 30 minutes and then stimulated with Ang-(1-9) (200nM) alone or in the presence of PD123,319 (500nM). Untransfected cells (NTC) control cells and cells stimulated with Ang-(1-9), and transfected cells unstimulated and stimulated with PD123,319 were used as negative controls. Images of the cells were taken at 15 minutes post stimulation of 5 individual areas per condition. Representative images shown of an experiment performed on 2 separate occasions. Scale bar = 10 µm.

4.2.5 Effect of Ang-(1-9) on vascular reactivity in intact vessels

Despite the data suggesting that Ang-(1-9) mediated NO release is via the AT_2R, previous unpublished data from Prof Robson Santos' group demonstrated that stimulation of pre-constricted aortic rings from $AT_2R^{-/-}$ mice with Ang-(1-9) resulted in significant vasorelaxation in an endothelium- and concentration-dependent manner (Prof Robson Santos, Federal University of Minas Gerais, Brazil, personal communication). This finding was confirmed in the present study where it was demonstrated that Ang-(1-9) promoted vasodilation of pre-constricted aortic rings from $AT_2R^{-/-}$ mice in a concentration and endothelium-dependent manner, resulting in 19.2 ± 6.3 % relaxation of the vessel at the maximal concentration of 1 μM (Figure 4.7 A). As Ang-(1-9) is reported to be converted to Ang-(1-7) (Donoghue *et al* 2000), which is widely accepted to promote vasodilation via Mas, vessels were incubated with A779 prior to stimulation with Ang-(1-9). A779 blocked the response to Ang-(1-9) and a negligible response to Ang-(1-9) was observed both in endothelium intact and denuded aortic rings in the presence of A779.

A similar set of experiments was also performed using mesenteric artery rings from $AT_2R^{-/-}$ mice to investigate if this phenotype also occurs in small resistance arteries. Similar to in the aorta, incubation with Ang-(1-9) mediated significant vasodilation of pre-constricted mesenteric artery rings in a concentration dependent manner (Figure 4.7 B). Importantly, this response was also found to be endothelium-dependent as vessels with an intact endothelium produced a 25.8 ± 6.6 % relaxation at the maximum concentration of Ang-(1-9) (1 μM) which was significantly different to the 5.7 ± 2.2 % relaxation observed when the endothelium was denuded. However, in contrast to the findings in aortic rings, A779 had no effect on the vasodilation induced by Ang-(1-9) in either endothelium intact or denuded vessels.

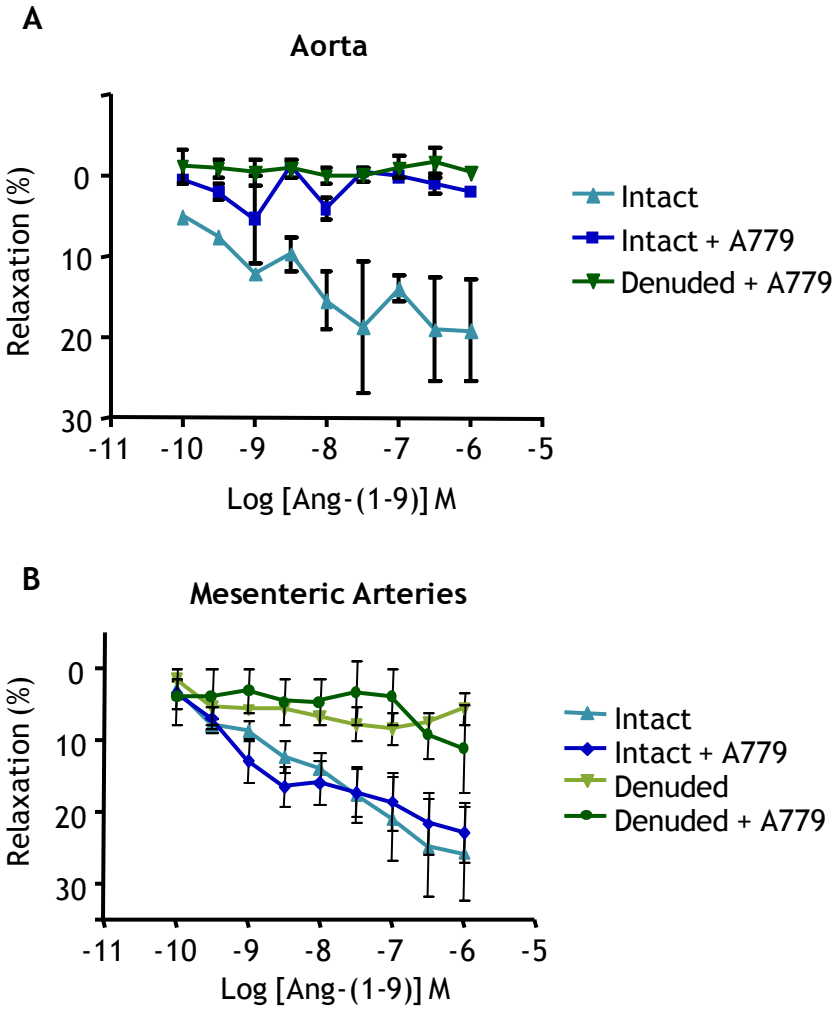

Figure 4.7 Effects of Ang-(1-9) on vascular tone in AT_2R knockout mice
Vasorelaxant response to increasing concentrations of Ang-(1-9) in $AT_2R^{-/-}$ (A) aortic or (B) mesenteric artery rings. Vessel rings were pre-contracted to phenylepherine (Phe) and vasorelaxant response to Ang-(1-9) assessed. Response is expressed as % relaxation of the Phe preconstriction. N=4-6.

4.3 Discussion

In this chapter, the effect of Ang-(1-7) and Ang-(1-9) on endothelial cell growth, migration and function were assessed. It was found that Ang II, Ang-(1-7) or Ang-(1-9) had no effect on growth or migration of primary, adult HSVEC. Furthermore, a direct effect of Ang-(1-9) on NO release from both HSVEC and a cell line that expressed the AT_2R (CHO) was demonstrated. *In vitro* Ang-(1-9) induced NO release in an AT_2R sensitive manner, however in vessels from $AT_2R^{-/-}$ mice the biological effect of Ang-(1-9) was maintained and led to vasodilation of both aortic and mesenteric artery rings.

The findings from this current data set demonstrate that Ang II, Ang-(1-7) or Ang-(1-9) do not influence endothelial cell proliferation or migration. The effects of Ang II on endothelial cell growth and migration have been extensively studied and Ang II has been demonstrated to have both pro- and anti-proliferative effects. For example, Ang II is a potent mediator of angiogenesis and has been demonstrated to promote proliferation and migration of endothelial cells, largely through increased VEGF expression, leading to vessel tube formation and neoangiogenesis (Herr *et al.*, 2008, Zhu *et al.*, 2013, Martini *et al.*, 2010). Conversely, Ang II has been demonstrated to promote endothelial cell apoptosis, largely through the production of ROS and pro-inflammatory cytokines, leading to enhanced endothelial dysfunction and vascular remodelling (Dimmeler *et al.*, 1997, Shan *et al.*, 2008, Liu *et al.*, 2013). These differential results are not entirely unexpected due to the vast array of cell signalling pathways induced by Ang II and are linked to differences in experimental protocols used to study different biological processes that occur in angiogensis and re-endothelialisation. There is currently no evidence from the literature to suggest that Ang-(1-7) or Ang-(1-9) would promote endothelial cell proliferation or migration and the majority of studies to date have focussed on the effects of these peptides on endothelial cell function (Sampaio *et al.*, 2007a, Sampaio *et al.*, 2007b, Jackman *et al.*, 2002).

In addition to endothelial damage, VSMC proliferation is a key process involved in late vein graft failure and in-stent restenosis. One therapeutic approach is targeting the VSMC proliferation that underlies the formation of the neointima through the use of cell cycle inhibitory drugs, such as rapamycin, paclitaxel or

cytochalasin D (Schachner *et al.*, 2004, Murphy *et al.*, 2007). While these drugs have been shown to have beneficial effects leading to reduced neointimal formation they have also been linked to impaired re-endothelialisation through inhibition of endothelial cell proliferation, leading to increased neointimal formation and late stage thrombosis which can have serious clinical implications (Karha and Topol, 2006, Murphy *et al.*, 2007, Luscher *et al.*, 2007, Douglas *et al.*, 2013). Therefore therapies that specifically target VSMC growth and migration, without preventing re-endothelialisation are optimal in vein graft failure and in-stent restenosis (Inoue and Node, 2009). As demonstrated in Chapter 3, both Ang-(1-7) and Ang-(1-9) prevent VSMC proliferation and migration. Combined with the findings in this present study that they do not effect endothelial cell proliferation or migration, this data set identifies these two peptides as potential therapeutics in these pathologies.

Re-growth of the endothelial layer is essential in acute vascular injury; however, the neo-endothelium is often dysfunctional due to an impaired ability to generate NO, which can lead to further vascular remodelling, increased inflammation and thrombosis (Kipshidze *et al.*, 2004, Weidinger *et al.*, 1990, Hamon *et al.*, 1995). Therefore, the optimal treatment strategy would not only allow complete re-endothelialisation but would also contribute to improved functioning of the neo-endothelium through increased NO production.

As discussed, Ang-(1-7) via Mas is known to increase NO production directly via increased eNOS activity and indirectly via interaction with the bradykinin system (Sampaio *et al.*, 2007b, Jackman *et al.*, 2002). This increased NO production has been linked to improved vascular endothelial function, vasodilation, and reduced ROS production, inflammation and thrombosis (Brosnihan *et al.*, 1996, Faria-Silva *et al.*, 2005, Stegbauer *et al.*, 2011). Importantly, Ang-(1-7) has also been linked to improved endothelial function as well as reduced vascular remodelling following stent implantation in rats, identifying this peptide as an attractive therapeutic agent in acute vascular injury (Langeveld *et al.*, 2008, Langeveld *et al.*, 2005).

Comparatively, there is much less known about Ang-(1-9) and for this reason the effect of Ang-(1-9) on NO release was assessed in this present study. Ang-(1-9) stimulated NO release in an AT_2R sensitive manner in both primary HSVEC and in

cells transfected with AT_2R. While this is the first *in vitro* evidence to suggest a direct effect of Ang-(1-9) signalling via the AT_2R leading to production of NO, this has previously been demonstrated *ex vivo* where it was found that aortic rings from SHRSPs infused with Ang-(1-9) displayed improved vasorelaxation which was blunted by the NOS inhibitor, L-NAME (Flores-Munoz *et al.*, 2012). Importantly, this effect was absent in SHRSP infused with Ang-(1-9) and PD123,319, suggesting Ang-(1-9) acts via the AT_2R. Furthermore, Ang-(1-9) induced vasodilation of rat arotic rings in an concentration- and endothelium-dependent manner, and was blocked by pre-incubation of the vessel with L-NAME or PD123,319, suggesting that Ang-(1-9) mediated vasodilation via the AT_2R and generation of NO (Ocaranza *et al.*, 2014).

In HSVEC, unstimulated cells displayed basal NO release, however, Ang-(1-9) stimulation led to increased NO release or generation, associated with a change in cell morphology and cellular location of NO. However, this change was difficult to quantify due to the level of basal NO activity and the lack of staining of different cellular structures, which would allow for a more in depth interpretation of the results observed. Additionally, a more thorough assessment of NO signalling pathways, over an extended time course, in Ang-(1-9) stimulated HSVEC through the use of western immunoblotting for key proteins involved, such as eNOS and Akt, is required to understand the mechanism of action of Ang-(1-9). This approach has previously been utilised to investigate the effects of Ang-(1-7) in primary endothelial cells and demonstrated that Ang-(1-7) signalling via Mas induces NO release through sustained Akt activation and reciprocal phosphorylation/dephosphorylation of eNOS at serine^{1177}/threonine^{495} (Sampaio *et al.*, 2007b). Furthermore, as HSVEC express AT_1R, AT_2R and Mas, it would be worthwhile to extend these findings to include a comparison of the effects of Ang II, Ang-(1-7) and Ang-(1-9) on NO signalling in HSVEC to allow for an in depth investigation of the functional interplay between the RAS peptides and receptors in primary endothelial cells.

Ang-(1-9) also stimulated release of NO from CHO cells expressing the AT_2R. While the use of cell lines has less translational impact than the experiments performed in primary HSVEC, an artificial approach is beneficial to study the Ang-(1-9)/AT_2R interaction and cell signalling pathways due to the fact that there are less variables. For example, there is no basal NO activity and the cells

only express the human form of the AT_2R, while the HSVEC express AT_1R, AT_2R and Mas, allowing for interaction between receptors and potential signalling via receptors other than AT_2R. In fact, the use of both primary cells and cell lines would be beneficial to investigate Ang-(1-9) signalling. This approach has previously been utilised to investigate the cell signalling mechanisms induced by Ang-(1-7) at Mas and to identify the functional effects of the novel RAS peptide alamandine and its receptor, MrgD (Sampaio *et al.*, 2007b, Lautner *et al.*, 2013). However, there are also some limitations of the cell line-based approach. For example, it is important to assess transfection efficiency of the receptor in each experiment. However, due to the lack of availability of specific AT_2R antibodies, it was not possible to determine the transfection efficiency in this current data set. One way to overcome this would be to design a plasmid to express the AT_2R with a fluorescent epitope tag, such as cyan fluorescent protein (CFP), which would allow the transfection efficiency to be determined parallel to visualisation of the fluorescent NO signal (Canals *et al.*, 2006). One major limitation to experiments based on transiently transfected cells is the large variability in expression level of the gene of interest among population of cells and between experiments (Ward *et al.*, 2011). Furthermore, due to differences in expression patterns this approach is not ideal to study receptor interactions. Therefore, it would be worthwhile to generate stable cell lines to express either the AT_2R alone or co-expressed with another receptor to allow for the study of receptor interactions. Stable cell lines remove both the variability between populations and experiments as they as designed so that all cells express the receptor and from one parental stock of cells (Ward *et al.*, 2011). Stable cell lines engineered to express the AT_1R and Mas have proven utility in aiding the understanding of ligand-mediated signalling at each receptor as well as allowing the effects of receptor-receptor interaction to be assessed (Kostenis *et al.*, 2005).

While the data discussed above demonstrate that Ang-(1-9) stimulates NO release in an AT_2R dependent manner *in vitro*, ongoing, unpublished studies from Prof. Robson A. Santos' research group revealed that Ang-(1-9) induced vasodilation in pre-constricted aortic rings from both wild type and $AT_2R^{-/-}$ mice in an endothelium dependent manner, suggesting that at least *ex vivo* Ang-(1-9) may act on the endothelium via an alternative receptor or mechanism. These findings in the $AT_2R^{-/-}$ mice were confirmed in this present study in both aortic

rings and mesenteric artery rings, demonstrating that the effect is present in both large conduit vessels and small resistance arteries. However, it is important to highlight the low number of vessels/animals used in this study, therefore, further replicates are required. Additionally, it would be worthwhile to investigate differences in expression of other RAS receptors or enzymes within the vessels of the two strains of mice in order to gain further insight into any potential differences in the vasorelaxant response to Ang-(1-9) in the presence and absence of the AT_2R.

The fact that Ang-(1-9) elicits direct biological effect independent of the AT_2R is somewhat unexpected as to date all previously published data suggests that Ang-(1-9) acts via this receptor (Flores-Munoz *et al.*, 2011, Flores-Munoz *et al.*, 2012, Ocaranza *et al.*, 2014, Cha *et al.*, 2013). One possible explanation for this phenotype is that in the absence of the AT_2R, Ang-(1-9) acts on an alternative receptor leading to vasodilation, however, further work would be required to investigate this fully. Another explanation is that Ang-(1-9) may be metabolised to an alternative peptide, such as Ang-(1-7) which also promotes vasodilation in an endothelium-dependent manner. As Ang-(1-7) has been shown to promote vasodilation via Mas this potential mechanism of action was explored in the current study using the Mas antagonist A779. A779 blocked the vasodilator effects of Ang-(1-9) in aortic rings but not mesenteric artery rings, suggesting that in the aorta, but not mesenteric artery, Ang-(1-9) may be converted to Ang-(1-7) which acts via Mas to induce vasodilation.

It is currently unclear as to why this differential mechanism of action of Ang-(1-9) between vascular beds occurs, one possibility is that levels of key enzymes involved in Ang-(1-9) metabolism, such as ACE, are differentially expressed within the aorta and mesenteric arteries of $AT_2R^{-/-}$ mice, and therefore future experiments should involve an assessment of ACE expression. Furthermore, the differential mechanism of relaxation is possibly linked to differences in the mechanisms of vasodilation in conduit and resistance vessels. In large conduit vessels such as the aorta, release of NO from the endothelium is the main mechanism of vasodilation, and in smaller resistance vessels, such as the mesenteric arteries, EDHF emerges as an important mediator of vasodilation in addition to NO. In fact, it has been demonstrated that the contribution of EDHF-mediated responses as a mechanism for endothelium-dependent relaxation

increases as the vessel size decreases (Shimokawa *et al.*, 1996), with the exception of the coronary and renal vasculature where EDHF plays a major role even in large conduit arteries (Feletou and Vanhoutte, 1988). In mice, EDHF-mediated responses in resistance vessels are at least as important as NO in mediating endothelium-dependent vasodilatation, as demonstrated by the fact that neither inhibition of or genetic deletion of eNOS attenuates vasodilator responses both *in vivo* and *in vitro* (Waldron *et al.*, 1999, Brandes *et al.*, 2000). Therefore, it is possible that in the absence of the AT_2R, in the aorta Ang–(1-9) is metabolised to Ang-(1-7), a peptide which has previously been demonstrated to promote vasodilation via NO, while in the mesenteric arteries Ang-(1-9) activates an alternative signalling pathway that is potentially involved in EDHF signalling. The findings from this data set highlight the need for accurate measurement of peptide levels and the use of a combination of RAS peptides and antagonists to fully understand the functional interplay involved.

While the current data set suggests that neither Ang-(1-7) nor Ang-(1-9) would enhance regeneration of the endothelial layer through proliferation or migration of resident endothelial cells, it is possible that these peptides may promote re-endothelialisation via an alternative mechanism. It has been suggested that while local endothelial cells would be sufficient to maintain vascular integrity throughout life in healthy circumstances, in the maintained presence of risk factors, loss of endothelial integrity would rapidly develop if local replication were the only repair mechanism (Op den Buijs *et al.*, 2004). It has become clear that circulating endothelial progenitor cells (EPC) are an alternative mechanism for maintenance and repair of the endothelium (Asahara *et al.*, 1997). These cells are recruited from the bone marrow, circulate in the peripheral blood, and can differentiate into mature cells with endothelial characteristics. In their fully developed state they are capable of endothelial outgrowth and tube or vascular sprout formation, and therefore are involved in re-endothelialisation and angiogenesis (Asahara *et al.*, 1997). Although under physiological conditions EPC do not contribute significantly to endothelial cell turnover of blood vessels in normal adult tissue, there is a robust contribution of circulating EPCs to endothelial cells within vessels one month following vascular injury (Crosby *et al.*, 2000). Several factors, including VEGF, NO, estrogen and drugs such as statins can regulate the number of EPC in the circulation (Takahashi *et al.*,

1999, Asahara *et al.*, 1999, Dimmeler *et al.*, 2001, Strehlow *et al.*, 2003, Aicher *et al.*, 2003).

In recent years the effects of the RAS, in particular Ang II, on EPC population and function has been explored. Evidence from the literature suggests that Ang II can be both detrimental and beneficial to EPC functioning. For example, Ang II via the AT_1R has been linked to reduced EPC population in a number of models, an effect which has been linked to inhibition of differentiation of bone marrow mononuclear cells (BM-MNC) to EPCs (Bahlmann *et al.*, 2005, Yu *et al.*, 2008, You *et al.*, 2008). For example, in BM-MNC cultured from SHRs, AT_1R inhibition increased the number of EPCs after a 7 day culture period (You, Cochain *et al.* 2008). Additionally, chronic AT_1R inhibition *in vivo* increased EPCs but not haematopoetic stem cells in diabetic patients, suggesting that the AT_1R may inhibit their differentiation to EPCs (Bahlmann, de Groot *et al.* 2005). Ang II has also been linked to increased EPC senescence through increased ROS production (You *et al.*, 2008, Imanishi *et al.*, 2005). Stimulation of EPC senescence occurred due to increased activation of NADPH oxidase signalling leading to increased ROS and $ONOO^-$ production. Increased oxidative stress was associated with down-regulation of the chromosomal telomere-extending enzyme telomerase, suggesting that Ang II may induce telomeric damage in EPC leading to senescence. However, in other cell types Ang II/ROS-induced senescence does not involve telomeres or telomerase activity, and therefore its effect could also be associated with global DNA damage (Herbert *et al.*, 2008). Further investigation into the mechanisms of Ang II induced senescence is therefore required. In contrast, it has also been demonstrated that Ang II improves EPC recruitment through stimulation of VEGF, which leads to increased eNOS signalling (Imanishi *et al.*, 2005, Qian *et al.*, 2009). However, while Ang II may have the potential to enhance the activity of EPC's, increased Ang II is detrimental during vascular injury and it would therefore be unsuitable to utilise this peptide as a therapy.

There is also mounting evidence to suggest a role for Ang-(1-7) in EPCs. For example, Ang-(1-7) has been shown to increase production of EPCs in a Mas dependent manner (Qian *et al.*, 2009). Furthermore, Ang-(1-7) has recently been demonstrated to improve EPC migration and functioning in diabetic patients (Jarajapu *et al.*, 2013). The mechanisms through which Ang-(1-7) increases EPC

population are still unknown; however, two potential pathways have been identified. First, Ang-(1-7) inhibits Ang II-induced ROS production through inhibition of c-Src signalling and therefore reduced NADPH oxidase activity (Sampaio *et al.*, 2007a). Second, Ang-(1-7) has been demonstrated to stimulate eNOS activity through Akt (Sampaio *et al.*, 2007b), and this pathway is also involved in VEGF signalling. Since these pathways play an important role in EPC senescence and angiogenic function, respectively, they may account for the stimulating effects of Ang-(1-7) on EPCs (Qian *et al.*, 2009).

An effect of Ang-(1-9) on EPC generation or function has yet to be identified, however, ACE2 has recently been linked to improved EPC function through increased eNOS expression and activity, and reduced production of ROS (Chen *et al* 2013). While the authors concluded that this was likely to be due to reduced Ang II and increased Ang-(1-7) production (Chen *et al.*, 2013), as Ang-(1-9) is also synthesised by ACE2 it is possible that this peptide may also play a role in the observed results. While the involvement of EPCs in the protective effects of Ang-(1-7) and Ang-(1-9) in acute vascular injury has not yet been explored, this novel interaction identifies a potential mechanism for enhanced re-endothelialisation.

4.4 Conclusion

In summary, the data demonstrate that Ang-(1-7) or Ang-(1-9) do not promote or inhibit proliferation or migration of primary HSVEC, suggesting that they would not limit re-endothelialisation following vascular injury. Furthermore, a direct role for Ang-(1-9) in production of NO has been identified, which appears to be mediated via the AT_2R *in vitro*. However, studies using $AT_2R^{-/-}$ mice revealed that Ang-(1-9) retained its biological functions in the absence of the AT_2R, suggesting that, at least in the absence of the AT_2R, Ang-(1-9) can either act at an alternative receptor or is metabolised to an alternative peptide which promotes vasodilation. Importantly, in the aorta the vasodilator effect of Ang-(1-9) was abolished by A779, indicating that in the aorta Ang-(1-9) may be converted to Ang-(1-7) to elicit its effects. Interestingly, this was not observed in the mesenteric arteries, identifying differential effects of Ang-(1-9) between vascular beds. While further work is required to fully elucidate the effects of Ang-(1-9) in the vasculature, this present study highlights both Ang-(1-7) and Ang-(1-9) as potential therapeutic targets in acute vascular injury.

Chapter 5

Effects of Ang-(1-7) and Ang-(1-9) on vascular remodelling *in vivo*

5.1 Introduction

As atheroscelerosis develops, advanced complex plaques are formed that largely occlude the affected artery resulting in reduced blood flow through the vessel and tissue ischaemia (Ross, 1999a). Restoration of blood flow through an occluded blood vessel, known as revascularisation, is most commonly achieved through CABG surgery or PCI (Goldman et al., 2004, British Heart Foundation, 2012, European Coronary Surgery Study Group, 1982, RITA-2 trial participants, 1997, King, 2005). However, the long-term success of these treatments are significantly limited by failure of conduit grafts in CABG and restenosis of stented vessels in PCI, caused by neointimal formation, impaired re-endothelialisation and thrombosis (Motwani and Topol, 1998, Mehilli *et al.*, 2011, Lopes *et al.*, 2012, Schwartz *et al.*, 1995, Mitra *et al.*, 2006).

Various cellular processes initiated during and immediately following engraftment of the vein to the arterial system underlie the pathogenesis of vein graft failure, both at an early and late stage. Surgical preparation of the vein causes significant denudation of the endothelial layer and immediately following engraftment to the arterial circulation the vein is exposed to a period of ischaemia following by reperfusion (Thatte and Khuri, 2001, Shi *et al.*, 2001, West *et al.*, 2001). Furthermore, the engrafted vein is subject to increased pressure and altered haemodynamic conditions within the arterial circulation resulting in further endothelial stress (Dobrin *et al.*, 1989). Together, this results in the generation of ROS, which in turn trigger an inflammatory response within the graft, leading to recruitment of platelets and various inflammatory cells (West *et al.*, 2001, Shi *et al.*, 2001). Under these conditions, the remaining endothelial cells become activated, allowing adhesion of the circulating platelets and inflammatory cells. This exposes the circulating blood to a highly thrombogenic surface, resulting in thrombosis, the main cause of early vein graft failure (Bryan and Angelini, 1994). These early events also predispose the vessel to neointimal formation and superimposed atherosclerosis, the main cause of reduced graft patency. Adherent platelets and leukocytes generate various growth factors and pro-inflammatory mediators which activate VSMC within the media, leading to VSMC migration to the intima where they proliferate and synthesise ECM, resulting in the formation of a large neointimal area (Lerner *et al.*, 1986, Cooper and Newby, 1991, Segel *et al.*, 2011). While VSMC migration

and proliferation is thought to be most prevalent within the first week following engraftment, these processes are key in vein graft patency and failure (Newby, 1997). Furthermore, this neointimal area is highly susceptible to an accelerated form of atherosclerosis due to the infiltration of inflammatory cells, which creates a cytotoxic environment within the plaque encouraging the uptake of lipids (Schwartz *et al.*, 1995). Adherent monocytes infiltrate the vessel wall and differentiate into resident macrophages which then transform into foam cells following lipid uptake. These resident macrophages also release various mediators that further enhance neointimal formation such as MMPs, growth factors and cytokines. The atherosclerotic plaques that form within the graft are diffuse and unstable due to poorly developed or absent fibrous caps, and are therefore more prone to rupture (Shelton *et al.*, 1988, Virmani *et al.*, 1988).

Within four weeks the endothelial layer of the engrafted vein is repaired due to proliferation and migration of endothelial cells and recruitment of bone marrow derived EPCs, a process which is thought to repress VSMC growth and proliferation, and therefore neointimal thickening (Cross *et al.*, 1988, Ku *et al.*, 1991). However, the function of the new endothelial layer is impaired, largely due to the increased arterial pressure and asymmetric neointimal area creating increased flow-induced stress, resulting in endothelial cell damage and enhanced platelet and leukocyte adhesion. This process ultimately leads to late stage thrombosis and enhanced neointimal thickening (Shimokawa *et al.*, 1987, Dobrin *et al.*, 1989, Caro *et al.*, 2002).

In the setting of in-stent restenosis, the neointimal growth that occurs is a multifactorial response to mechanical vessel injury at the time of stent deployment. The force required to dilate the vessel and deploy the stent results in denudation of the endothelium and often dissection of the medial layer of the arterial wall (Costa and Simon, 2005). Furthermore, the atherosclerotic plaque is compressed and the fibrous cap disrupted, exposing the sub-endothelial pro-inflammatory core of the plaque to the circulating blood (Inoue and Node, 2009). This initial damage stimulates an acute inflammatory response within the vessel, resulting in expression of adhesion molecules, recruitment and infiltration of various inflammatory cells and a cascade of cytokine and growth factor release (Costa and Simon, 2005, Welt and Rogers, 2002). The pro-inflammatory environment within the vessel is sustained for several weeks

following injury and leads to migration and growth of VSMC, processes which underlie the pathogenesis of restenosis and neointimal growth (Libby *et al.*, 1992). While the VSMC closest to the region of injury undergo apoptosis, the surviving VSMC migrate from the media to the intima (Perlman *et al.*, 1997, Hanke *et al.*, 1990). Activated platelets at the site of injury and resident VSMC secrete growth factors promoting VSMC proliferation and migration (Walker *et al.*, 1986). VSMC proliferation is thought to be maximal at 5 to 7 days after injury with around 10 to 20% of the total medial VSMC proliferating (Rogers *et al.*, 1998). VSMC in the synthetic state are also key producers of ECM proteins, such as collagens and proteoglycans, resulting in increased neointimal volume due to the high rate of ECM synthesis which lasts for a number of months following injury (Inoue and Node, 2009, Lee *et al.*, 1993).

Re-endothelialisation of the vessel is thought to occur within three to four weeks following PCI with growth factors such as VEGF facilitating endothelial cell proliferation and migration (Brindle, 1993). While re-growth of the endothelial layer has been demonstrated to repress neointimal thickening and the occurrence of thrombosis, the new endothelial layer may also be dysfunctional, resulting in decreased vascular integrity, increased permeability and impaired vasodilation (Weidinger *et al.*, 1990, Hamon *et al.*, 1995, Kipshidze *et al.*, 2004).

One interventional approach to prevent late vein graft failure and in-stent restenosis is targeting the VSMC proliferation that underlies the formation of the neointima through the use of cell cycle inhibitory drugs, such as paclitaxel or sirolimus (Htay and Liu, 2005, Murphy *et al.*, 2007). In the case of CABG surgery the vein graft can be incubated with these drugs prior to engraftment and several studies in animal models have shown that adopting this approach results in reduced neointimal formation, at least in the early phase of disease (Schachner *et al.*, 2004, Murphy *et al.*, 2007). For example, incubating pig saphenous veins for one hour with paclitaxel, sirolimus or cytochalasin D reduced neointimal formation at one but not three months following implantation (Murphy *et al.*, 2007). Additionally, sirolimus reduced re-endothelialisation and increased the incidence of thrombosis (Murphy *et al.*, 2007). In an attempt to circumvent the issue of restenosis following stent employment drug eluting stents were developed which were coated with polymers to elute the cell cycle inhibitory drugs (Htay and Liu, 2005). Drug eluting stents have been shown to

inhibit restenosis in comparison to bare metal stents, confirming that inhibition of VSMC proliferation is beneficial in this setting (Schampaert *et al.*, 2006, Stone *et al.*, 2009, Caixeta *et al.*, 2009), however they do not come without complications of their own. Several studies have reported that drug eluting stents are associated with an increased risk of late stent thrombosis (Karha *et al.*, 2006, Joner *et al.*, 2007) as the drugs also inhibit endothelial cell proliferation, thereby inhibiting regeneration of the endothelial layer, enhancing neointimal formation and increasing the incidence of late in-stent thrombosis (Douglas *et al.*, 2012). Therefore therapies that specifically target VSMC growth and migration, without preventing re-endothelialisation are optimal in vein graft failure and in-stent restenosis (Inoue and Node, 2009).

As discussed in Chapter 3, Ang-(1-7) and Ang-(1-9) prevent VSMC migration and proliferation, via the Mas receptor and AT_2R, respectively. Importantly, as shown in Chapter 4, these peptides did not affect endothelial cell proliferation and migration, indicating that they may prevent neointimal formation but not inhibit re-endothelialisation of the vasculature following injury *in vivo,* and are therefore attractive novel therapeutic targets in the setting of vascular remodelling. In this chapter the role of Ang-(1-7) and Ang-(1-9) *in vivo* in a mouse model of vascular remodelling, the carotid artery wire injury model, has been investigated. This model was first developed by Lindner *et al* in 1993 and involves isolation of the carotid artery and advancement of a guide wire into the vessel towards the aorta (Lindner *et al.*, 1993). This causes endothelial denudation and in turn promotes intimal and medial VSMC proliferation and migration, and rapid platelet adherence to the subendothelial matrix, resulting in the formation of a large neointima within 2 to 4 weeks (Lindner *et al.*, 1993). Repair of the endothelial layer is expected to be complete at 4 weeks following injury (Lindner *et al.*, 1993). Based on the effects of Ang-(1-7) and Ang-(1-9) *in vitro* and the fact that this will be the first study to assess the role of Ang-(1-9) in this setting, this model was deemed to be ideal to investigate the role of Ang-(1-7) and Ang-(1-9) in vascular remodelling.

Additionally, this study includes an investigation into the involvement of the AT_2R and Mas receptor in this setting. Ang-(1-7) is widely accepted to be the endogenous ligand at the Mas receptor (Santos *et al.*, 2003) has been previously shown to exert its inhibitory effects on neointimal formation in rats by

interaction with Mas (Zeng *et al.*, 2009) and therefore a group of animals were administered both Ang-(1-7) and A779 following vascular injury to assess this interaction in the mouse carotid artery injury model. The protective effects of Ang-(1-9) has been previously shown to be mediated via the AT_2R and independent of conversion to Ang-(1-7) and signalling at Mas (Flores-Munoz *et al.*, 2012, Flores-Munoz *et al.*, 2011) (Chapters 3 and 4). However as very little is known about this peptide in the vasculature, one group of animals received Ang-(1-9) and PD123, 319, and another Ang-(1-9) and A779 to investigate the possible conversion to Ang-(1-7). All peptide and antagonist combinations were delivered subcutaneously via osmotic mini pumps resulting in delivery of the peptide to the systemic circulation at a constant rate over the 28 day post-operative period.

Furthermore, an additional study was performed to assess the effectiveness of locally delivered peptide in the mouse carotid artery wire injury model. Here the peptides were applied directly to the vessel immediately following injury to allow for local delivery through the use of Pluronic F127 gel. Pluronic F127 is a commercially available copolymer that when dissolved in aqueous solution displays the unique characteristic of reverse thermal gelation, as at room temperature Pluronic F127 solution is a viscous liquid which is transformed to a semisolid gel at body temperature (Schmolka, 1972). Therapies can therefore be dissolved in the Pluronic F127 solution when aqueous then be delivered locally to the animal to form a gel based depot of the therapeutic at the site of administration. This approach has never been used to deliver Ang-(1-7) or Ang-(1-9), and confirmation of delivery of the peptides was required prior to investigating their therapeutic potential. Therefore a pilot study was performed using an antibody against Ang-(1-7) and a biotinylated form of Ang-(1-9) to confirm their delivery prior to investigating the therapeutic effects of locally delivered peptides.

5.1.1 Aims

The aims of this chapter were:

- To determine the extent of neointimal formation and vascular remodelling following carotid artery injury in C57BL/6 mice.

- To investigate the effect of subcutaneous delivery of Ang-(1-7) and Ang-(1-9), and the role of Mas and the AT_2R, on neointimal formation and vascular remodelling following carotid artery injury in C57BL/6 mice.

- To assess the efficacy of Pluronic F127 gel as a method for local delivery of Ang-(1-7) and Ang-(1-9).

- To determine the effects of local delivery of Ang-(1-7) and Ang-(1-9) on neointimal formation and vascular remodelling following carotid artery C57BL/6 mice.

5.2 Results

5.2.1 Neointimal formation in C57BL/6 mice following vascular injury

Total body weight was monitored throughout the wire injury study and no difference was observed between sham and injured mice (Table 5.1). Heart and kidney weight were assessed at 28 days and expressed a percentage of total body weight; no differences in heart or kidney were observed between sham and injured mice (Table 5.1).

Table 5.1 Body and organ weight measurements following sham and vascular injury procedures.

	Sham	Wire Injury
Start weight (g)	23.26 ± 0.27	23.24 ± 0.39
Final weight (g)	26.46 ± 0.41	27.46 ± 0.56
Heart/Body weight (%)	0.56 ± 0.02	0.58 ± 0.03
Kidney/Body weight (%)	0.68 ±0.01	0.61 ± 0.03

Organ weight was taken as a percentage of the body weight, n=6-8

Histological analysis of EVG stained sections from carotid arteries at 28 days following wire injury revealed a significant increase in vascular remodelling in injured carotid arteries in comparison to sham operated arteries (Figure 5.1). This remodelling was associated with the formation of a significant neointimal area (23608.3 ± 5100.1 μm^2 wire injury vs. 176.9 ± 136.4 μm^2 sham; $P<0.01$), an increase in medial area (57300.5 ± 16051.3 μm^2 wire injury vs. 23842.9 ± 1376.7 μm^2 sham; $P<0.05$) and neotintima/media (NI/MA) ratio (0.49 ± 0.13 wire injury vs.0.01 ± 0.01 sham; $P<0.01$) (Figure 5.1). Additionally, injured carotid arteries had a more disorganized elastic lamina in comparison to sham operated animals. The neointimal area observed in wire injured vessels appeared to have a large proportion of ECM as evidenced by the large proportion of elastin (black) and collagen (pink) staining (Figure 5.1).

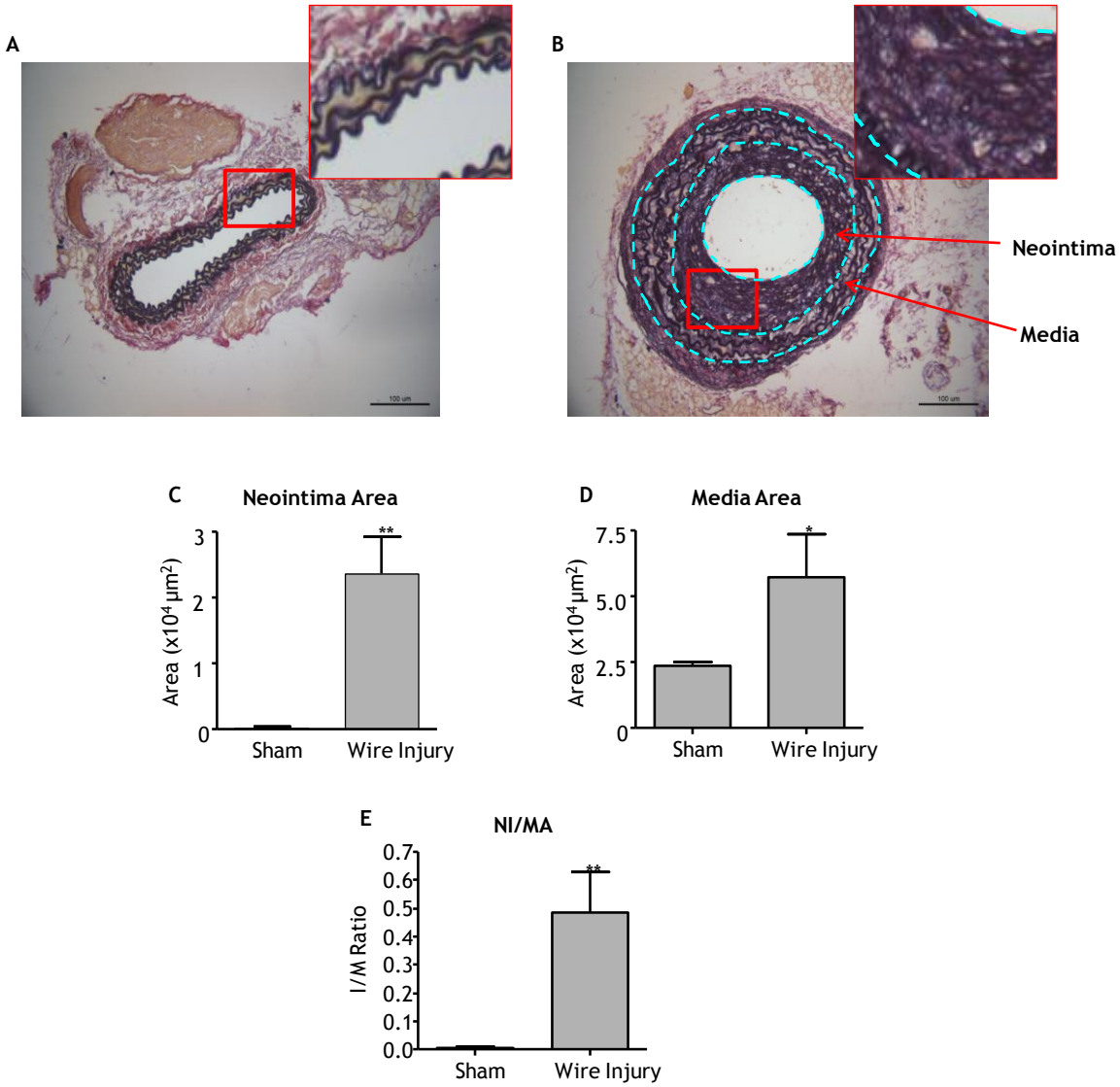

Figure 5.1 Neointimal growth in the carotid artery 28 days after sham and vascular injury procedure.

Representative histological sections of the left carotid artery from (A) sham and (B) injured mice using EVG staining. Elastin appears black and collagen pink. (C) Neointimal area (NI), (D) media area (MA), and (E) NI/MA ratio was assessed. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 µm, magnification x20, red box = 3x zoom. ***P<0.001, **P<0.01, P<0.05 vs. sham. n=6-8.

5.2.2 Effects of subcutaneous delivery of Ang-(1-7) and Ang-(1-9) on vascular remodelling, in the absence and presence of Mas or AT_2R inhibition,

Total body weight was monitored throughout the study and no difference was observed between the experimental groups (Table 5.2). Heart and kidney weight was assessed at 28 days and expressed a percentage of total body weight; no differences in heart or kidney were observed between the experimental groups (Table 5.2).

Table 5.2 Effects of subcutaneous delivery of water, Ang-(1-7) and Ang-(1-9) on body and organ weight measurements following vascular injury

	Control (water)	Ang-(1-7)	Ang-(1-9)	Ang-(1-7) + A779	Ang-(1-9) + PD	Ang-(1-9) + A779
Start weight (g)	25.35 ± 0.87	25.54 ± 0.78	26.18 ± 0.31	25.76 ± 0.59	27.16 ± 0.43	26.08 ± 0.52
Final weight (g)	27.55 ± 0.99	27.29 ± 0.76	28.61 ± 0.29	27.85 ± 0.51	28.94 ± 0.54	27.93 ± 0.52
Heart/Body weight (%)	0.60 ± 0.03	0.61 ± 0.03	0.61 ± 0.04	0.60 ± 0.02	0.67 ± 0.02	0.63 ± 0.01
Kidney/Body weight (%)	0.58 ±0.03	0.67 ± 0.02	0.65 ± 0.04	0.63 ± 0.01	0.62 ± 0.01	0.64 ± 0.01

Organ weight was taken as a percentage of the body weight, n=8-10

EVG staining was also performed to assess the extent of vascular remodelling following subcutaneous delivery of Ang-(1-7) and Ang-(1-9), in the absence and presence of Mas or AT_2R inhibition (Figure 5.2). Delivery of Ang-(1-7) resulted in a trend towards reduced neointimal area (16538.0 ± 3487.3 μm^2 Ang-(1-7) vs. 30609.7 ± 2771.3 μm^2 control) and a significant reduction in NI/MA ratio (0.40 ± 0.07 Ang-(1-7) vs. 0.80 ± 0.07 control; $P<0.05$) in comparison to control vessels (Figure 5.2). Ang (1-7) had no effect on medial area (39178.3 ± 6051.23 μm^2 Ang-(1-7) vs. 38132.8 ± 2708.8 μm^2 control) (Figure 5.2). To assess the involvement of Mas in the effect of Ang-(1-7), Ang-(1-7) and A779 were co-infused for 28 days following vascular injury. A779 abolished the effects of Ang-(1-7), resulting in a similar neointimal area as control vessels (28617.1 ± 3479.9 μm^2 Ang-(1-7) +A779; $P>0.05$ vs control) (Figure 5.2).

Ang-(1-9) also reduced vascular remodelling in comparison to control vessels, resulting in a significant reduction in neointimal area (6577.7 ± 3090.0 μm^2 Ang-

(1-9) vs. 30609.7 ± 2771.3 μm^2 control; P<0.001) and NI/MA ratio (0.17 ± 0.10 Ang-(1-9) vs. 0.80 ± 0.07 control; P<0.001), suggesting it may be more efficacious than Ang-(1-7) (Figure 5.2). Media area was unaltered by Ang-(1-9) (50664.8 ± 4847.1 μm^2 Ang-(1-9) vs. 38132.8 ± 2708.8 μm^2 control; P>0.05 vs control) (Figure 5.2).

To assess the involvement of the AT_2R in the effects of Ang-(1-9), Ang-(1-9) was co-infused with PD123,319 for 28 days following vascular injury. Co-infusion of PD123,319 with Ang-(1-9) blocked its effects, resulting in a significant increase in neointima area compared to Ang-(1-9) (32912.6 ± 6432.3 μm^2 Ang-(1-9)+PD123,319 vs 6577.7 ± 3090.0 μm^2 Ang-(1-9); p<0.001) and NI/MA (0.58 ± 0.13 Ang-(1-9)+PD123,319 vs 0.17 ± 0.10 Ang-(1-9);P,0.05) in comparison to animals who received Ang-(1-9) alone (Figure 5.2). PD123,319 blocked the effects of Ang-(1-9) on neointima area and NI/MA to similar levels as control vessels (Figure 5.2). Furthermore, co-infusion of Ang-(1-9) and PD123,319 resulted in a significant increase in media area in comparison to control (61230.0 ± 7993.2 μm^2Ang-(1-9) + PD123,319 vs 38132.8 ± 2708.8 μm^2 control; P<0.05) (Figure 5.2).

Ang-(1-9) was also co-infused with A779 to address the possibility of conversion to Ang-(1-7) and/or interaction with Mas. A779 did not alter the anti-remodelling effects of Ang-(1-9), resulting in a comparable neointima area (13751.1 ± 2709.4 μm^2) and NI/MA (0.34 ± 0.06) to Ang-(1-9) treated groups; both parameters were significantly different to control vessels (neointima area P<0.05 and NI/MA P<0.01 vs control). Co-infusion of Ang-(1-9) and A779 resulted in an equivalent medial area to control and Ang-(1-9) treated vessels [42175.2 ± 3019.6 μm^2; P>0.05 vs control or Ang-(1-9)] (Figure 5.2).

During histological analysis of the vessels it was also observed that while co-infusion of Ang-(1-7) with A779, and Ang-(1-9) with PD123,319 prevented the anti-remodelling effects elicited by the peptides alone, a large proportion of these two groups had increased vessel remodelling, largely within the media, in comparison to all other groups (Figure 5.3). This increased remodelling presented in the form of more complex lesions and was associated with positive vessel remodelling, medial hyperplasia, and stretching and often disruption of the elastic lamina (Figure 5.3).

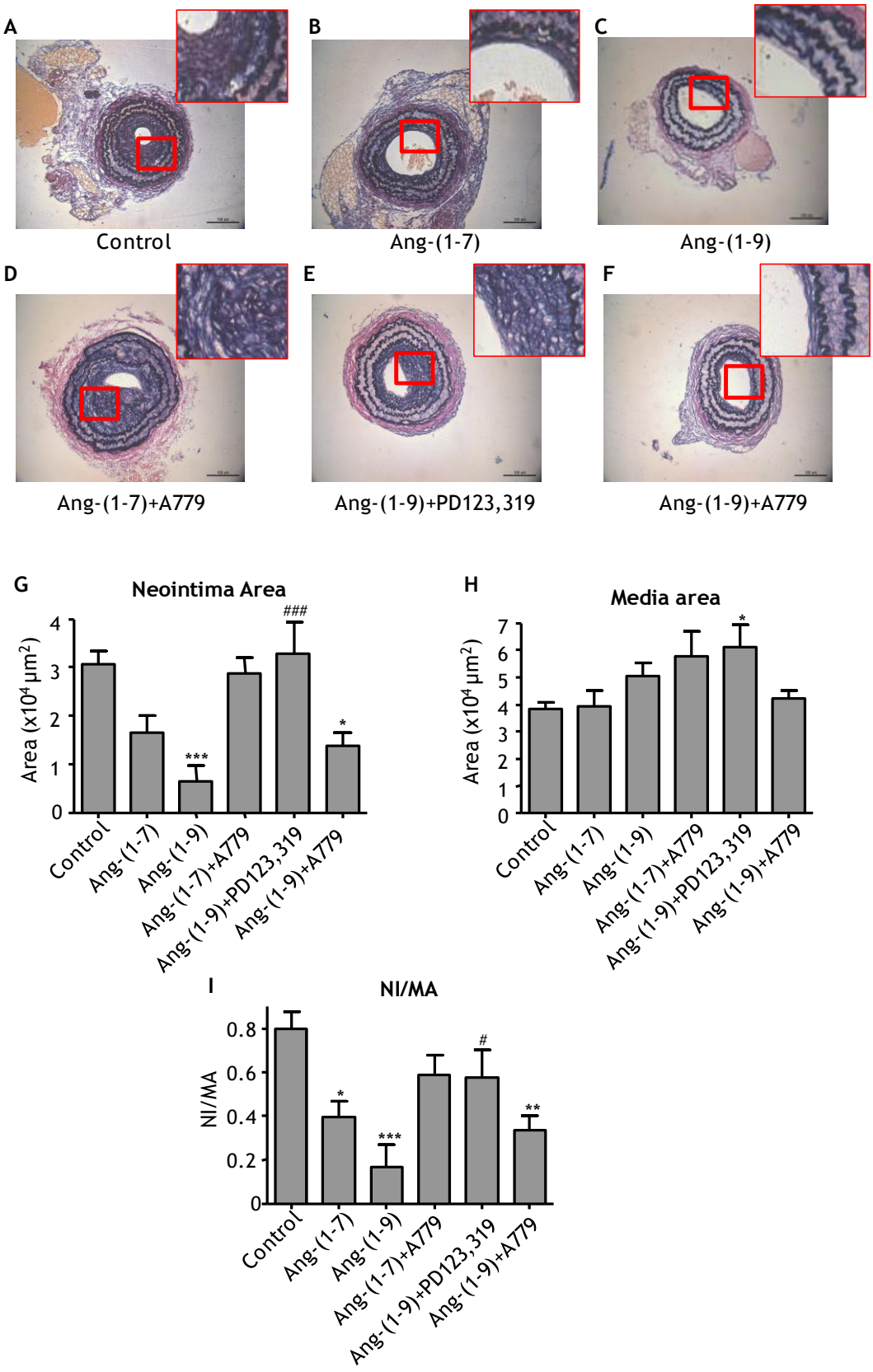

Figure 5.2 Effects of subcutaneous delivery of Ang-(1-7) and Ang-(1-9) on neointima formation in the carotid artery at 28 days following vascular injury, in the absence and presence of Mas or AT_2R inhibition.

Representative histological sections of the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 stained using EVG staining. Elastin appears black while collagen is stained pink. (G) Neointima area, (H) media area and (I) NI/MA ratio was assessed. Scale bar =100 µm, magnification x20, red box = 3x zoom. ***P<0.01 **P<0.01, P<0.05 vs. control (water); $^{###}$P<0.001, $^{#}$P<0.05 vs Ang-(1-9). N= 6-10 animals per group.

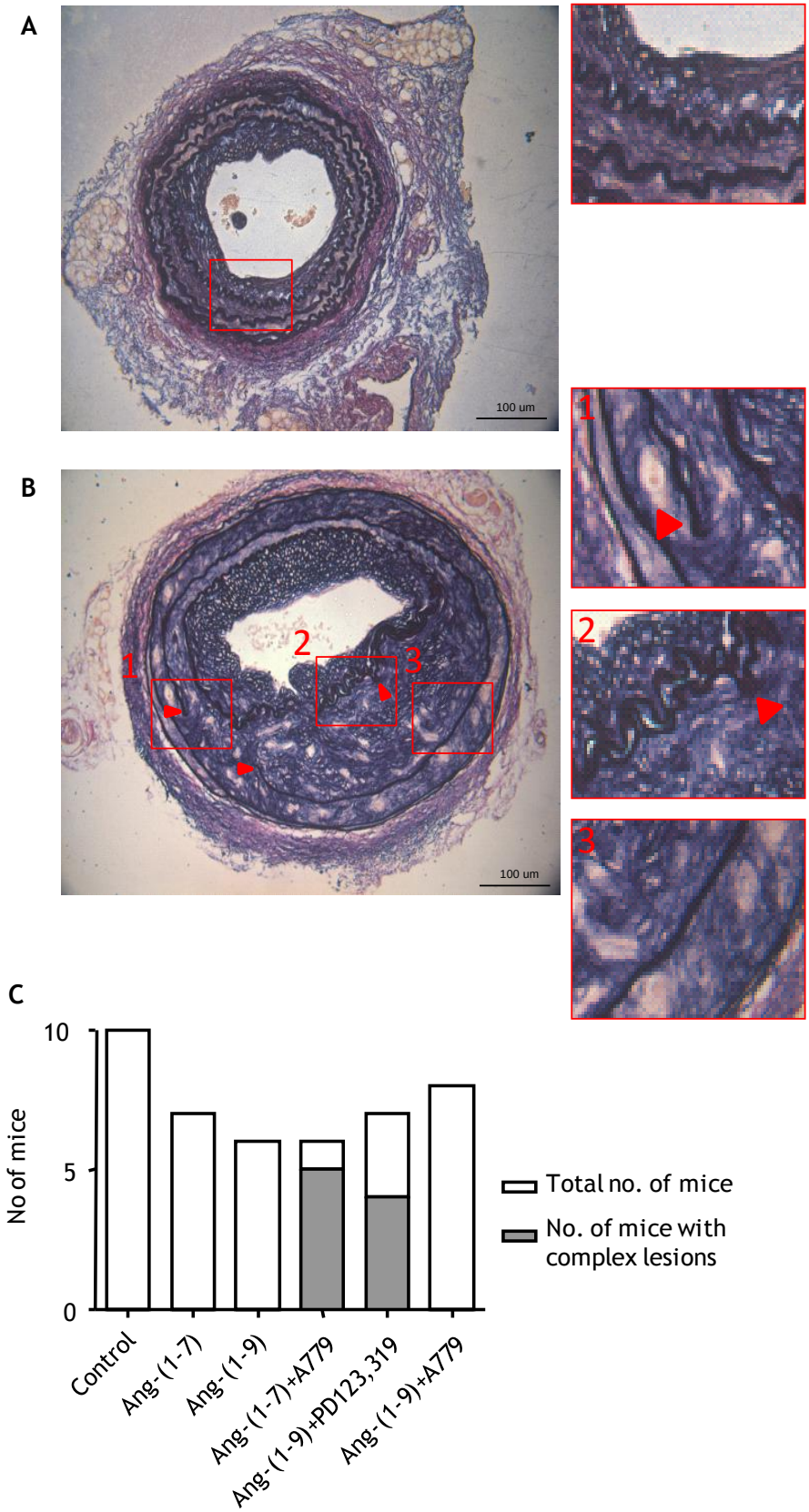

Figure 5.3 Incidence of increased vessel remodelling
Representative histological sections of the injured left carotid artery exhibiting (A) normal and (B) more complex vascular remodelling at 28 days post injury stained with EVG. Complex lesions presented with positive outward vessel remodelling and stretching of and/or disruption of the elastic lamia, as indicated by red arrows. (C) Number of mice per group with complex vessel remodelling at 28 days following vascular injury. Scale bar =100 µm, magnification x20. Red box = 3x zoom.

5.2.2.1 Structural composition of remodelled vessels.

To further investigate the effects of Ang-(1-7) and Ang-(1-9) on vascular remodelling following injury the composition of the lesions was assessed using further histological analysis.

5.2.2.1.i Extracellular matrix

EVG staining was performed to assess elastin content within the vessel (Figure 5.2). At 28 days post injury, large areas of neointimal formation were present in control and Ang-(1-7) + A779 and Ang-(1-9) + PD123, 319 infused animals, with comparatively smaller neointimal area in Ang-(1-7), Ang-(1-9) and Ang-(1-9) +A779 treated animals. The neointima areas contained large amounts of ECM as indicated by the high degree of elastin staining. In control vessels, a high degree of elastin staining was observed within the media, this was unaltered in animals infused with Ang-(1-7) or Ang-(1-7)+A779, however reduced medial elastin content was observed in animals infused with Ang-(1-9) alone or Ang-(1-9) and PD123,319 or A779.

Picrosirus red staining was used to assess collagen content within the remodelled vessels at 28 days post injury (Figure 5.4). Picrosirus red staining was present within the neointima, and a higher degree of positive staining was observed within the neointima compared to the media. However, positive collagen staining was similar in colour to the elastic lamina and much less than staining observed in the periadventitial connective tissue, therefore under these conditions it is unclear if the pink staining is truly new collagen deposition or whether stained elastic fibres were contributing to the observed results.

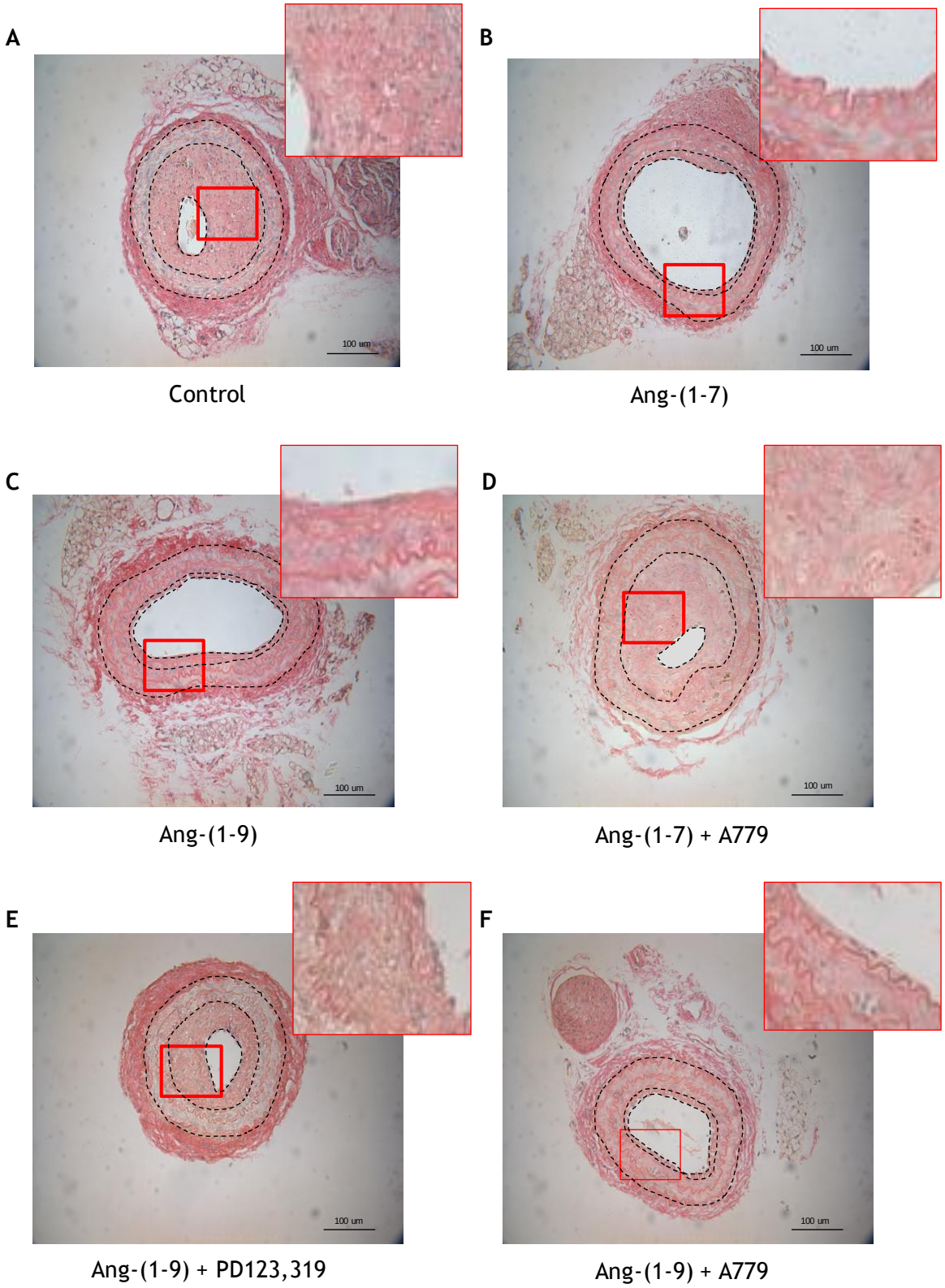

Figure 5.4 Picrosirus red staining of left carotid arteries at 28 days following vascular injury. Representative histological sections of the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 stained with picrosirus red and counterstained with Weigarts haemotoxylin. Collagen appears pink/red and nuclei appear purple/blue. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20. Red box = 3x zoom. N = 6-10 animals per group.

5.2.2.1.ii. Vascular smooth muscle cell content

As migration and proliferation of VSMC are key processes in neointimal formation, the contribution of VSMC to vascular remodelling following injury was assessed via the presence of α-SMA, a SMC marker (Figure 5.5). Vessels from all groups at 28 days displayed positive staining for α-SMA within both the media and neointima. Large neointimal lesions were observed in control, Ang-(1-7)+A779 and Ang-(1-9)+PD123, 319 treated animals and these neointimal areas were largely composed of VSMC, as indicated by the high degree of positive detection of α-SMA. Overall these vessels contained a larger number of α-SMA positive cells due to the presence of a large neointima in comparison to vessels from animals infused with Ang-(1-7), Ang-(1-9) or Ang-(1-9) + A779.

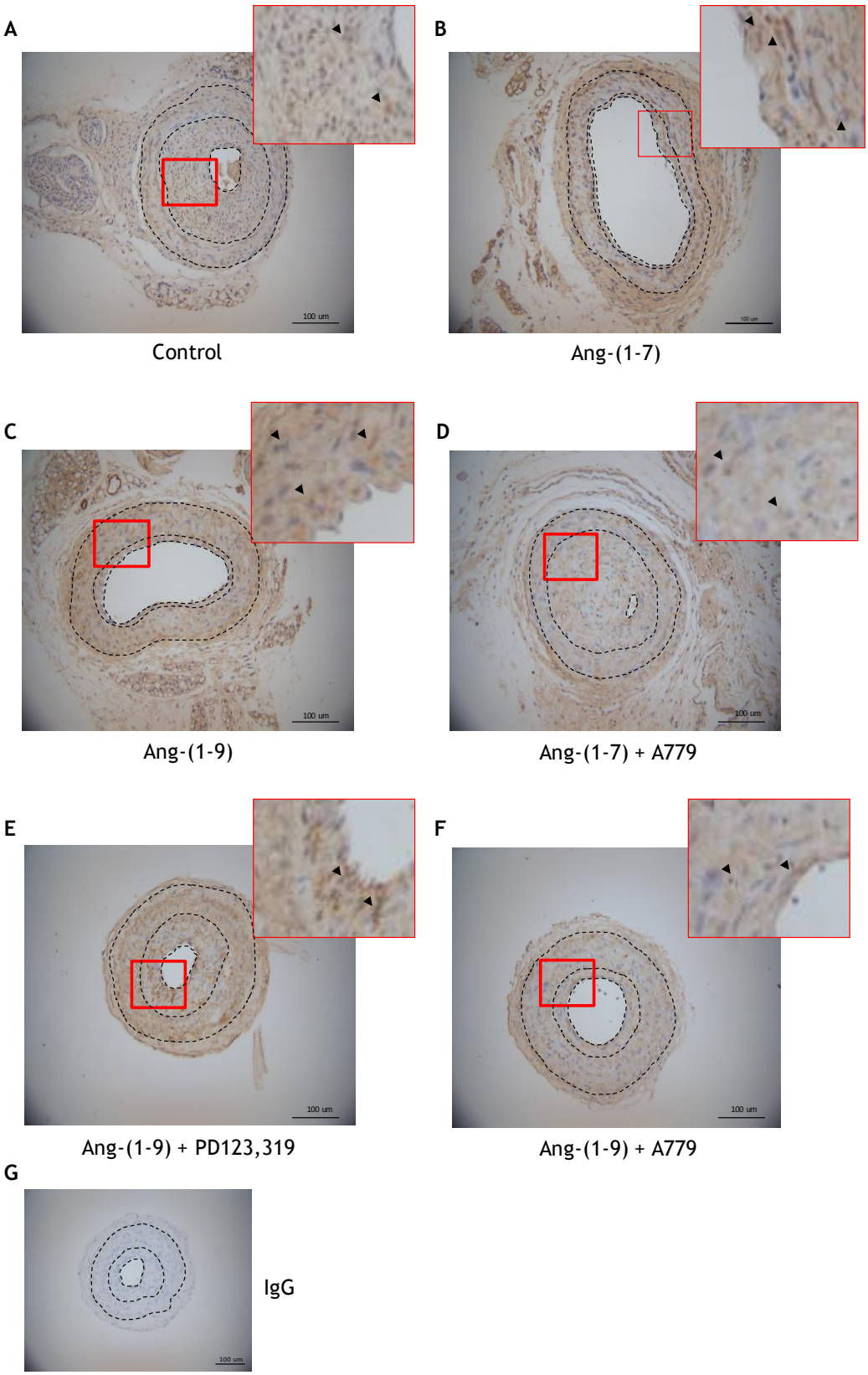

Figure 5.5 α-SMA content of the left carotid artery at 28 days following vascular injury. IHC was performed using an anti-α-SMA antibody to assess VSMC expression within the injured vessel. DAB chromagen was used to detect α-SMA positive cells (brown), indicated by arrow head, and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of α-SMA content in the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 via osmotic minipump. (G) An isotype matched IgG was used as a control. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-10 animals per group.

5.2.2.1.iii. Re-endothelialisation

Re-endothelialisation of the vessels following vascular injury was assessed via immunostaining for CD31, an endothelial cell marker. At 28 days post-injury an intact endothelial cell layer was prevalent in all groups, as indicated by the brown staining surrounding the lumen (Figure 5.6).

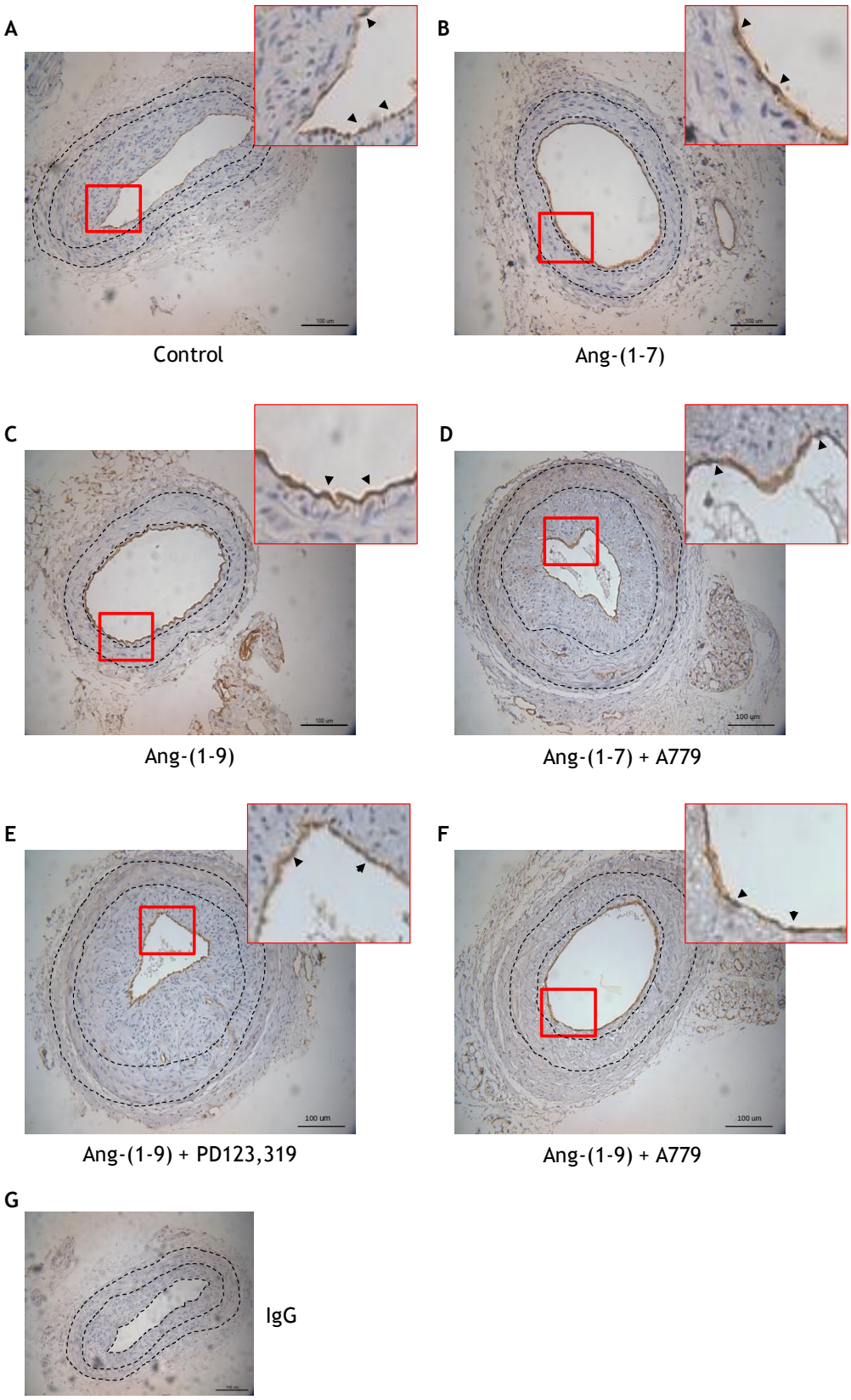

Figure 5.6 CD31 content of the left carotid artery at 28 days following vascular injury. IHC was performed using an anti-CD31 antibody to assess endothelial cell expression within the injured vessel. DAB chromagen was used to detect CD31 positive cells (brown), indicated by arrow head, and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of CD31 content in the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 via osmotic minipump. (G) An isotype matched IgG was used as a control. Outer dotted line = external elastic lamina, inner dotted line = internal elastic lamina. Lumen is not outlined due to positive CD31 detection in this region. Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-10 animals per group.

5.2.2.1.iv. Proliferation

The degree of cell proliferation within the injured vessel at 28 days post injury was assessed using the proliferation marker proliferating-cell nuclear antigen (PCNA) (Figure 5.7). In control vessels and vessels from mice infused with Ang-(1-7) or Ang-(1-9), few PCNA positive cells were detected, closely associated with the sub-endothelial cell layer. However, in vessels from animals co infused with Ang-(1-7) and A779 there was a significant number of PCNA positive cells observed in both the media and the neointimal area, however the highest proportion was within the neointima. A similar effect was observed in Ang-(1-9) + PD123, 319 treated mice where a high proportion of PCNA positive cells was observed, particularly within the neointima. PCNA positive cells were also observed in vessels co-infused with Ang-(1-9) + A779, however this was to a much lesser extent than in mice co-infused with Ang-(1-9) + PD123,319 and where present was observed within the media.

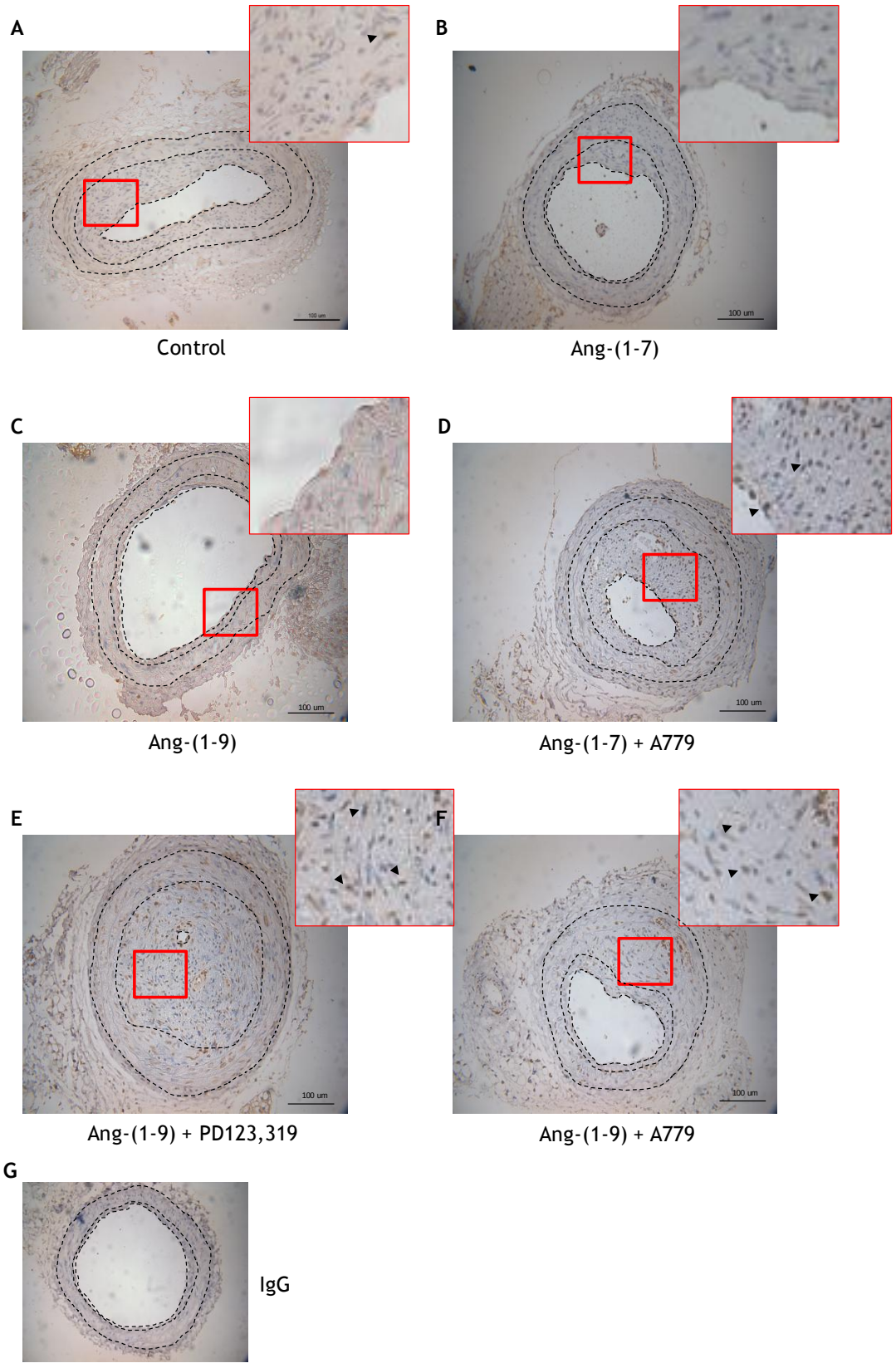

Figure 5.7 PCNA content of the left carotid artery at 28 days following vascular injury. IHC was performed using an anti-PCNA antibody to assess cell proliferation within the injured vessel. DAB chromagen was used to detect PCNA positive cells (brown), indicated by arrow heads, and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of PCNA content in the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 via osmotic minipump. (G) An isotype matched IgG was used as a control. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-10 animals per group.

5.2.2.1.v. Inflammation

To assess the presence of macrophages as a measure of inflammation within the remodelled vessels at 28 days post injury, immunostaining for the macrophage marker macrophage antigen-2 (MAC-2) was performed (Figure 5.8). In control vessels, a small number of MAC-2 positive cells were observed within the media and neointima. In Ang-(1-7) and Ang-(1-9) infused mice no significant differences were observed, however positive immunostaining did appear to be reduced in comparison to control animals. The effects of Ang-(1-7) were blocked by co-infusion of A779 as there was a higher proportion of MAC-2 positive cells than both control or Ang-(1-7) infused mice. In Ang-(1-7) + A779 infused animals the largest proportion of MAC-2 positive cells were observed within the media, however, positive cells were also detected within the neointima. The effects of Ang-(1-9) were unaltered by co-infusion of A779 as vessels from animals infused with Ang-(1-9) + A779 displayed similar levels of MAC-2 positive cells to that observed in control or Ang-(1-9) infused mice. Conversely, co-infusion of Ang-(1-9) + PD123,319 appeared to increase MAC-2 content within the injured vessels to a higher extent than control or Ang-(1-9) infused animals, as a higher number of MAC-2 positive cells were observed within the media and to a lesser extent in the neointima.

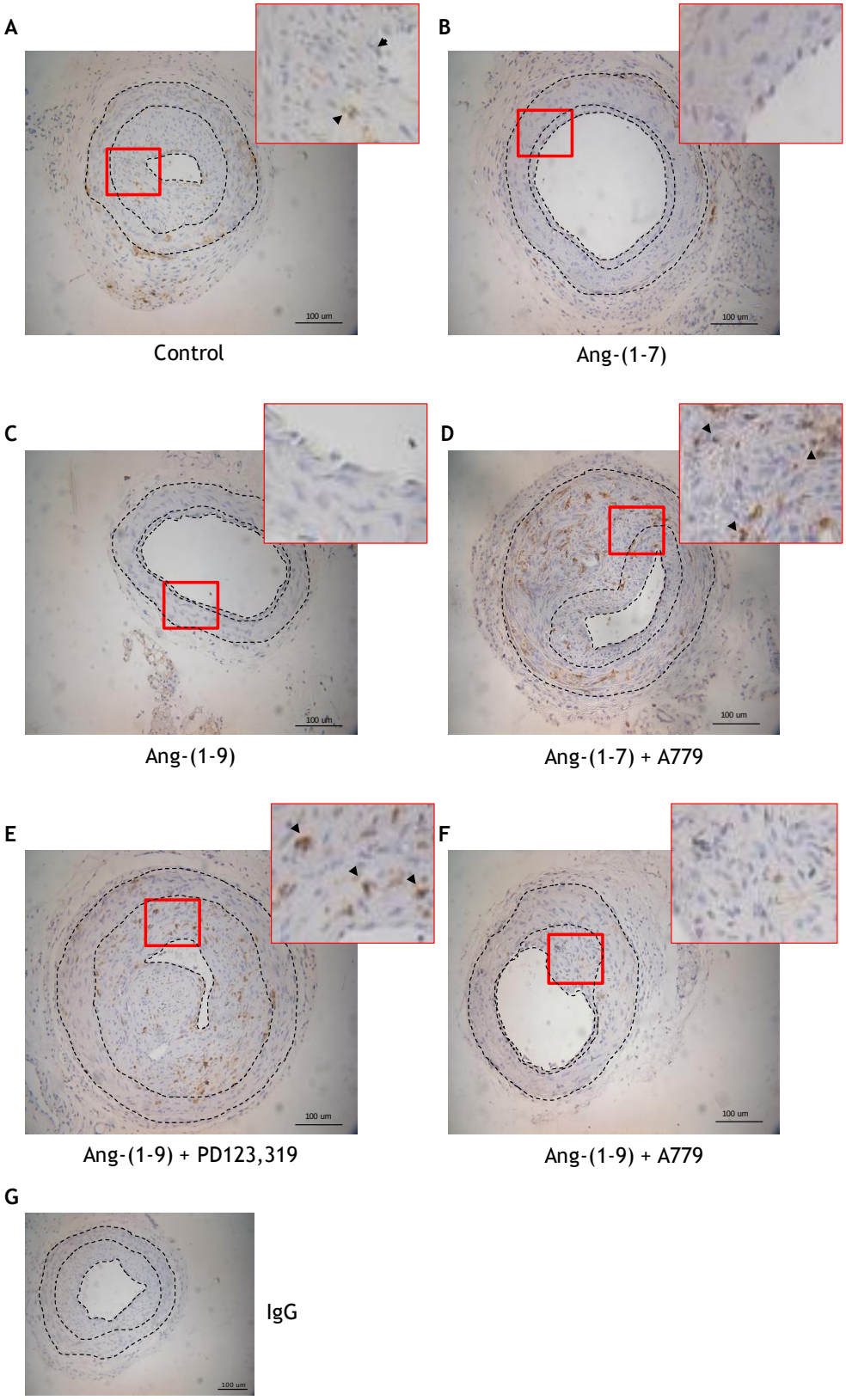

Figure 5.8 Mac2 content of the left carotid artery at 28 days following vascular injury. IHC was performed using an anti-Mac2 antibody to assess macrophage content within the injured vessel. DAB chromagen was used to detect Mac2 positive cells (brown), indicated by arrow head, and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of Mac2 content in the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 via osmotic minipump. (G) An isotype matched IgG was used as a control. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-10 animals per group.

5.2.2.1.vi. Apoptosis

To assess the extent of cell apoptosis within the injured vessel, immunostaining was performed against the apoptosis marker active-caspase 3 (Figure 5.9). At 28 days post injury negligible active caspase 3 activity was observed in all groups and no differences were apparent between any of the groups.

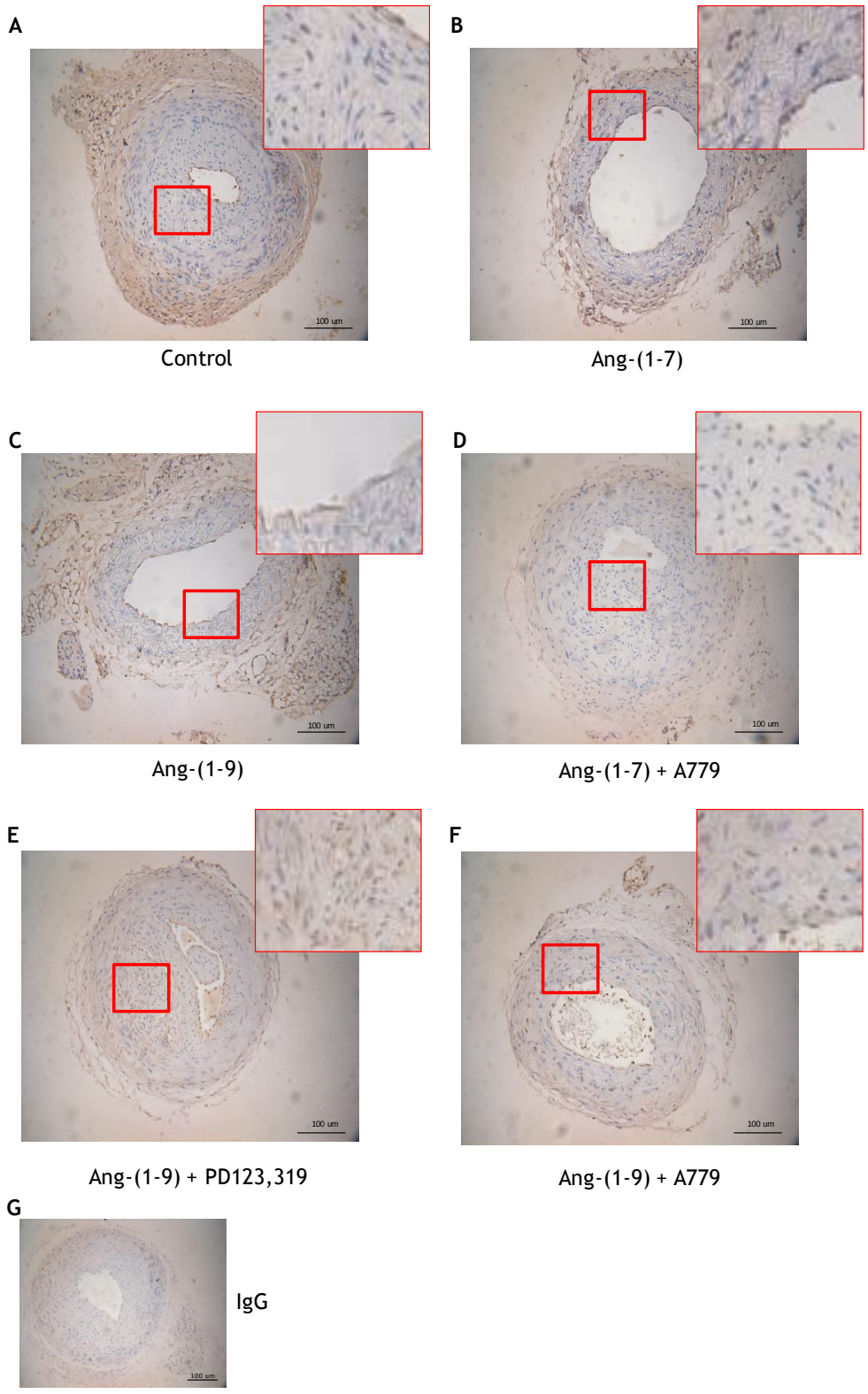

Figure 5.9 Active caspase-3 content of the left carotid artery at 28 days following vascular injury.
IHC was performed using an anti-active caspase-3 antibody to assess cell apoptosis within the injured vessel. DAB chromagen was used to detect active caspase-3 positive cells (brown) and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of active caspase-3 content in the injured left carotid artery from (A) control animals (water) and animals administered (B) Ang-(1-7), (C) Ang-(1-9), (D) Ang-(1-7)+A779, (E) Ang-(1-9)+PD123,319 or (F) Ang-(1-9)+A779 via osmotic minipump. (G) An isotype matched IgG was used as a control. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-10 animals per group.

5.2.2.2 Structural composition of complex lesions

As detailed in section 5.2.2, it was observed that a large proportion of vessels from animals co-infused with Ang-(1-7) and A779, or Ang-(1-9) and PD123,319 developed complex lesions with increased vessel remodelling, largely within the media, in comparison to other groups (Figure 5.3). To investigate the structural composition of these complex lesions the histology stains and IHC described in section 5.2.2.1 were employed. For all components of the vessels investigated there was no difference in the composition of the complex lesions observed in animals infused with Ang-(1-7)+A779 or Ang-(1-9)+PD123,319, therefore the following analysis describes the common features of the complex lesions which were found consistently in both groups.

The complex lesions contained a high proportion of ECM content, as revealed by EVG staining for elastin and picrosirus red staining for collagen (Figure 5.10). Many vessels contained an intact internal elastic lamina, therefore it was clear to distinguish between the neointima and the media. However, even in vessels where the elastic lamina was disrupted the neointima could still be distinguished due to differences in the structural organisation. Similar to vessels without complex lesions, the neointima contained large amounts of elastin which appeared highly organised, more compact and matrix-like in structure. However, EVG staining within the medial area of the complex lesions was far less uniform in structure and composition. Picrosirus red staining revealed a similar pattern, with a higher degree of more organised positive staining for collagen within the neointima compared to the media. However, as outlined previously this staining was similar in colour to the elastic lamina and lighter than the periadventitial connective tissue, so it was unclear whether this is elastin or collagen.

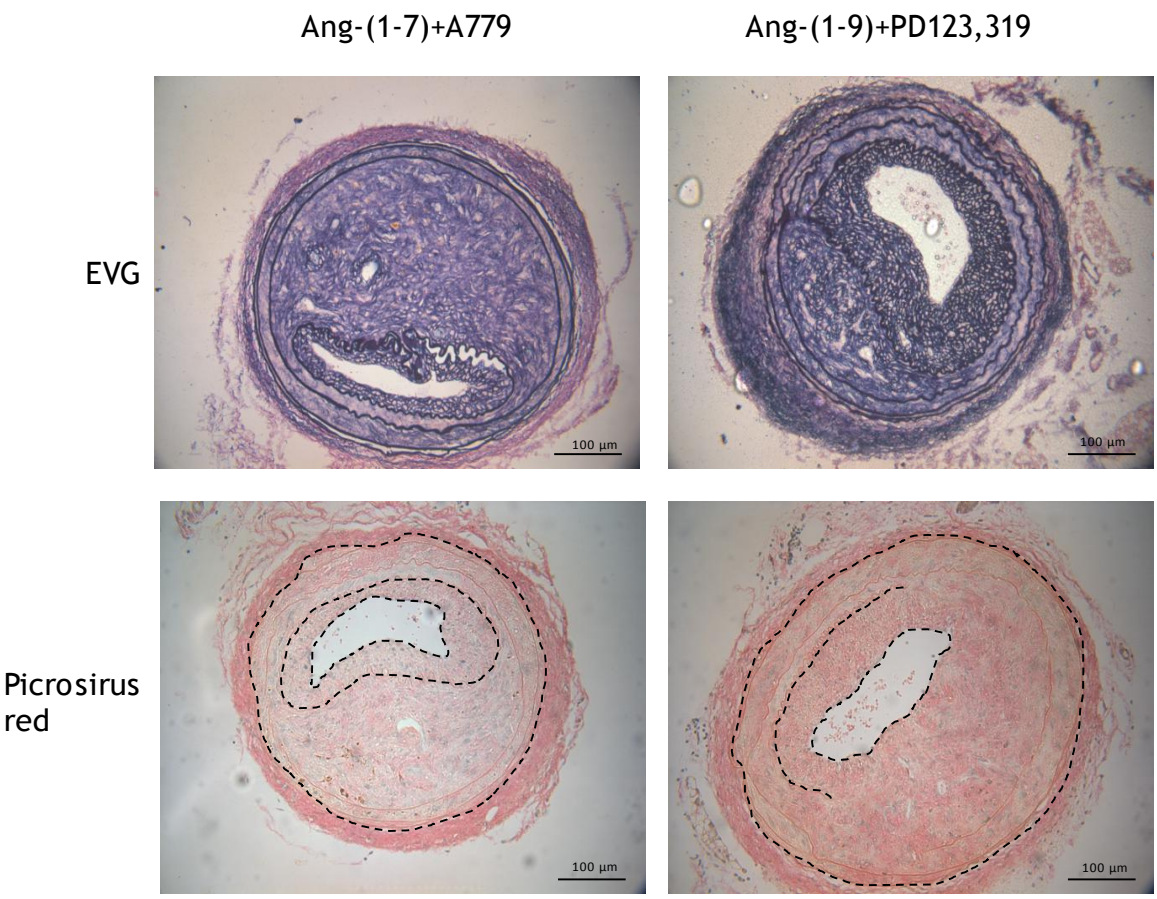

Figure 5.10 Elastin and collagen content of the complex lesions observed in the injured vessels of Ang-(1-7) + A779 and Ang-(1-9) + PD123,319 infused mice.
Representative histological sections of the complex lesions observed in the injured vessels of Ang-(1-7) + A779 and Ang-(1-9) + PD123,319 infused mice stained with EVG and picrosirus red to assess elastin and collagen content within the vessel, respectively. In EVG stained sections elastin appears black while collagen is stained pink. In picrosirus red stained sections collagen appears pink/red and nuclei appear purple/blue due to counterstaining with Weigarts haemotoxylin. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Incomplete dotted line indicates disruption of elastic lamina. Scale bar =100 µm, magnification x20. n = 6-10 animals per group.

Immunostaining for VSMC using α-SMA revealed that the complex lesions contained a high proportion of VSMC within the media and neointima (Figure 5.11).

Re-endothelialisation of the complex lesions was confirmed by immunostaining for CD31 (Figure 5.11). An intact endothelial cell layer was present in all vessels, as indicated by the brown staining surrounding the lumen. However, CD31 positive cells were also present within the media of the complex lesions, and appeared in a pattern of circular areas, indicative of small vessel formation with lumens, suggesting neovascularisation had occurred within the complex lesion.

A high proportion of proliferating cells were present within the complex lesion, as indicated by positive immunostaining for PCNA (Figure 5.11). Most PCNA cells were localised within the media of the complex lesion and to a lesser extent within the neointima. However, the number of PCNA positive cells within the neointima of vessels with complex lesions was higher than in those without. Additionally, a number of PCNA positive cells were observed in areas of apparent neovascularisation.

The complex lesions also contained a high number of cells positive for MAC-2, indicating increased presence of macrophages (Figure 5.11). Similar to cell proliferation, macrophage content was highest within the medial area and to a lesser extent within the neointima, and was higher in both areas than in vessels without complex lesions.

Very few vessels with a complex lesion contained cells positive for active caspase-3 activity, and in any vessels where it was observed, the number of positive cells were very few and localised within the media of the lesion (Figure 5.11).

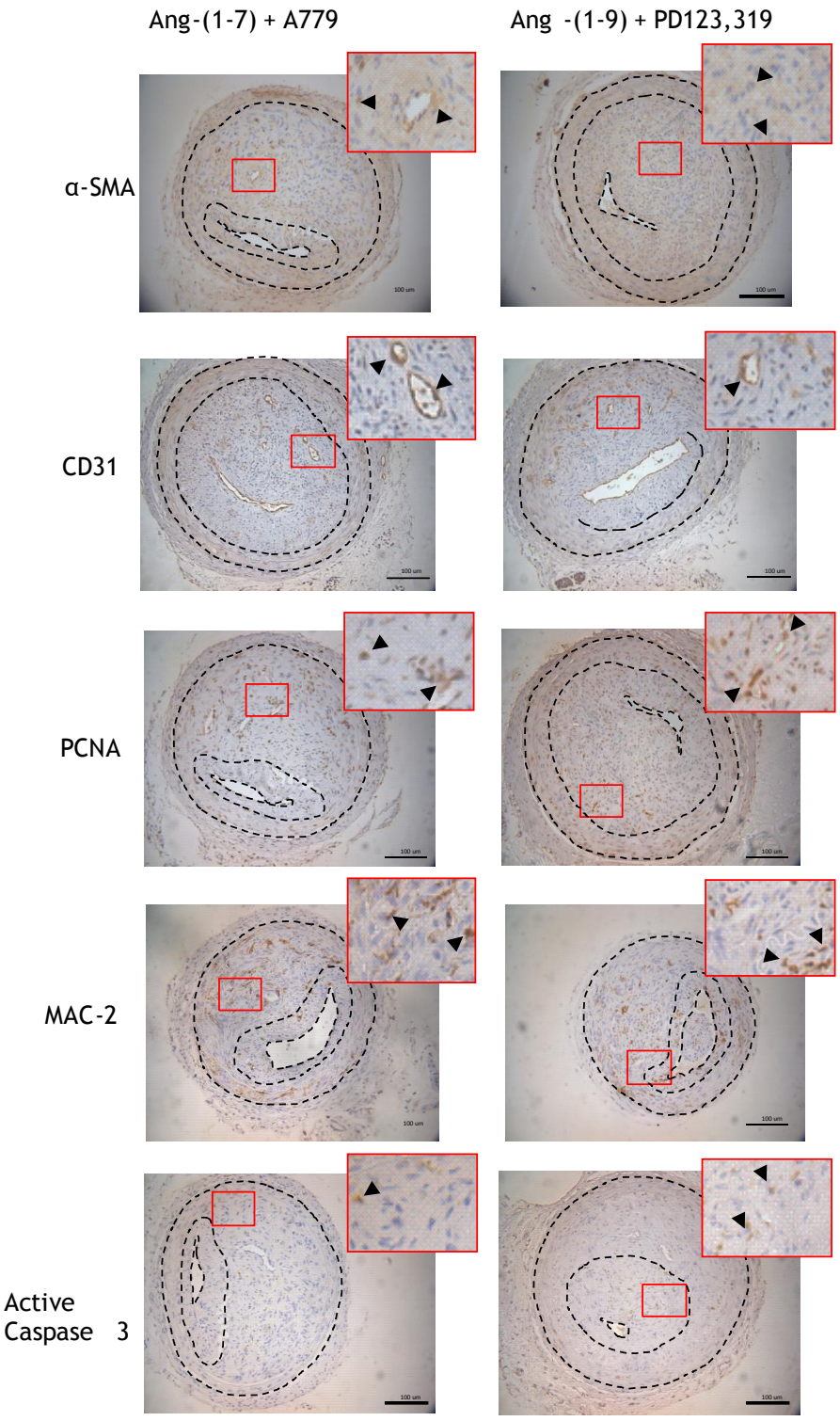

Figure 5.11 Cellular composition of the complex lesions observed in the injured vessels of Ang-(1-7) + A779 and Ang-(1-9) + PD123,319 infused mice.
Representative images of the IHC analysis of the cellular composition of the complex lesions observed in the injured vessels of Ang-(1-7) + A779 and Ang-(1-9) + PD123,319 treated mice was performed using anti-α-SMA, anti-CD31, anti-PCNA, anti-Mac2 and anti-active caspase-3 antibodies to detect the expression of VSMC, VEC, proliferating cells, macrophages and cell apoptosis, respectively. DAB chromagen was used to detect positive antigen binding (brown), indicated by arrow heads, and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen, with the exception of CD31 images where the inner dotted line = internal elastic lamina and luminal outline is absent due to positive CD31 cells within this region. Incomplete dotted line indicates disruption of the elastic lamina. Scale bar =100 μm, magnification x20, red box = 3x zoom. n = 6-7 animals per group.

5.2.3 Local delivery of Ang-(1-7) and Ang-(1-9) via Pluronic F-127 gel

Prior to investigating the functional effects of local delivery of Ang-(1-7) or Ang-(1-9) via Pluronic F-127 gel, the efficacy of delivery via this method was assessed. Mice whose vessels were exposed to gel only were used as controls. Animals were sacrificed at 7, 14 and 28 days post-wire injury and peptide delivery to the vessel assessed via IHC, using a specific antibody for Ang-(1-7) and streptavadin for the biotin-labelled Ang-(1-9).

Ang-(1-7) was detected in control and Ang-(1-7) treated vessels at all time points, as indicated by brown staining, and the level of detection was similar between both groups throughout the study (Figure 5.12). Detection of Ang-(1-7) was most prevalent in the adventitia and periadventitial connective tissue. Ang-(1-7) was also detected in the neointimal of injured vessels, particularly in control vessels at 28 days post injury. Ang-(1-7) was not detected in the media of vessels from either group at any time point. While Ang-(1-7) was detected in the adventitia and neointima of vessels from all groups, the staining was diffuse and did not appear to be localised to specific cells. Furthermore, some positive staining was observed in the lumen in areas which were acellular, suggesting unspecific binding of the primary antibody. No positive staining was observed in the PBS control group.

Positive detection of biotin-labelled Ang-(1-9), as indicated by brown staining, was observed at all time points; no positive staining was observed in control vessels (Figure 5.13). However, biotin-labelled Ang-(1-9) was only detected in the periadventitial connective tissue. Furthermore, detection levels declined over time, with the highest level of positive staining at 7 days post injury and application, and the lowest levels observed at 28 days.

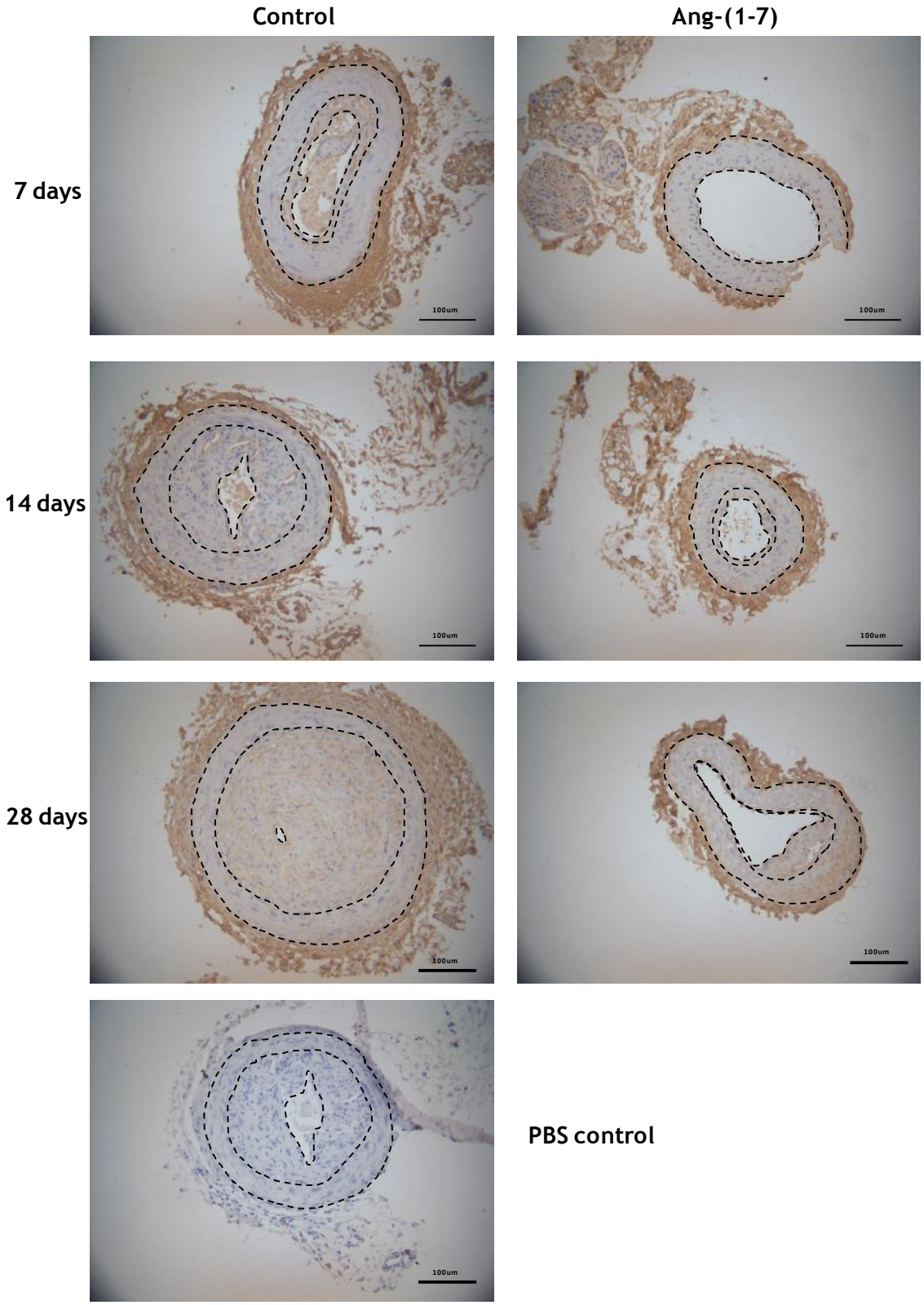

Figure 5.12 Detection of Ang-(1-7) in left carotid arteries at 7, 14 and 28 days post vascular injury.

IHC was performed using an anti-Ang-(1-7) antibody to assess peptide levels within the injured vessel following application of Ang-(1-7)-containing Pluronic F-127 gel. Tissue sections were incubated with PBS in place of primary antibody as a control. DAB chromagen was used to detect bound antigen (brown) and tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of Ang-(1-7) in the injured left carotid artery from control animals and animals administered Ang-(1-7) at 7, 14 and 28 days post application via Pluronic F-127 gel. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20. N = 3 animals per group.

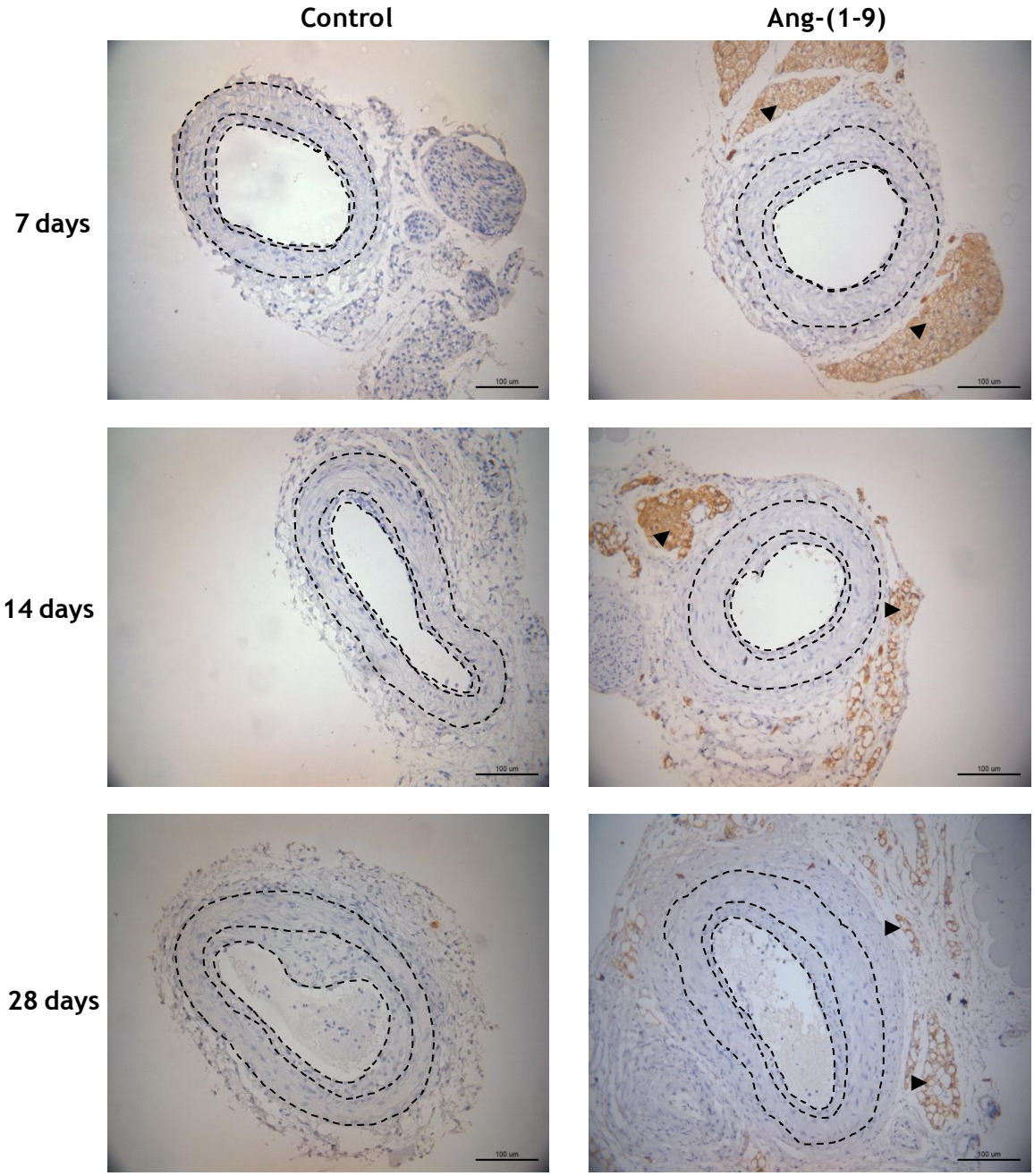

Figure 5.13 Detection of biotinylated-Ang-(1-9) in left carotid arteries at 7, 14 and 28 days post vascular injury

Delivery of biotinylated-Ang-(1-9) via Pluronic F-127 gel was assessed by incubation of tissue sections with ExtrAvadin peroxidase and the peptide detected using DAB chromogen (brown), indicated by arrow heads. Tissue sections were counterstained with haemotoxylin to visualise nuclei (blue). Representative histological sections of biotin-Ang-(1-9) detection in the injured left carotid artery from control (gel only) and peptide treated animals at 7, 14 and 28 days post application via Pluronic F-127 gel. Outer dotted line = external elastic lamina, middle dotted line = internal elastic lamina, inner dotted line = lumen. Scale bar =100 μm, magnification x20. N = 3 animals per group.

5.2.4 Effects of local delivery of Ang-(1-7) and Ang-(1-9) on vascular remodelling

Total body weight was monitored throughout the local peptide delivery study and no difference was observed between the experimental groups (Table 5.3). Heart and kidney weight was assessed at 28 days and expressed as a percentage of total body weight; no differences in heart or kidney weight were observed between the experimental groups (Table 5.3).

Table 5.3 Effects of local delivery of Ang-(1-7) and Ang-(1-9) on body and organ weight measurements following vascular injury

	Control (gel only)	Ang-(1-7)	Ang-(1-9)
Start weight (g)	24.0 ± 0.9	23.2± 0.2	24.0 ± 0.7
Final weight (g)	26.8 ± 1.0	25.5 ±0.7	26.3 ± 0.7
Heart/Body weight (%)	0.7 ± 0.02	0.6 ± 0.03	0.7 ± 0.02
Kidney/Body weight (%)	0.7 ±0.01	0.7 ± 0.02	0.7 ± 0.02

Organ weight was taken as a percentage of the body weight, n=8.

EVG staining was performed to assess the extent of vascular remodelling at 28 days post vascular injury, following local delivery of Ang-(1-7) or Ang-(1-9) via Pluronic F-127 gel. Vessels coated in gel only were used as a control. The study was unblinded following morphometric analysis of the stained sections and revealed that there were no differences between control, Ang-(1-7) or Ang-(1-9) treated vessels for all parameters measured (Figure 5.14). Neointima formation was modest and similar in area in all experimental groups. Similarly, no differences were observed in medial area, and therefore NI/MA. There were no apparent differences in ECM deposition in the injured vessels of all three groups as indicated by the similar degree of elastin (black) and collagen (pink staining) (Figure 5.14).

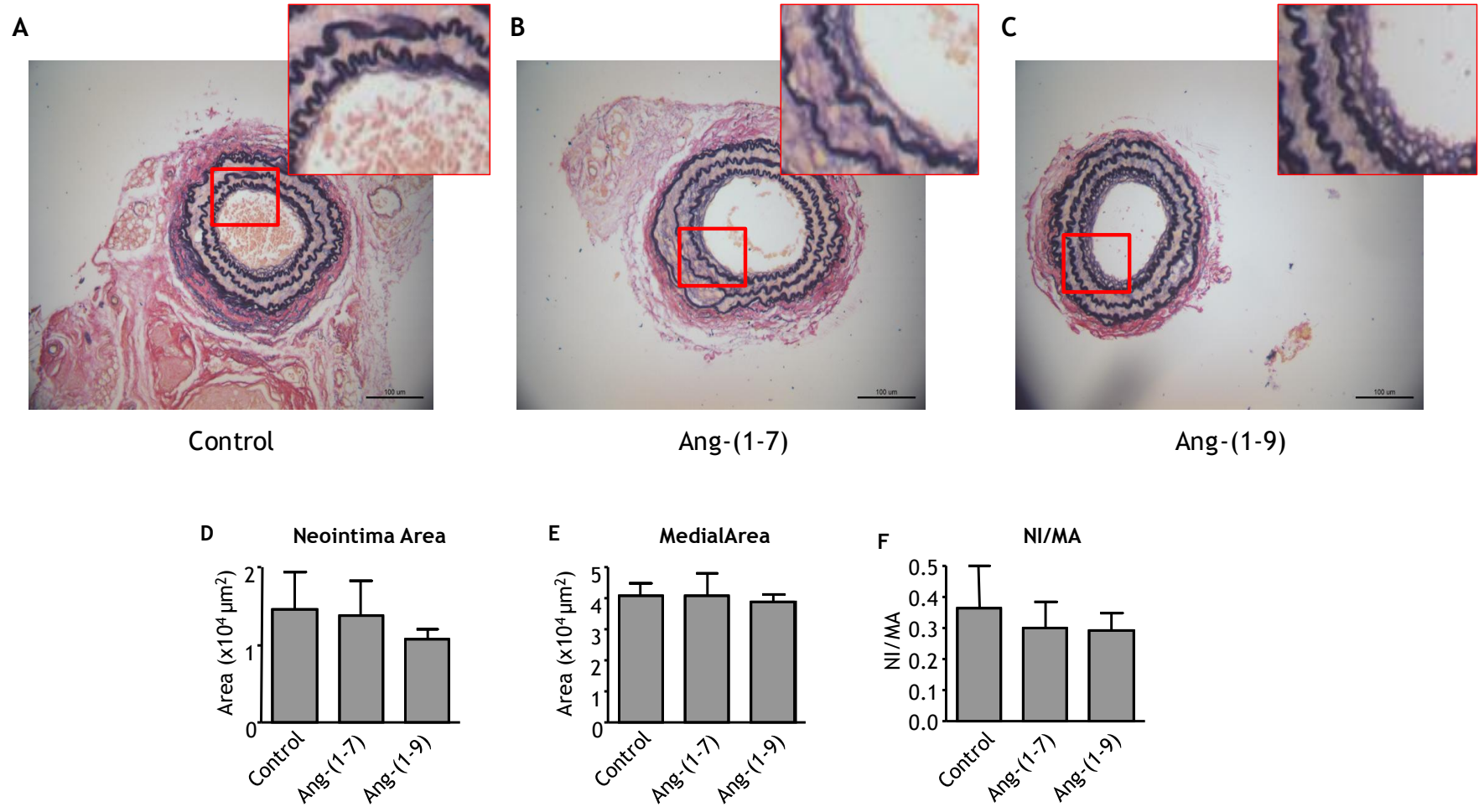

Figure 5.14 Effects of local delivery of Ang-(1-7) and Ang-(1-9) on neointimal formation in the carotid artery at 28 days following vascular injury.
Representative histological sections of the injured left carotid artery from (A) control animals and animals administered (B) Ang-(1-7), (C) Ang-(1-9) via Pluronic F-127 gel stained using EVG stain. Elastin appears black while collagen is stained pink. Neointimal area (D), media area (E) and NI/MA ratio (F) was assessed. Scale bar =100 µm, magnification x20, red box = 3x zoom. n= 7-8 animals per group.

5.3 Discussion

Prior to assessing the effects of Ang-(1-7) and Ang-(1-9) in vascular remodelling, a carotid artery wire injury model in mice was established. This is a commonly used model of acute vascular injury, where a nylon wire is inserted into the vessel causing endothelial denudation and mechanical stretching of the vessel wall, resulting in the formation of a neointima within 2 to 4 weeks (Lindner *et al.*, 1993). Consistent with previous findings it was demonstrated that at 28 days post injury there was a significant increase in vascular remodelling in injured vessels compared to non-injured vessels (Lindner *et al.*, 1993). This remodelling was associated with an increase in medial area and formation of a large neointimal area which was highly composed of ECM.

In this present study it was found that Ang-(1-7) infusion reduced vascular injury in comparison to control vessels, as evidenced by the reduction in neointimal formation and NI/MA ratio. Ang-(1-7) had no effect on the medial area. Importantly, blockade of Mas using the pharmacological inhibitor A779 abolished the anti-remodelling effects of Ang-(1-7), resulting in a similar neointimal area and NI/MA ratio as control vessels. These findings are consistent with previous studies in various rodent models of vascular injury where Ang-(1-7) was shown to inhibit neointimal growth (Strawn *et al.*, 1999, Langeveld *et al.*, 2005, Langeveld *et al.*, 2008, Zeng *et al.*, 2009, Wu *et al.*, 2011). For example, in a rat model of carotid artery injury induced by balloon embolectomy catheter, Ang-(1-7) infusion via osmotic mini pump was proven to reduce neointimal area without affecting medial area (Strawn *et al.*, 1999). Furthermore, it was demonstrated that these effects were independent of the AT_1R or AT_2R (Strawn *et al.*, 1999). In another study, where vascular injury in rabbits was induced by aortic angioplasty, Ang-(1-7) infusion, again via osmotic mini pump, resulted in inhibition of neointimal formation but no change in media area (Zeng *et al.*, 2009). Importantly, these effects were blocked by A779, indicating that Ang-(1-7) acted via Mas. More recently, using the rat autologous jugular vein graft model it was demonstrated that Ang-(1-7) reduced vascular remodelling and neointimal formation within the vein graft (Wu *et al.*, 2011).

The findings from this study show for the first time a direct action of Ang-(1-9) in vascular remodelling. Here we show that Ang-(1-9) reduced neointimal

formation and NI/MA ratio following wire injury to the mouse carotid artery. Importantly, the effects of Ang-(1-9) were demonstrated to be via the AT_2R, as PD123,319 blocked the effects of Ang-(1-9), resulting in a significant increase in neointima area, media area and NI/MA in comparison to animals who received Ang-(1-9) alone. Conversely, co infusion of A779 with Ang-(1-9) produced similar results to those observed with Ang-(1-9) alone, indicating that the Ang-(1-9) mediated inhibition of neointimal formation was achieved via a direct effect of Ang-(1-9), as opposed to conversion to Ang-(1-7) and signalling via Mas. No previous studies have been performed to assess the role of Ang-(1-9) in acute vascular injury, however, Ang-(1-9) has recently been shown to indirectly contribute to reduced aortic remodelling associated with hypertension (Ocaranza *et al.*, 2011). In hypertensive deoxycorticosterone acetate (DOCA)-salt rats, it was demonstrated that inhibition of the RhoA/Rho-associated, coiled-coil containing protein kinase (ROCK) signalling pathway using Fausidil resulted in increased activity and expression of ACE2 in the aorta, and increased Ang-(1-9) plasma levels, as well as reduced blood pressure and reduced expression of key genes related to vascular remodelling, such as TGF-β1, PAI-1 and MCP-1 (Ocaranza *et al.*, 2011). While this study identifies a potential novel pathway of Ang-(1-9) in the vasculature and an avenue for exploration in the current study it is worthwhile to highlight that the vascular remodelling observed in the study by Ocaranza *et al* was very mild as it was induced by hypertension as opposed to direct vessel injury (Ocaranza *et al.*, 2011).

The results from this present study indicate that the effects of Ang-(1-9) are mediated via the AT_2R. While further work is required to fully understand the vasculoprotective effects of the Ang-(1-9)/AT_2R interaction and corroborate these findings, there is evidence in the literature which supports a protective role for the AT_2R in vascular injury. For example, over-expression of the AT_2R following balloon injury in the rat results in attenuation of neointimal formation (Nakajima *et al.*, 1995). Similarly, in an alternative model of vascular injury where a cuff is placed around the femoral artery to induce neointima formation, absence of the AT_2R resulted in increased neointimal formation (Akishita *et al.*, 2000, Wu *et al.*, 2001). Furthermore, following cuff placement $AT_2R^{-/-}$ mice have reduced apoptosis within the neointima, consistent with increased neointimal area (Suzuki *et al.*, 2002). The AT_2R has also been linked to reduced

atherosclerosis as deletion of the AT_2R from Apo $E^{-/-}$ mice results in a worsened atherosclerotic phenotype associated with increased VSMC content within the lesion (Sales *et al.*, 2005). Taken together these results demonstrate that the AT_2R is important in the development of atherosclerosis and vascular remodelling following injury.

Remodelling of the ECM and deposition of ECM proteins such as elastin and collagen occurs during the remodelling process and largely contributes to neointimal formation. This has been shown to be the case in the present study where large areas of neointimal formation containing a high proportion of ECM following vascular injury were observed. While Ang-(1-7) reduced neointimal formation there were no differences in the organization of the ECM within the media or small neointimal area present. The effects of Ang-(1-7) were shown to be achieved via Mas and these findings were consistent with previously published results suggesting that Ang-(1-7) reduces vascular remodelling in part through inhibition of ECM synthesis and deposition (Strawn *et al.*, 1999, Langeveld *et al.*, 2005, Zeng *et al.*, 2009). Similarly, Ang-(1-9) reduced neointimal formation and therefore ECM synthesis, an effect which was shown to be via the AT_2R and independent of conversion to Ang-(1-7) and signalling via Mas. However, Ang-(1-9) also reduced medial elastin content in comparison to control vessels, an effect which was unaffected by either AT_2R or Mas inhibition. Although there is no information in the literature to suggest Ang-(1-9) can reduce ECM synthesis and deposition in the vessel in response to injury, it has been shown that Ang-(1-9) can inhibit collagen expression in the heart in an Ang II infusion model of hypertension (Flores Munoz *et al*, unpublished) and in the SHRSP (Flores-Munoz *et al.*, 2012). In the studies by Flores Munoz *et al*, these effects were demonstrated to be via the AT_2R. Currently little is known about the cell signalling mechanisms employed by Ang-(1-9) and it is possible that in the vasculature this peptide reduces ECM deposition via an alternative receptor to the AT_2R or can be metabolised to an alternative peptide that may lead to this reduced ECM content within the media following vascular injury.

VSMC migration and proliferation is largely involved in the development of neointimal formation following vascular injury (Costa and Simon, 2005). The results from this current study confirm that vessels from all groups are largely composed of VSMC. Vascular injury results in the formation of a large neointimal

area, highly composed of VSMC, this neointimal formation is blocked by both Ang-(1-7) and Ang-(1-9) and therefore reduced VSMC content is observed within the total vessel. The effects of Ang-(1-7) were shown to be via Mas while the effects of Ang-(1-9) appeared to be via the AT_2R and independent of conversion to Ang-(1-7) and signalling through Mas. While this anti-vascular remodelling effect of Ang-(1-9) has not previously been demonstrated, the anti-proliferative and anti-migratory role of Ang-(1-7) has been observed in numerous rodent models of vascular disease. Ang-(1-7), via Mas, reduces neointimal formation following balloon injury (Strawn *et al.*, 1999), stent implantation in rats (Langeveld *et al.*, 2005), and angioplasty in rabbits (Zeng *et al.*, 2009) and has been associated with reduced atherosclerotic lesion size in Apo $E^{-/-}$ mice (Jawien *et al.*, 2012).

VSMC proliferation is one of the most important processes involved in the development of neointimal formation. In this present study, very little cell proliferation was observed at 28 days following vascular injury in control, Ang-(1-7) and Ang-(1-9) groups. However, this is consistent with previous findings and likely to be linked to the 28 day time point studied. Cell proliferation is thought to be maximal within the first 5 to 14 days following vascular injury and then decline as the vessel becomes re-endothelialised, findings which are in line with this current study (Lindner *et al.*, 1993). While no differences were observed in cell proliferation between control or Ang-(1-7) treated vessels at 28 days post injury, this may not have been the case at earlier time points where VSMC proliferation has been shown to be maximal, such as 5 to 14 days post-injury, and further investigation of these times would be required to assess the anti-proliferative effects of Ang-(1-7). It has previously been demonstrated that Ang-(1-7) infusion reduced VSMC proliferation within both the media and neointimal of normotensive Sprague-Dawley rats in comparison to saline infused animals at 12 days following vascular injury (Strawn *et al.*, 1999). However, in contrast to the findings of this present study, VSMC proliferation following rat autologous vein graft surgery was present at 28 days following vascular injury. The number of proliferating cells was reduced in comparision to control vessels and this was demonstrated to be via inhibition of the ERK1/2 and p38 MAPK pathways (Wu *et al.*, 2011).

Increased cell proliferation was observed in vessels from animals co-infused with Ang-(1-7) + A779 or Ang-(1-9) + PD123, 319, and to a lesser extent Ang-(1-9)+ A779, in comparison to control vessels. It is unclear as to the reason for this as there were no differences in reendothelialisation however, a similar trend is observed in macrophage content of the vessels suggesting a link between the two. This is plausible since macrophages secrete a number of growth factors and cytokines which would in turn act on the VSMC causing cell proliferation (Assoian *et al.*, 1987). Ang-(1-7) signalling via Mas has previously been linked to reduced macrophage content within injured and atherosclerotic vessels. For example, Ang-(1-7) has been demonstrated to reduce macrophage content within atherosclerotic lesions of Apo $E^{-/-}$ via Mas, thereby contributing to reduced VSMC content and plaque stability (Yang *et al.*, 2013). While a role for Ang-(1-9) in reduced inflammation within the vasculature has yet to be demonstrated, administration of CGP42112, an AT_2R agonist, to Apo $E^{-/-}$ mice resulted in reduced macrophage content within the plaque and increased plaque stability (Kljajic *et al.*, 2013). Despite the fact that little macrophage expression was observed in control vessels, is it possible that inhibition of both endogenous and exogenous Ang-(1-7) or Ang-(1-9) signalling via Mas and the AT_2R, by A779 and PD123,319, respectively, has led to increased expression of macrophages within the vessel. However, further investigation would be required to fully assess this.

During vascular injury the endothelial lining of the vessel is denuded, with re-endothelialisation complete by 28 days post injury (Lindner *et al.*, 1993). The results from this study are consistent with these findings; an intact endothelial cell layer was present in all groups. As re-endothelialisation was complete by this stage it is unclear if Ang-(1-7) or Ang-(1-9) affect the rate at which re-endothelialisation occurs and earlier time points would be required to investigate this further. Furthermore, the new endothelial cell layer is often markedly dysfunctional, so it would be worthwhile to explore whether of Ang-(1-7) or Ang-(1-9) improved the function of the re-endothelialised vessel. Ang-(1-7) has been shown to increase NO release from endothelial cells, thereby acting as a vasodilator and improving vascular endothelial function (Brosnihan *et al.*, 1996, Faria-Silva *et al.*, 2005). Increase of NO is achieved directly via Mas-mediated stimulation of eNOS and sustained Akt phosphorylation, or indirectly via production of bradykinin and receptor cross talk with the BK_2R (Jackman *et*

al., 2002, Sampaio *et al*., 2007b). Additionally, in the rat stent model, Ang-(1-7) infusion improved endothelial function through enhanced prostaglandin function (Langeveld *et al*., 2005). Similarly, Ang-(1-9) has also recently been shown to improve endothelial function (Flores-Munoz *et al*., 2012). Ang-(1-9) infusion in the SHRSP improved aortic vasorelaxation and NO bioavailability via the AT_2R (Flores-Munoz *et al*., 2012). While the mechanisms involved are currently unknown it is possible that Ang-(1-9) may increase NO bioavailability by stimulating bradykinin release, as previously documented in cardiac endothelial cells (Jackman *et al*., 2002), or by enhancing the activity of eNOS, as has been shown for Ang-(1-7) (Sampaio *et al*., 2007b).

Cell apoptosis is a key process in vascular remodelling, however, apoptosis was not apparent within the injured vessel in any group. This may be due to the 28 day time point studied. Various studies have been performed in models of vascular injury that may provide more insight into the role of apoptosis. Following balloon injury in $ApoE^{-/-}$ mice, cell apoptosis was found to be elevated within the first 24 hrs (Matter *et al*., 2006). Similar findings were observed in balloon injured rabbits where it was demonstrated VSMC apoptosis was highest within 24 hrs and was via a caspase 3-dependent mechanism (Spiguel *et al*., 2010). These findings are consistent with those in the present study where cell apoptosis was minimal at the later time points studied. An assessment of the involvement of apoptosis as a mechanism for reduced neointimal formation at earlier time points would be worthwhile, particularly as the AT_2R-induced apoptosis has previously been linked to reduced neointimal formation. For example, in the cuff injury model of vascular injury, $AT2R^{-/-}$ mice displayed increased neointimal formation associated with reduced VSMC apoptosis and increased VSMC proliferation in comparison to wild type mice at 14 days post injury (Suzuki *et al*., 2002).

An interesting, yet unexpected finding from this present study was that a large proportion of vessels from animals co-infused with Ang-(1-7) and A779, or Ang-(1-9) and PD123,319 developed more complex lesions with increased vessel remodelling and neovascularisation, largely within the media, in comparison to all other groups. The formation of complex lesions was associated with positive vessel remodelling and stretching, often disruption, of the elastic lamina. Analysis of the structural composition of these complex lesions revealed that

they were largely composed of disorganised ECM and were highly cellular, containing a large number of VSMC, macrophages and proliferating cells. Re-endothelialisation had occurred on the lumenal lining of the vessels and neovascularisation of the complex lesion was also observed. There was no difference in the composition of the complex lesions observed in animals infused with Ang-(1-7) and A779 or Ang-(1-9) and PD123, 319. It is unclear what has led to the development of these complex lesions, however, based on their histological profile one possible explanation could be that it was due to the presence of an intra-mural thrombus. During the wire injury procedure, the endothelial layer of the vessel is denuded, exposing the blood to a highly thrombogenic surface. Additionally, the vessel is stretched, which can cause disruption of the internal elastic lamina. Normally, the elastic lamina is repaired within 7 days, however in circumstances with high thrombogenesis; the thrombus can become encapsulated within the damaged vessel, more often in the medial area (Wilensky *et al.*, 1995). This is known as an intramural thrombus. Within the media, VSMC and macrophages migrate into the thrombus and begin to proliferate, resulting in the intramural thrombus becoming highly cellular (Wilensky *et al.*, 1995). While this proliferative stage is most prevalent within 7 to 14 days post injury, due to the presence of inflammatory cells various growth factors and cytokines are secreted which may explain why there is still a high degree of proliferation within these complex lesions in this present study. Within the intramural thrombus the VSMC can differentiate into myofibroblasts, and together the VSMC and myofibroblasts synthesise and deposit large proportions of ECM (Wilensky *et al.*, 1995). Both VSMC and myofibroblasts are positive for α-SMA, and the high proportion of positive staining observed within the vessels may be indicative of expression of both cell types (Shi *et al.*, 1997). Re-endothelialisation of the vessel is found to be complete by around 4 weeks in most models of vascular injury, and this was also found to be the case in vessels with complex lesions (Lindner *et al.*, 1993, Zou *et al.*, 1998, Ali *et al.*, 2007). However, it was also noted that neovessels had formed within the complex lesions, as indicated by the positive CD31 staining within the vessels. This phenomenon of neovessel formation has previously been described in atherosclerotic vessels and from failed human saphenous vein bypass graft tissue, and has been demonstrated to be associated with atherosclerotic lesion formation and intramural thrombus (Bobryshev *et al.*,

2001, Motwani and Topol, 1998, Nielsen *et al.*, 1997). Intimal neovascularisation in atherosclerosis has been linked to the development of complications such as intimal haemorrhage, plaque rupture and the formation of an occlusive thrombosis (Zhang *et al.*, 1993, O'Brien *et al.*, 1994, Ignatescu *et al.*, 1999). Furthermore, the newly formed vessels are associated with inflammatory cell infiltration and increased cell proliferation (Kumamoto *et al.*, 1995). While in this current study the vessels did not contain atherosclerotic plaques, the appearance of the neovessels is consistent with those found in atherosclerosed vessels, in that cell proliferation and macrophage expression are found to be associated with these neovessels, suggesting that they may be formed in a similar manner. Investigation into the formation of neovessels within atherosclerotic arteries has revealed that VEGF plays an important role in this process, in fact advanced atherosclerotic plaques containing a large number of VEGF-positive cells are much richer in neovascularisation than atherosclerotic lesions containing fewer VEGF-positive cells (Couffinhal *et al.*, 1997, Inoue *et al.*, 1998, Chen *et al.*, 1999). Therefore, to gain further insight into the mechanisms involved in the formation of complex lesions and neovascularisation observed in this present study it would be worthwhile to assess the expression of VEGF within these vessels.

It is unlikely that these complex lesions have occurred as a result of either the antagonists or peptides singularly, as no complex lesions were observed in mice infused with the peptides on their own or co infused with Ang-(1-9) + A779. However, as only one group of animals received PD123,319, it cannot be ruled out that this compound is not having an effect on its own. Furthermore, while it is widely used as an AT_2R antagonist, PD123, 319 has also been demonstrated to have non AT_2R- specific effects, for example it has recently been shown to be an agonist of the MrgD receptor (Lautner *et al.*, 2013). Furthermore, in a mouse model of abdominal aortic aneurysm (AAA), PD123,319 was demonstrated to augment Ang II-induced AAA formation through an AT_2R-independent mechanism (Daugherty *et al.*, 2013).

In addition to blocking the effects of Ang-(1-9), PD123,319 will block endogenous AT_2R activity, potentially contributing to increased vessel remodelling as the AT_2R has been linked to reduced vascular injury, as previously discussed (Wu *et al.*, 2001, Suzuki *et al.*, 2002, Akishita *et al.*, 2000). Furthermore, the AT_2R has

been demonstrated to possess constitutive activity, leading to ligand-independent activation of various cell signalling pathways, particularly those linked to increased cell apoptosis, a processes which could contribute to reduced vascular remodelling (Miura and Karnik, 2000, Jin *et al.*, 2002). It is therefore possible that PD123,319 could contribute to increased vascular remodelling via inhibition of the constitutive activity of the AT_2R. One possible explanation is that due to blockade of their endogenous receptors, the peptides are converted to alternative peptides or interact with an alternative receptor, which enhances thrombus formation. For example, while Ang-(1-7) has been demonstrated to have anti-thrombotic effects (Kucharewicz *et al.*, 2002, Heitsch *et al.*, 2001, Fraga-Silva *et al.*, 2011, Fraga-Silva *et al.*, 2008), it may be converted to an alternative peptide with potential prothrombotic effects, such as Ang-(3-7). While a direct pro-thrombotic role for Ang-(3-7) has not yet been established, it has been reported to interact with the AT_4R, which is known to upregulate PAI-1, a key protein involved in thrombogenesis (Gesualdo *et al.*, 1999, Numaguchi *et al.*, 2009). Furthermore, AT_1R activation is widely accepted to be prothrombotic, and while Ang-(1-9) appears to display functional selectivity at the AT_2R, is has been shown to have equal affinity at both the AT_1R and AT_2R (Flores-Munoz *et al.*, 2011). In a recent study by Kramkowski *et al* (2010) it was indicated that Ang-(1-9) enhances electrically stimulated thrombosis and increases platelet aggregation in rats via the AT_1R (Kramkowski *et al.*, 2010). However, it is worthwhile to point out that electrical stimulation was used to injure the vessel and initiate thrombosis which is somewhat different to the wire injury model. Also, the pro-thrombotic effect of Ang-(1-9) was much lower than that of Ang II, and was in fact found to be due to direct metabolism of Ang-(1-9) to Ang II (Kramkowski *et al.*, 2010, Drummer *et al.*, 1988, Singh *et al.*, 2005).

While the current data set highlights the potential of Ang-(1-7) and Ang-(1-9) as therapeutic agents in the setting of vascular remodelling, one factor limiting the translational impact of these results is the delivery method used. Subcutaneous delivery via osmotic mini pump results in peptide delivery to the systemic circulation and although minimum infusion is a commonly used drug delivery method in animal models, which ensures that the drug is delivered at a constant rate over a set period of time without the need to cause the animal additional stress by daily dosing, this would not represent the situation in the clinic

(Theeuwes and Yum, 1976). Therefore, to represent a more clinically relevant delivery approach it was also investigated whether Ang-(1-7) or Ang-(1-9) could be delivered locally to the vessel via Pluronic F-127 gel immediately following vascular injury, and if so what effect this would have on the resultant remodelling.

Delivery of Ang-(1-7) detected using an antibody for the peptide and it was shown that peptide levels were comparable between control and Ang-(1-7) at all time points, and Ang-(1-7) detection did not change over time. If Ang-(1-7) was delivered efficiently via Pluronic F127 gel it would be expected that higher levels of peptide would be detected in the Ang-(1-7) treated vessels and the levels would decline towards control levels over the course of the study. It is possible that the Ang-(1-7) had already been fully delivered by the time points chosen, as previous studies have shown that drug delivery via this method is optimal within the first week following application (Villa *et al.*, 1995, Grassia *et al.*, 2010, Shi *et al.*, 2014). Therefore in this present data it is unclear if Ang-(1-7) was delivered to the vessel. Furthermore, some positive immunostaining for Ang-(1-7) was observed in the lumen in areas which were acellular, suggesting that there may be some unspecific binding of the primary antibody. Importantly no positive staining was observed in sections treated with PBS instead of the antibody, suggesting that this is not due to the presence of residual unbound secondary antibody. However, it has previously been demonstrated that this antibody is specific for Ang-(1-7) in different tissues such as the heart and kidney, therefore further testing of the antibody specificity, by preincubation of vessel sections with Ang-(1-7) peptide, for example, is required (Giani *et al.*, 2012, Calo *et al.*, 2010a, Zhang *et al.*, 2010a).

At the time of this study an Ang-(1-9) antibody was not commercially available, therefore, delivery of this peptide was assessed using a biotinylated Ang-(1-9). One benefit to this approach over antibody based detection is that only the exogenous biotinylated Ang-(1-9) will be detected, therefore it can be distinguished from endogenous Ang-(1-9) within the vessel. Ang-(1-9) was only detected in vessels which had the peptide delivered and levels declined over time, with the highest level of positive staining observed at 7 days post-application. However, Ang-(1-9) was only detected in the periadventitial tissue, with no positive staining observed within the media or neointimal area, where

present. Therefore it is unclear if the peptide was effectively delivered to the vessel. Pluronic F-127 gel has previously been reported to mediate sustained release and reduced degradation of peptides such as gonadotropin-releasing hormone (GnRH) and deslorlin (a GnRH agonist) both *in vitro* and *in vivo* (Wenzel *et al.*, 2002), thereby providing sustained delivery over time. While this increased bioavailability has yet to be demonstrated for angiotensin peptides, one possible explanation for the observed results is that while in the gel Ang-(1-9) is more stable, but once dissolved from the gel to the vessel it is rapidly metabolised, and therefore unlikely to be observed within the different layers of the vessel. Biotinylated Ang-(1-9) was still present in the periadventitial tissue, although at very low levels, at 28 days post application, and while previous studies have suggested that Pluronic F-127 gel is dissolved within 3 to 7 days *in vivo* and that drug delivery is maximal within this time, this finding of sustained drug levels post-gel degradation is not unheard of. In a study where the cell cycle inhibitor cilostazol was delivered via Pluronic F-127 gel following balloon injury in rats it was found that while the gel was dissolved by 7 days post application, tissue levels of the drug were still increased within the vessel and surrounding tissue at 14 days (Ishizaka *et al.*, 1999). Similarly, in a study investigating the effects of NF-κB inhibition on neointimal formation following wire injury, Grassia *et al* delivered a biotinylated NF-κB essential modulator-binding domain peptide, an antagonist of the inhibitor of kappa b (IκB) kinase complex, via Pluronic F-127 gel and found that while the gel was dissolved at 3 days post application, the biotinylated peptide was still observed at the termination of the study at 14 days (Grassia *et al.*, 2010).

In summary, from this pilot study, it is unclear if Ang-(1-7) and Ang-(1-9) can be delivered to the vessel via Pluronic F-127, however, the decline in Ang-(1-9) levels over time suggests that this may be possible. To provide more conclusive evidence it would be beneficial to measure peptide levels directly within the vessel, surrounding tissue and in the systemic circulation. This can be achieved through the use of high-performance liquid chromatography-based radioimmunoassay with amino-terminal-directed antisera, a method which has been demonstrated to effectively measure angiotensin peptide levels in various settings (Campbell *et al.*, 1995, Ocaranza *et al.*, 2006, Lawrence *et al.*, 1990).

While it could not be proven conclusively that the peptides had been delivered to the vessel via Pluronic F-127 gel, the results from the Ang-(1-9) portion of this study suggested that it may have been possible. Therefore, a full blinded study was performed to assess the effect of local application of Ang-(1-7) and Ang-(1-9) on vascular remodelling and neointimal formation. However, in this study very little neointimal formation was observed in gel only control vessels. As these vessels were a vehicle control it would be expected that a similar neointimal area would be observed to that of the control animals in the minipump study, however, both neointimal area and NI/MA ratio of the gel only controls were significantly lower than those of the minipump controls. This finding was unexpected as this gel based delivery approach has been utilised in a number of studies using various *in vivo* models of vascular disease/remodelling and has been demonstrated to have no effect on neointimal formation (Ishizaka *et al.*, 1999, Grassia *et al.*, 2010, Wenzel *et al.*, 2002, Shi *et al.*, 2014). While vessel morphology in the previous study to determine peptide delivery to the vessel was not quantified due to low numbers, neointimal area was present in gel only treated vessels at all time points, particularly at 28 days, therefore there was no indication that this finding would be expected in this present study. It is unclear as to why this impaired neointimal formation has occurred within this group, however as this was the only study where minimal neointimal area was present in control vessels it may be due to variability in batches of animals. Ultimately, due to the limited neointimal formation in the control groups it could not be determined if the small neointimal area in the Ang-(1-7) and Ang-(1-9) treated vessels was as a result of the peptides.

There are a number of advantages to the mouse carotid artery wire injury model, namely that it is highly reproducible, results in the formation of a large neointimal area and is low cost. However, this is a very simple model of vascular remodelling and as with all rodent models, has a number of limitations. While the injury induced is similar to that of PCI this model involves disruption of the flow of blood due to the ligation, an effect which would not occur clinically. Furthermore, alternative, more clinically relevant animal models of vascular injury exist; however, these also are not without limitation. Traditionally porcine models have been used to investigate vascular injury in response to CABG surgery or stenting due to the fact that pigs are physiologically a lot closer

to human than rodents and that their blood vessels and response to injury are very similar to those in humans, however, these studies are extremely expensive and perhaps more relevant for preclinical work as opposed to an early stage investigation (Taylor *et al.*, 2001, Miller *et al.*, 1996, Angelini *et al.*, 1990). Mouse models of vascular disease are therefore advantageous due to the high throughput of these animals, the low cost and the ability to use genetically modified strains. Mouse models of stenting and vein grafting have been developed which are more representative of the type of damage observed in humans compared to wire injury (Zou *et al.*, 1998, Ali *et al.*, 2007), however both are very complex to perform, are expensive due to the requirement for a donor mouse and come with their own disadvantages. The mouse model of vein grafting was developed by Zou *et al* in 1998 and involved the engraftment of the vena cava or external jugular vein from a donor mouse to the carotid artery of a recipient mouse (Zou *et al.*, 1998). While this model is useful for studying the mechanisms involved in vein graft failure and closely represents the disease observed in humans, there are some important considerations. For example, the formation of accelerated atherosclerosis, which is present in human grafts beyond the first year after CABG surgery, would be difficult to investigate in the mouse model due to the shorter life span and the fact that mice do not develop spontaneous atherosclerosis (Zou *et al.*, 1998). The mouse stent model was developed by Ali *et al* and involves the deployment of a small stent into the aorta of the donor mouse, which is then engrafted to the carotid artery of a recipient mouse (Ali *et al.*, 2007). However, in contrast to human disease this model does not involve stenting of an atherosclerotic plaque or contain the inflammatory component associated with atherosclerosis in humans, and due to the absence of a plaque the vessel is not restored to its original diameter, but expanded to a lot wider than normal. Additionally, in the wire injury model it is easy to obtain a large length of carotid artery that has been injured to use process for histological analysis, however, due to the small size of the stent there is a limited amount of tissue available and the presence of metal struts makes it very difficult to process (Bradshaw *et al.*, 2009). This greatly reduces the amount of sections available for analysis and therefore can restrict the depth of the investigation. Taking into consideration the aforementioned advantages and disadvantages of each model and the fact that the studies presented here were the first exploration of the effects of the peptides on

vascular remodelling, it was decided the wire injury model was the most appropriate.

5.4 Conclusion

In summary, the data demonstrates that the carotid artery wire injury model is a highly reproducible model of neointimal formation to act as a platform to investigate the effects of Ang-(1-7) and Ang-(1-9) on vascular remodelling. In this study it was demonstrated that infusion of Ang-(1-7) via osmotic minipump prevents wire injury induced vascular remodelling via the Mas receptor. Importantly, this study also demonstrates for the first time that infusion of Ang-(1-9) via osmotic minipump is similarly capable of inhibiting wire injury induced vascular remodelling and that its effects are via the AT_2R, and independent of conversion to Ang-(1-7) and signalling via Mas. This reduction in vascular remodelling observed following treatment with Ang-(1-7) or Ang-(1-9) was associated with reduced neointimal formation and reduced proliferation and migration of VSMC. Importantly, reendothelialisation of the vessel was unaltered by either peptide, although further investigation is required to assess the functioning of this new endothelial cell layer. While further studies are required to fully understand the signalling mechanisms involved in this Ang-(1-9)/AT_2R interaction within the vasculature, these results highlight a novel action of Ang-(1-9) that may be exploited therapeutically.

Furthermore, it was also observed that infusion of Ang-(1-7) during Mas blockade or Ang-(1-9) during AT_2R blockade resulted in the formation of complex lesions which were potentially indicative of the formation of an intramural thrombus. As this only occurred within these two groups it suggests that this phenomenon may occur as a result of conversion of Ang-(1-7) and Ang-(1-9) to an alternative peptide and/or interaction with an alternative receptor that enhances the occurrence of thrombosis. However, further work is required to fully investigate the mechanisms leading to the formation of these complex lesions.

Chapter 6

General Discussion

6.2 Overall summary

The main focus of this thesis was to investigate the interaction of Ang II and the counter-regulatory peptides Ang-(1-7) and Ang-(1-9) in the vasculature using primary HSVSMC and HSVEC and an *in vivo* model of acute vascular injury. First, a model of HSVSMC proliferation was established where it was demonstrated that addition of serum induced HSVSMC proliferation in a concentration-dependent manner. Stimulation with Ang-(1-7) or Ang-(1-9) inhibited serum-induced proliferation of HSVSMC via Mas and AT_2R, respectively. While Ang II was unable to induce proliferation of HSVSMC at the concentration used, it was demonstrated to have potent pro-migratory effects. Ang II-induced HSVSMC migration via the AT_1R, and this effect was inhibited by Ang-(1-7) or Ang-(1-9) via Mas or the AT_2R, respectively. Further investigation into the functional interplay of Ang II, Ang-(1-7) and Ang-(1-9) in HSVSMC migration identified alterations in ERK1/2 activity and, MMP2 and MMP9 expression as potential mechanisms contributing to the observed results.

Additionally, as Ang II has recently been demonstrated to regulate expression of the miR-132/-212 cluster in rat aortic VSMC, thereby regulating a number of target genes involved in VSMC migration including PTEN, MCP-1 and RASA-1, this pathway was also assessed in this present study (Jin *et al.*, 2012). The data demonstrate that Ang II-mediated HSVSMC migration was associated with an increase in miR-132 but not miR-212 expression, and a decrease in PTEN expression at the mRNA level. These changes were found to be via the AT_1R and were inhibited following addition of Ang-(1-7) or Ang-(1-9); the effects of Ang-(1-7) and Ang-(1-9) were partially attenuated by antagonism of Mas or the AT_2R, respectively, suggesting a role for these receptors. However, PTEN protein levels were unchanged. Similarly, there was no change in MCP-1 expression at mRNA or Akt at protein levels, both of which are key proteins involved in the downstream signalling pathways of PTEN, suggesting these changes in gene expression were not converted to protein within these experimental conditions. Additionally, in contrast to the findings in rat aortic VSMC, no changes in RASA-1 were observed.

The role of miRNA-132 in Ang II induced HSVSMC migration was further investigated through the use of a miR-132 inhibitor, which blocks miR-132 activity, and through siRNA-mediated downregulation of DICER, a key enzyme

involved in miRNA biogenesis. It was found that miR-132 or regulation of an alternative miRNA via DICER is not essential for Ang II induced HSVSMC migration. However, inhibition of miR-132 or DICER enhanced basal migration of unstimulated HSVSMC. While highlighting a potential mechanism involved in HSVSMC migration, these results also demonstrate key differences in the miRNA response to Ang II between rat and human VSMC.

Next, the effect of Ang II, Ang-(1-7) and Ang-(1-9) on endothelial cell growth, migration and function was assessed. Ang II, Ang-(1-7) or Ang-(1-9) had no effect on growth or migration of primary adult HSVEC. A direct effect of Ang-(1-9) on NO release from both HSVEC and a cell line transiently expressing the AT_2R was demonstrated. Although in cell culture Ang-(1-9) induced NO release in an AT_2R sensitive manner, it was found that in vessels from $AT_2R^{-/-}$ mice the biological effect of Ang-(1-9) was maintained and lead to vasodilation of both aortic and mesenteric artery rings. Further investigation revealed that Ang-(1-9) induced relaxation of $AT_2R^{-/-}$ aortic rings, but not mesenteric artery rings was blocked by A779, suggesting that in large vessels Ang-(1-9) may mediate is vasodilatory effects via conversion to Ang-(1-7) and signalling via Mas, while in small resistance vessels of $AT_2R^{-/-}$ mice Ang-(1-9) promotes vasodilation through an unknown mechanism.

Since data from the previous chapters demonstrated that Ang-(1-7) and Ang-(1-9) blocked VSMC proliferation and migration, without affecting endothelial cell growth or migration, they could be potential therapeutic agents in vascular injury and remodelling. Prior to assessing the effects of Ang-(1-7) and Ang-(1-9) on vascular remodelling, a carotid artery wire injury model in mice was established. Injury to the carotid artery using a synthetic nylon fibre induced significant vessel injury, manifesting in the production of a large neointimal area and increase medial remodelling at 28 days post injury. The effects of Ang-(1-7) or Ang-(1-9) in this setting were assessed by delivery of the peptide for 4 weeks via subcutaneously implanted osmotic minipumps. It was observed that Ang-(1-7) infusion reduced vascular injury in comparison to control vessels, as evidenced by the reduction in neointimal formation and NI/MA ratio. Ang-(1-7) had no effect on the medial area. Importantly, blockade of Mas using the pharmacological inhibitor A779 abolished the anti-remodelling effects of Ang-(1-7). Similarly, Ang-(1-9) reduced neointimal formation and NI/MA ratio following

wire injury to the mouse carotid artery and interestingly, Ang-(1-9) appeared to be more efficacious than Ang-(1-7). Importantly, the effects of Ang-(1-9) were demonstrated to be via the AT_2R, as PD123,319 blocked its effects. Conversely, co infusion of A779 with Ang-(1-9) produced similar results to those observed with Ang-(1-9) alone, indicating that the Ang-(1-9) mediated inhibition of neointimal formation was achieved via a direct effect of Ang-(1-9), as opposed to conversion to Ang-(1-7) and signalling via Mas.

An interesting, yet unexpected finding from the *in vivo* study was that a large proportion of vessels from animals co-infused with Ang-(1-7) and A779, or Ang-(1-9) and PD123,319 developed more complex lesions with increased vessel remodelling and neovascularisation, largely within the media, in comparison to all other groups. The formation of complex lesions was associated with positive vessel remodelling and stretching, often disruption, of the elastic lamina. Analysis of the structural composition of these complex lesions revealed that they were largely composed of disorganised ECM and were highly cellular, containing a large number of VSMC, macrophages and proliferating cells. Re-endothelialisation had occurred on the luminal lining of these vessels and neovascularisation of the complex lesion was also observed.

6.3 Future perspectives

Ang II is a key peptide involved in the development and progression of vascular remodelling, largely through promoting VSMC proliferation and migration (Mehta and Griendling, 2007, Touyz and Schiffrin, 2000). In this present study it was observed that when utilising the same concentration, Ang II enhanced migration but not proliferation of quiescent HSVSMC. These divergent effects of Ang II reveal potential differences in the cell signalling mechanisms employed in HSVSMC; however this has not yet been assessed and further investigation is required. While it is important to study the processes of proliferation and migration in isolation to gain a more in depth understanding of the mechanisms involved, *in vivo* these processes occur concurrently and together contribute to remodelling of the vasculature. Furthermore, as one of the main aims of this thesis was to investigate the interaction of Ang II and the counter-regulatory peptides Ang-(1-7) and Ang-(1-9) in HSVSMC, an *in vitro* assay where Ang II induced both proliferation and migration of VSMC would be of great use. One

such model is the *ex vivo* HSV organ culture model, where both HSVSMC proliferation and migration contribute to the development of a neointimal layer within 14 days (Soyombo *et al.*, 1990). Importantly, Ang II has been shown to increase HSVSMC migration and proliferation within this model via the AT_1R (Ibrahim *et al.*, 2000). Therefore, further investigation of the inhibitory effects of Ang-(1-7) and Ang-(1-9) on Ang II signalling in HSVSMC would benefit from the use of the *ex vivo* organ culture model.

In the vasculature, Ang II activates various signalling pathways, via the AT_1R, resulting in both acute responses such as vasoconstriction and increased blood pressure, and more long term responses such as cell proliferation and structural remodelling. While the signalling mechanisms of Ang II have been well defined [reviewed extensively by (Touyz and Schiffrin, 2000, Mehta and Griendling, 2007)] less is known about the signalling mechanisms employed by Ang-(1-7) and Ang-(1-9). For example, in VSMC Ang-(1-7) has been shown to inhibit Ang II-induced VSMC proliferation and migration largely through inhibition of Ang II-mediated ERK1/2 activation and this was also demonstrated to be the case in this present study. However, the signalling mechanisms evoked by Ang-(1-7) upstream of ERK1/2 in the MAPK signalling pathway are unknown (Tallant and Clark, 2003, Zhang *et al.*, 2010b). Similarly, in this present study Ang-(1-9) has also been demonstrated for the first time to regulate ERK1/2 activity in HSVSMC; at early time points (5 minutes post stimulation) Ang-(1-9) inhibits Ang II induced ERK1/2 activation, however, at later time points (60 minutes post stimulation) Ang-(1-9) enhanced ERK1/2 activation. Similarly, a role for Ang-(1-7) in NO release from endothelial cells has previously been reported (Sampaio *et al.*, 2007a), and while this has been demonstrated to involve eNOS phosphorylation, again the upstream signalling pathways have yet to be detailed. In this present study Ang-(1-9) was found to increase NO release from both primary HSVEC and cell lines via the AT_2R. Therefore it is of great importance that future studies focus on defining the cell signalling pathways involved in the observed effects of Ang-(1-7) and Ang-(1-9) in primary vascular cells in order to gain an understanding of their mechanisms of actions. One of the main reasons there is little known about the signalling mechanisms involved in the effects of these peptides is that, to date, only western blot-based studies have been used to interrogate Ang-(1-7) signal transduction. While this western-blot based

approach has a number of advantages, such as high-sensitivity and specificity if good antibodies are available, it also has a number of limitations (Verano-Braga *et al.*, 2012). For example, the number of simultaneously analysed proteins is limited using this approach, and only a relatively few phospho-specific antibodies with high specificity exist (Verano-Braga *et al.*, 2012). Furthermore, discovery of unknown components of a signalling pathway by western blot analysis alone is not possible. Alternative methods of signalling pathways analysis, for example through the use of commercially available protein arrays or mass-spectrometry-based phosphoproteomics have emerged as alternative approaches to overcome these issues (Kopf *et al.*, 2005, Yaghooti *et al.*, 2010, Verano-Braga *et al.*, 2012, Christensen *et al.*, 2010). Protein arrays allow for the analysis of multiple proteins simultaneously on both a small and a large scale. Small scale protein arrays, such as the PathScan Antibody Array from Cell Signalling Technology, allows for the investigation of activation of distinct signalling pathways, including tyrosine kinase, EGFR and Akt signal transduction. These small scale array kits allow for the simultaneous detection of around 30 phosphorylated proteins and are based upon the sandwich ELISA principle (Kopf and Zharhary, 2007). Cells can be stimulated with ligands and then the cell lysates are incubated on the array slide followed by incubation with a biotinylated detection antibody cocktail. Bound antibody is then visualised by chemiluminescence and can be accurately quantified (Yaghooti *et al.*, 2010, Ketsawatsomkron *et al.*, 2010). This approach has been effectively utilised to investigate Ang II-induced MAPK and Akt signalling pathways in monocytes and rat VSMC, respectively (Yaghooti *et al.*, 2010, Ketsawatsomkron *et al.*, 2010).

Larger scale protein arrays allow for analysis of a vast number of proteins and numerous signalling pathways, for example the Panorama array from Sigma contains over 200 antibodies for key cell proteins involved in signal transduction (Kopf *et al.*, 2005). In this type of array, cell lysates are directly labelled with a fluorophore or biotin, applied to the protein array and then bound proteins are visualised. This approach has yet to be used to assess RAS signalling, however, has been proven to be extremely useful in investigating key signalling pathways involved in cancer (Kopf and Zharhary, 2007). While this method is advantageous to assess the involvement of a large number of signalling proteins, this method is less sensitive than the sandwich ELISA method and is not quantitative (Kopf and

Zharhary, 2007), therefore any positive proteins must be confirmed using traditional methods such as western Blotting.

Mass-spectrometry-based phosphoproteomics has been identified as a powerful method for analysis of cell signalling pathways (Christensen *et al.*, 2010, Verano-Braga *et al.*, 2012, Mann *et al.*, 2002). This approach has previously been employed to study Ang II and Ang-(1-7), in cell lines and primary human endothelial cells, respectively, and revealed a number of previously unidentified signalling pathways that may be involved in the signalling mechanisms of these peptides (Verano-Braga *et al.*, 2012, Christensen *et al.*, 2010). In summary, the use of these non-biased approaches would be greatly advantageous to investigate the cell signalling mechanisms of Ang-(1-9) in both HSVSMC and HSVEC, and would allow for an in depth comparison between the signal transduction pathways of Ang-(1-7) and Ang-(1-9).

Evidence from the literature and the results of this present study in primary HSVSMC and HSVEC suggest that Ang-(1-9) mediates its effects in the cardiovascular system via the AT_2R (Flores-Munoz *et al.*, 2011, Flores-Munoz *et al.*, 2012, Ocaranza *et al.*, 2014, Cha *et al.*, 2013). However, evidence of this interaction has relied on the use of the pharmacological antagonist of the AT_2R, PD123,319 which has been demonstrated to have non-AT_2R effects *in vitro* (Lautner *et al.*, 2013). For example, alamandine binding to MrgD and alamandine induced-vasodilation of aortic rings from $AT_2R^{-/-}$ mice were inhibited by PD123,319, suggesting that in addition to being an AT_2R antagonist, PD123,319 is also a MrgD antagonist/ligand (Lautner *et al.*, 2013). Therefore, future studies to confirm the functional interaction of Ang-(1-9) at the AT_2R using non pharmacological approaches would be beneficial. One approach would be to repeat these experiments following knockdown of AT_2R expression through the use of a specific siRNA. However, there are a number of limitations with this approach. For example, confirmation of the level of knockdown of the AT_2R at the protein level is limited due to poor antibody specificity at GPCRs (Michel *et al.*, 2009). Additionally, while the scratch assay is an accurate method to study cell migration, only a limited number of cells in each well are analysed and therefore it is imperative that these cells are efficiently transfected with the siRNA (Liang *et al.*, 2007). Together, the advantages and disadvantages of each

approach highlight the need for a combination of experimental approaches to fully investigate the interaction of Ang-(1-9) and the AT_2R.

It has also been demonstrated that Ang-(1-9) retains its biological function in isolated vessels from $AT_2R^{-/-}$ mice, suggesting that at least *ex vivo* Ang-(1-9) may act on the vasculature via an alternative receptor or mechanism. Ang-(1-9) induced vasodilation in pre-constricted aortic rings from both wild type and $AT_2R^{-/-}$ mice in an endothelium dependent manner (Prof R. Santos, Federal University of Minas Gerais, Brazil, personal communication, 2013). These findings in the $AT_2R^{-/-}$ mice were confirmed in this present study in both aortic rings and mesenteric artery rings, demonstrating that this effect is present in both large conduit vessels and small resistance arteries.

One possible explanation for this finding in $AT_2R^{-/-}$ mice is that in the absence of the AT_2R, Ang-(1-9) acts on an alternative receptor leading to vasodilation. While further work is required to assess the involvement of an alternative receptor, this has previously been demonstrated to be the case with Ang-(1-7) (Bosnyak *et al.*, 2011, Walters *et al.*, 2005). For example, in stable cell lines generated to express either AT_1R or AT_2R, but not the endogenous receptor for Ang-(1-7) Mas, Ang-(1-7) was found to bind the AT_2R with higher affinity than the AT_1R (Bosnyak *et al.*, 2011). The Ang-(1-7)/AT_2R interaction has also been observed *in vivo*. In isolated mouse hearts exposed to the AT_2R antagonist PD123, 319, Ang-(1-7) increased perfusion pressure, an effect not observed following Ang-(1-7) infusion alone and which was independent of both the AT_1R and Mas. It was also observed that in the presence of AT_1R blockade, Ang-(1-7) reduced blood pressure in both normotensive rats and the SHRSP, an effect mediated via the AT_2R (Walters *et al.*, 2005). Therefore, it is possible that Ang-(1-9) may also engage an alternative receptor under certain experimental conditions. One approach to investigate this would be to assess the affinity of Ang-(1-9) at receptors previously identified to function as part of, or alongside, the RAS leading to vasodilation, such as Mas, MrgD and the BK_2R, through the use of radioligand binding assays (Hornig *et al.*, 2003, Gorelik *et al.*, 1998, Lautner *et al.*, 2013, Santos *et al.*, 2003). However, as little is known about the signalling mechanisms induced by Ang-(1-9) it is possible that this peptide may engage a receptor that has not been implicated in the RAS and therefore a less targeted approach may be required, for example, through the use of a GPCR

microarray. This technology is based upon assessment of binding of a fluorescently labelled ligand to a large panel of GPCRs, and has been used to assess binding of novel ligands such as ICI118,551, a drug compound designed to target adrenergic receptors and screening of functional ligands at orphan GPCRs (Fang *et al.*, 2002, Fang *et al.*, 2003, Hong *et al.*, 2006). The use of a GPCR microarray would enable an assessment of the affinity of Ang-(1-9) at a number of receptors and possibly identify hit targets that could be validated further through the use of functional cell signalling assays. Ang-(1-9) has previously been included as a candidate ligand in a previous GPCR array to identify natural ligands for orphan GCPRs, however no positive binding results were observed for Ang-(1-9) (Southern *et al.*, 2013). However, in this screen the functional read out for GPCR activation relied on ß-arrestin recruitment only and analysis of other GPCR signalling mechanisms is required (Southern *et al.*, 2013).

Alternatively, Ang-(1-9) may be metabolised to an alternative peptide, such as Ang-(1-7), which also promotes vasodilation in an endothelium-dependent manner (Brosnihan *et al.*, 1996, Faria-Silva *et al.*, 2005). As Ang-(1-7) has been shown to promote vasodilation via Mas this potential mechanism of action was explored in the current study using the Mas antagonist A779. Here we show that A779 blocked the vasodilator effects of Ang-(1-9) in aortic rings but not mesenteric artery rings, suggesting that in the aorta, but not mesenteric artery, Ang-(1-9) may be converted to Ang-(1-7) which acts via Mas to induce vasodilation. As ACE is the main enzyme involved in the conversion of Ang-(1-9) to Ang-(1-7), future myography experiments would benefit from the inclusion of an ACE inhibitor, such as captopril, to further investigate the potential involvement of Ang-(1-7) (Donoghue *et al.*, 2000). While it appears that in large vessels of $AT_2R^{-/-}$ mice, such as the aorta, Ang-(1-9) may elicit its biological effects via conversion to Ang-(1-7), it is entirely possible that within mesenteric arteries Ang-(1-9) is converted to an alternative, unidentified peptide that also promotes vasodilation. With the emergence of the RAS as a system of interconverted peptides generated by a number of multifunctional enzymes including ACE, ACE2, and numerous aminopeptidases, carboxypeptidases and endopeptidases, this is a possibility (Santos *et al.*, 1992, Donoghue *et al.*, 2000, Nagata *et al.*, 2006, Lautner *et al.*, 2013, Zini *et al.*, 1996, Ackerly *et al.*, 1976). However, with the exception of Ang-(1-7) (Donoghue *et al.*, 2000), other

functional peptide metabolites of Ang-(1-9) have yet to be identified and therefore further investigation would be required. One method used previously to assess generation of novel angiotensin peptides is through the use of matrix-assisted laser desprption/ionisation mass spectrometry (MALDI-MS) (Jankowski *et al.*, 2007). The use of this technique has identified the generation of Ang A from Ang II, and Alamandine from Ang A and Ang-(1-7) (Jankowski *et al.*, 2007, Lautner *et al.*, 2013). Importantly, this technique allows for the measurement of peptide levels in samples following organ/tissue perfusion, and therefore it would be possible for the vessels used in this present study to be incubated with Ang-(1-9) prior to sample analysis (Jankowski *et al.*, 2007).

It is currently unclear as to why this differential mechanism of action of Ang-(1-9) between vascular beds occurs, however it is possibly linked to the differences in the mechanisms of vasodilation in conduit and resistance vessels. While in large conduit vessels such as the aorta, release of NO from the endothelium is the main mechanism of vasodilation, in smaller resistance vessels, such as the mesenteric arteries, EDHF emerges as an important mediator of vasodilation in addition to NO (Shimokawa *et al.*, 1996, Waldron *et al.*, 1999, Brandes *et al.*, 2000). Therefore it is possible that in the absence of the AT_2R, in the aorta Ang-(1-9) is metabolised to Ang-(1-7), a peptide which has previously been demonstrated to promote vasodilation via NO, while in the mesenteric arteries Ang-(1-9) activates an alternative signalling pathway that is potentially involved in EDHF signalling. However, further investigation is required in order to elucidate the mechanisms of relaxation in response to Ang-(1-9) and future studies involving the use of pharmacological antagonists of NO and EDHF signalling, such as L-NAME and potassium channel antagonists [including apamin (an inhibitor of small-conductance Ca^{2+}-activated K^+ channels) and charbybdotoxin (an inhibitor of large- and intermediate-conductance Ca^{2+}-activated K^+ channels)], respectively, would contribute to a more thorough understanding of the signalling mechanisms involved (Hinton and Langton, 2003).

As discussed in Chapter 3, Ang-(1-7) and Ang-(1-9) prevented VSMC migration and proliferation, via the Mas receptor or AT_2R, respectively. Importantly, as shown in Chapter 4, these peptides did not affect endothelial cell proliferation and migration, indicating that they may prevent neointimal formation but not inhibit re-endothelialisation of the vasculature following injury *in vivo*, and are

therefore attractive novel therapeutic targets in the setting of vascular remodelling. While Ang-(1-7) has previously been demonstrated to reduce neointimal formation in a number of acute models of vascular injury (Strawn *et al.*, 1999, Langeveld *et al.*, 2005, Langeveld *et al.*, 2008, Zeng *et al.*, 2009, Wu *et al.*, 2011), this present study is the first to characterise the role of Ang-(1-9) in vascular remodelling following acute vascular injury *in vivo* and provides a direct comparison between Ang-(1-7) and Ang-(1-9) in this setting. Using the mouse carotid artery wire injury model we demonstrated that Ang-(1-7) or Ang-(1-9) inhibited neointimal formation at 28 days post-injury via Mas or the AT_2R, respectively. However, the link between Ang-(1-9) and the AT_2R was again demonstrated through the use of the antagonist PD123,319, which, in addition to its effects at the MrgD *in vitro* and *ex* vivo, has also been demonstrated to have AT_2R-independent effects *in vivo*. For example, in a mouse model of abdominal aortic aneurysm (AAA), PD123,319 was demonstrated to augment Ang II-induced AAA formation through an AT_2R-independent mechanism (Lautner *et al.*, 2013, Daugherty *et al.*, 2013). Furthermore, through the use of PD123,319 the Ang-(1-9)/AT_2R interaction was in fact demonstrated to lead to the development of enhanced vascular remodelling through the development of complex lesions in response to injury in this present study. Due to the fact that no complex lesions were observed in animals infused with Ang-(1-9) or co-infused with A779, this phenotype is not due to Ang-(1-9) and therefore either occurs as a consequence of the Ang-(1-9)/AT_2R interaction, or due to PD123,319 and therefore this study would benefit from the inclusion of a group of animals infused with PD123,319 alone.

As discussed previously, Ang-(1-9) was demonstrated to retain its biological function in $AT_2R^{-/-}$ mice, therefore future *in vivo* studies in these mice to assess the effects of Ang-(1-9) on neointimal formation would be valuable. However, it is well known that the AT_2R plays an important role in the foetal and early post-natal periods, and that the absence of the AT_2R negatively affects cardiac and renal maturation and growth, limiting the use of the $AT_2R^{-/-}$ mouse (Price *et al.*, 1997, Biermann *et al.*, 2012). Thus, further studies using tissue-specific and inducible AT_2R knockout mice would be of value. Alternatively, use of a combination of wild type and $AT_2R^{-/-}$ mice would be of great benefit in other models of acute vascular injury such as the mouse model of vein graft failure or

in-stent restenosis, both of which rely on the use of a donor vessel (Ali *et al.*, 2007, Zou *et al.*, 1998). In these models the vein or stented artery from $AT_2R^{-/-}$ mice could be engrafted to the recipient vessel of the wild type mouse, and vice versa, as VSMC from both the donor and the recipient vessel have been demonstrated to contribute to neointimal formation (Hu *et al.*, 2002). This would enable a more in depth mechanistic investigation of the role of this receptor within the remodelled vessel.

Osmotic mini pumps have been a useful tool to investigate the physiological and pathophysiological role of various pharmacological agents *in vivo*. Subcutaneous delivery via osmotic mini pump results in drug delivery to the systemic circulation at a constant rate over a set period of time without the need to cause the animal additional stress by daily dosing (Theeuwes and Yum, 1976). Osmotic mini pumps have been widely used to study the effects of angiotensin peptides *in vivo* in a number of different models of cardiovascular disease which has greatly contributed to our knowledge of the RAS (Flores-Munoz *et al.*, 2012, Ocaranza *et al.*, 2010, Grobe *et al.*, 2006, Langeveld *et al.*, 2005). Osmotic mini pumps have not only expanded our knowledge of the effects of administration of Ang II in a number of different *in vivo* models of cardiovascular disease, but have also led to the development of the Ang II-infusion model of hypertension. In this model, infusion of Ang II induces increased blood pressure, accompanied with remodelling of both the heart and vasculature. Ang-(1-7) has also been delivered through osmotic mini pumps in several studies, enhancing our knowledge of the *in vivo* effects of this peptide in hypertension, atherosclerosis and remodelling of numerous organs such as the heart, kidney and blood vessels (Tesanovic *et al.*, 2010, Zeng *et al.*, 2009, Langeveld *et al.*, 2005, Grobe *et al.*, 2006, Grobe *et al.*, 2007, Stegbauer *et al.*, 2011). Furthermore, the first studies outlining a functional biological effect of Ang-(1-9) *in vivo* involved the use of osmotic mini pumps and demonstrated that delivery of Ang-(1-9) via this route effectively reduced cardiac fibrosis and hypertrophy (Flores-Munoz *et al.*, 2012, Ocaranza *et al.*, 2010). Additionally, data from this thesis demonstrate that osmotic mini pump-mediated infusion of Ang-(1-7) and Ang-(1-9) inhibit neointimal formation following vascular injury.

However, one disadvantage of osmotic minipumps is their limited translational impact, as this delivery approach would not represent the situation in the clinic

(Theeuwes and Yum, 1976). One delivery approach utilised clinically is local delivery of therapeutic agents directly to the vessel; this approach enhances drug delivery at the site of action and reduces off target effects at other organs/tissues (Inoue and Node, 2009). For example in the case of CABG surgery the vein graft can be incubated with therapeutic agents prior to engraftment and several studies in animal models have shown that adopting this approach results in reduced neointimal formation, at least in the early phase of disease (Schachner *et al.*, 2004, Murphy *et al.*, 2007). For example, incubating pig saphenous veins for one hour with paclitaxel, sirolimus or cytochalasin D reduced neointimal formation at one but not three months following implantation (Murphy *et al.*, 2007). Therefore, to represent a more clinically relevant delivery approach the findings of this present study were extended to assess if Ang-(1-7) or Ang-(1-9) could be delivered locally to the vessel immediately following vascular injury through the use of Pluronic F-127 gel, and if so what effect this had on the resultant remodelling. However, it could not be confirmed that Ang-(1-7) or Ang-(1-9) was in fact delivered to the vessel and it was found that very little neointimal formation was observed in gel only control vessels; for this reason an assessment of the effects of locally applied Ang-(1-7) or Ang-(1-9) directly to the injured vessel could not be performed within this present study. This finding was unexpected as this gel based delivery approach has been utilised in a number of studies using various *in vivo* models of vascular disease/remodelling and has been demonstrated to have no effect on neointimal formation (Bennett *et al.*, 1994, Abe *et al.*, 1994, Ishizaka *et al.*, 1999, Grassia *et al.*, 2010, Shi *et al.*, 2014). Despite the advantages of locally applied therapeutic agents the use of this approach is limited to invasive procedures and the therapeutic window of the drug is often limited due to the fact that it is applied once at the time of surgery.

While oral delivery of therapeutic agents for cardiovascular disease is optimal, many protein and peptide drugs cannot be administered orally because they are degraded by stomach and intestinal digestive enzymes (Uekama, 2004). In an attempt to address this limitation, an oral formation of Ang-(1-7) has recently been developed where Ang-(1-7) has been incorporated into a cyclodextrin compound [HPβCD/Ang-(1–7)] (Lula *et al.*, 2007). Cyclodextrins are pharmaceutical tools that are used to enhance drug stability, absorption across

biological barriers and provide gastric protection (Uekama, 2004). This oral formulation of Ang-(1-7) has been shown to effectively deliver this peptide to the systemic circulation where it retains its biological function. For example, administration of HPβCD/Ang-(1–7) results in improved cardiac function and remodelling following MI in rats, reduced thrombosis in the SHRSP, improved diabetic outcomes in Type 2 diabetic rats and improved plaque stability in atherosclerotic mice (Marques *et al.*, 2012, Santos *et al.*, 2014, Fraga-Silva *et al.*, 2011, Fraga-Silva *et al.*, 2014). An oral formation of Ang-(1-9) is currently being developed and while its efficacy *in vivo* has yet to be demonstrated this represents an attractive therapeutic agent which could potentially be used so further investigate the effects of Ang-(1-9) in the vasculature.

6.4 Conclusion

In summary the data from this thesis demonstrates for the first time a direct biological role for Ang-(1-9) in the vasculature through inhibition of HSVSMC migration and proliferation, and increase NO bioavailability from HSVEC *in vitro* and reduced neointimal formation in an *in vivo* mouse model of vascular injury. Furthermore, this study provides a direct comparison of Ang-(1-9) and Ang-(1-7) in the vasculature and while the end biological effects are similar, they act via different receptors, the AT_2R or Mas, respectively and differences exist in their signal transduction mechanisms. Further work is required to dissect the cell signal transduction pathway of Ang-(1-7) and Ang-(1-9) in the vasculature and assess their effects in more translational *in vivo* models of vascular disease; this study highlights Ang-(1-7) and Ang-(1-9) as potential therapeutic agents in vascular remodelling.

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