Development of targeted gene delivery vectors to assess cardiac overexpression of ACE2 *in vivo*

By

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Division of Cardiovascular and Medical Science

Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself with the exception of home office licensed procedures (Dr L.M Work and Mrs N Britton), echocardiography (Dr K Gilday and Mrs E Beattie), myography (Mrs A Spiers) and rAAV6 vector large-scale production (Dr J.M Allen and Dr P Gregorevic). It has not been submitted previously for a higher degree. The research was carried out in the Division of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Professor A H Baker.

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Oral Presentations

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List of abbreviations and symbols

AAV	Adeno-associated virus
ACE (2)	Angiotensin converting enzyme (2)
ACE_i	ACE inhibitors
Ad	Adenovirus
ADA	Adenosine deaminase deficiency
Ang	Angiotensin
ANP	Atrial naturetic peptide
APC	Antigen presenting cells
ApoE	Apolipoprotein E
ARB	Angiotensin II receptor blockers
AT_{1A}	Angiotensin receptor 1a homologue
AT_1	Angiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
AWT	Anterior wall thickness
BC-10	Bladder cancer-associated protein homologue
BCA	Bicinchoininc acid
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CABG	Coronary bypass graft
CAR	Coxsackievirus and adenovirus receptor
cDNA	Complementary DNA
cGMP	Guanosine cyclic 3', 5' monophosphate
CHD	Coronary heart disease
CMV	Cytomegalovirus
CO	Cardiac output
CPRG	Chlorophenol red β-d-galactopyranoside
CRIP-II	Cystein rich protein
CsCl	Caesium chloride
CTL	Cytotoxic T lymphocyte
CVD	Cardiovascular disease
d	diastole

DAB	Diaminobenzidine
DMEM	Dulbecco’s minimal essential media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECG	Electrocardiogram
ECL	Chemiluminescence
E.coli	Escherichia coli
EDTA	Ethylenediamine tetra-acetic acid
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor-1
FH	Familial hypercholesterolemia
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FLT-1	FMS-Related tyrosine kinase 1
FPS VI	Fluorogenic Peptide Substrate VI
FS	Fractional shortening
g	g-force
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GITC	Guanidine isothiocyanate
GTAC	Gene therapy advisory committee
HCSMC	Human coronary artery smooth muscle cells
HIV	Human immunodeficiency virus
HK	Human kallikrien
HO-1	Heme oxygenase-1
hPLAP	Human placental alkaline phosphatase
HR	Heart rate
HRE	Hypoxia response element
HRP	Horseradish peroxidase

HSPG	Heparan sulphate proteoglycans
HSV	Herpes simplex virus
HUCAEC	Human coronary artery endothelial cells
HUVEC	Human umbilical vein endothelial cells
ICAM-2	Intercellular adhesion molecule-2
ICP4	Infected-cell polypeptide 4
IFN-γ	Interferon-gamma
Ig	Immunoglobin
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
I/R	Ischemia-reperfusion
ISWT	Interventricular septal wall thickness
ITPG	Isopropyl thiogalactopyranoside
ITR	Inverted terminal repeats
LamR	Laminin receptor
LB	Luria broth
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LH-R	Human luteinizing receptor
L-NAME	NG -nitroarginine methyl ester
LTR	Long terminal repeats
LVEDD	Left ventricular end diastolic diameter
LVEDV	Left ventricular end diastolic volume
LVESV	Left ventricular end systolic volume
LVH	Left ventricular hypertrophy
LVMI	Left ventricular mass index
MEM	Minimal essential media
mg	Milligram
MHC	Myosin heavy chain
miRNA	MicroRNA
MLC-2v	Myosin light chain 2v
mM	Milli molar
MMPs	Matrix metalloproteinases

MOI	Multiplicity of infection
MPCII-3	Similar to integral membrane protein CII-3
MW	Molecular weight
NAD(P)H	Nicotinamide dinucleotide phosphate
NEP	Neutral endopeptidase
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Nuclear pore complex
ORF	Open reading frame
OTCD	Ornithine transcarboxylase deficiency
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PCP	Prolylcarboxypeptidase
PCSMC	Porcine coronary artery smooth muscle cells
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene glycol
PEP	Prolylendopeptidase
PFU/ml	Plaque forming unit / millilitre
Phage	Bacteriophage
PLA2	Phospholipase A2
PMSF	Phenylmethanesulfonylfluoride
Q-PCR	Quantitative PCR
RAC	Recombinant DNA advisory committee
RAS	Renin angiotensin system
RGD	Arginine-Glycine-Asparate
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RSV	Rous sarcoma virus
RT-PCR	Real-time polymerase chain reaction
s	systole

SARS-CoV	Severe acute respiratory syndrome-associated coronavirus
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SERCA2a	Sarco-endoplasmic reticulum calcium ATPase pump
SHR	Spontaneously hypertensive rat
SHRSP	Stroke prone spontaneously hypertensive rat
SMC	Smooth muscle cell
TIA	Transient ischemic attack
TIMPs	Tissue inhibitors of metalloproteinases
TK	Thymidine kinase
TNF-α	Tumour necrosis factor-α
UPDRS	Unified Parkinson's disease rating scale
VEGF	Vascular endothelial growth factor
VP	Virus Particle
WKY	Wistar-Kyoto rat
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside
X-SCID	X-linked severe combined immunodeficiency

Summary

The renin angiotensin system is often manipulated clinically for the treatment of hypertension and heart failure. This pathway is of major clinical importance and it thus a major target for therapy. The incidence of cardiovascular diseases continues to increase worldwide, highlighting the need for new therapies to treat these conditions. Gene therapy for the treatment of cardiovascular diseases is currently being developed. Gene therapy is by definition the treatment or prevention of disease by means of gene transfer. The efficiency of gene transfer will determine how successful the gene therapy application will be. Before the full potential of gene therapy can be reached, many limitations common to all methods of gene delivery must be overcome. The current lack of suitable vectors capable of transducing cells of the vasculature or of the myocardium is a major rate-limiting step, but may be overcome by increasing the specificity of gene therapy vectors. This may be achieved through the isolation of new viral serotypes that can be developed into vectors, or the creation of new vectors by the alteration of the tropism of existing ones.

This thesis aimed to assess the effect of ACE2 overexpression *in vivo* on heart function and blood pressure. In order to achieve cardiac gene transfer, we first had to identify an efficient cardiac gene delivery vector. This was approached by the application of two main techniques; (1) the use of phage-display identified peptides to retarget viral vectors and (2) the comparison and optimisation of rAAV6 and rAAV9 mediated gene delivery to myocardium *in vivo* in a rat disease model.

The initial aim of the project was to produce viral vectors that are highly efficient and selective at transducing cells of the cardiovascular system. Novel targeting ligands, incorporated into the capsid protein of viral vectors may help to achieve site-specific gene delivery. Candidate heart targeting peptides identified through phage display, in which the heart vasculature was probed for heart-specific endothelial markers, were evaluated. Four potential candidates were identified as CRPPR, CSGMARTKC, CRSTRANPC and CPKTRRVPC. All four showed an increase in ability to home to the heart when compared to insertless phage. Once shown to be selective for the heart tissues, these peptides were used to modify Ad5, Ad19p and AAV2 vectors to assess if they increased the selectivity of these vectors to endothelial cells of the vasculature.

Phage-display derived targeting peptides proved disappointing in the context of viral vector retargeting, with the tropism of peptide-modified vectors remaining unchanged.

Alternative viral vectors have been developed in recent years for cardiovascular gene therapy as a result of the poor uptake of existing vectors. RAAV6 vectors have been shown to display a powerful natural tropism for skeletal and cardiac muscle. One of the most recently identified AAV serotypes is AAV9, which has also been shown to display cardiac tropic characteristics achieving high cardiac transduction rates following systemic injection. Thus we aimed to identify an efficient cardiac gene delivery vector by comparison and optimisation of AAV6 and AAV9-mediated gene delivery to myocardium *in vivo* in SHRSPs. Whilst rAAV6 and rAAV9 vector-mediated gene transfer were both found to be high in heart, rAAV6 vectors were found to exhibit the most favourable profile for cardiac gene delivery.

The incorporation of a transcription-regulating element that limits transgene expression to the muscle of interest would reduce transgene expression in non-target cells. Thus rAAV6 vectors were tested under the control of a cardiac-specific promoter to achieve both selective targeting of myocardial cells and selective transgene expression in these cells. The promoter chosen was myosin light chain 2v (MLC-2v), which is abundant in skeletal and cardiac muscles and is the ventricular form of myosin light chain. This promoter has not previously been characterised in rAAV6 vectors. However, disappointingly, we found that systemic injection of AAV6 under the control of the MLC2v promoter led to the uptake, but to no expression of the transgene in the heart.

Gene therapy vectors have been developed with the ultimate aim of efficiently and selectively inducing appropriate transgene expression for a clinically beneficial outcome. Therefore, we aimed to assess the effect of ACE2 overexpression *in vivo* on heart function and blood pressure. Overexpression was achieved through the exploitation of the cardiac delivery profile of rAAV6 vectors. We demonstrated that sustained (11 week) rAAV6-mediated ACE2 overexpression in the SHRSP exerts detrimental effects on cardiac structure and function whilst increasing basal NO

bioavailability, suggesting both positive and negative effects of overt ACE2 overexpression *in vivo*.

Through the comparsion of gene delivery vectors through different genetic approaches, this thesis identified a suitable candidate vector for targeted gene delivery to the myocardium of the SHRSP. This vector, rAAV6 was used in an appropriate disase model to define the effects of overexpression of ACE2, an important pharmacological target. The findings of this have immense cardiac pharmacological importance.

Introduction

1.1 Gene Therapy

Advancement in the understanding of molecular therapeutics has allowed the development of novel treatments to prevent and treat many diseases. Gene therapy is one of these novel strategies and involves the delivery of genes into a host's cells to express a therapeutic gene and ultimately achieve beneficial effects. The science behind gene therapy was first shown possible in 1977 when the thymidine kinase (TK) gene was transferred into TK^- L mammalian cells (Wigler, Silverstein et al. 1977). The genetic deficiency of these cells was corrected by the transfer of a single copy of a functional copy of the TK gene (Anderson, Killos et al. 1980). *In vivo* expression in a mouse was soon obtained using the retroviral vector N2 expressing the NeoR marker gene (Eglitis, Kantoff et al. 1985), which was then used to tag tumour infiltrating lymphocytes in the first human clinical trial in skin cancer patients (Rosenberg, Aebersold et al. 1990).

Originally conceived for the treatment of inherited monogenic disorders such as Duchenne's muscular dystrophy and haemophilia where gene replacement should restore a normal phenotype, gene therapy approaches can now be applied to the treatment of more complex acquired diseases including cardiovascular diseases and cancers. A variety of approaches have been developed to enable this, including the replacement or correction of missing or functionally impaired genes, the addition of a new function to a cell and the inhibition of proteins with undesirable effects. Gene therapy has enormous potential to provide novel treatments in areas which are currently lacking in suitable therapies. However, before the full potential of gene therapy can be reached, many limitations common to all methods of gene delivery must be overcome. The efficiency and selectivity of gene transfer will determine how successful the gene therapy application will be. This can be considered a major rate-limiting step. In all cases, a 'vector' must be used to carry the therapeutic or corrective genetic sequence in order to efficiently deliver it to the patient's target cells or tissue in such a way that the gene can be expressed at a beneficial level and for a suitable duration. Difficulties in achieving sustained gene expression in the target

tissue or cell has resulted in limited clinical benefits from gene therapy to date. The success of gene therapy is restricted by the relative lack of suitable vectors and will depend on the ability of researchers to address a number of still unsolved problems. This can be approached by either the isolation of new viral serotypes that can be developed into vectors or the creation of new vectors by the modification of existing ones.

1.2 Current Status of Gene Therapy

Developments in the field of gene therapy have been rapid. Since 1990, over 1300 clinical trials have been approved worldwide (www.advisorybodies.doh.gov.uk) with 11.5% of these being conducted in the UK (www.wiley.co.uk/genmed/clinical). The majority of clinical trials are for the treatment of cancers (66.5%), with the second biggest field in gene therapy being for the treatment of cardiovascular diseases (9.1%) (Figure 1.1). Viral vectors remain the current vectors of choice. In 1990, the first clinical trial of human gene therapy to correct the genetic disorder adenosine deaminase (ADA) deficiency in two children was initiated (Blaese, Culver et al. 1995). Retroviral-mediated transfer of the ADA gene into T-cells led to the normalisation of immune responses, both cellular and humoral, and demonstrated for the first time the enormous clinical potential of gene therapy.

In 2006, the success of a clinical trial to treat patients with progressive metastatic melanoma was reported (Morgan, Dudley et al. 2006). Retrovirus vectors encoding a T-cell receptor were used to genetically engineer peripheral blood lymphocytes *ex vivo*. These transduced cells were then re-infused into the patients. Tumour recognition abilities were conferred onto the autologous lymphocytes and caused the destruction of tumour cells *in vivo*. All 15 patients tolerated the engraftment of lymphocytes for at least 2 months post-infusion. 2 patients were found to have maintained a high level of genetically modified cells at 1 year post-infusion and both these patients displayed regression of metastatic melanoma lesions, as assessed by standard criteria. This trial is an example of successfully bringing treatment from the laboratory to the clinic.

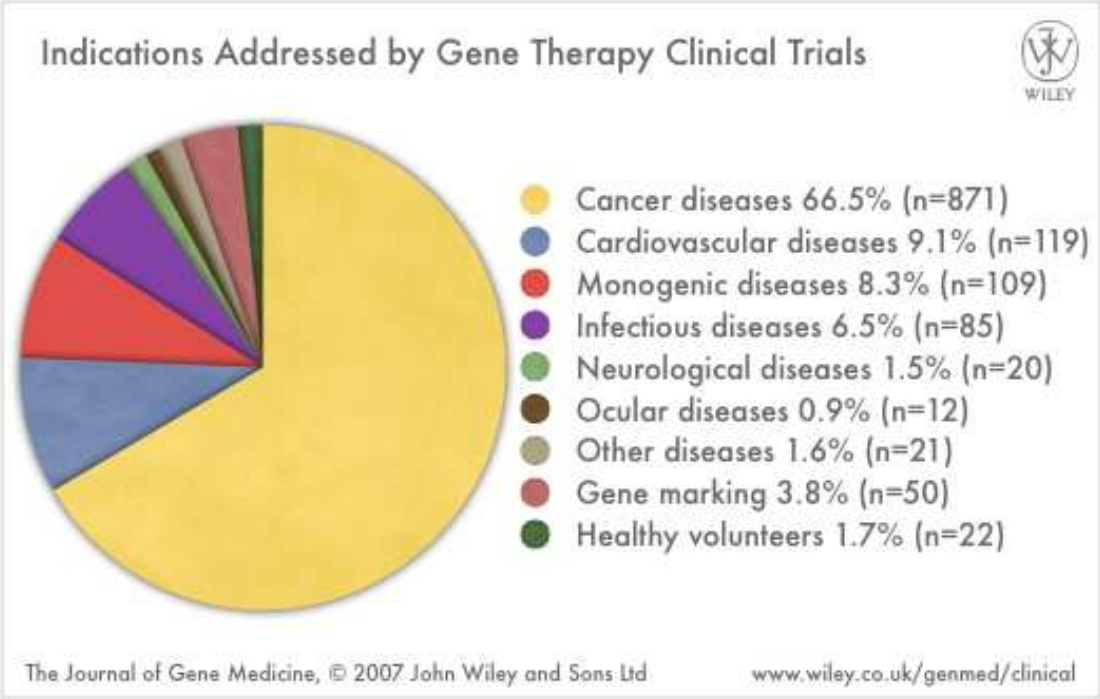

Figure 1.1 Gene therapy clinical trials.

Disease targets for gene therapy presented as a proportion of ongoing clinical trials. Data taken from www.wiley.co.uk/wileychi/genmed/clinical

Additional positive results can be seen in the recent phase I clinical trial of 12 patients to check the safety and tolerability of an AAV2 vector for use in the treatment of Parkinson's disease (Kaplitt, Feigin et al. 2007). The AAV2 vector used in the trial expresses the glutamic acid decarboxylase (GAD) gene and has generated some encouraging results (Kaplitt, Feigin et al. 2007). The vector was injected into one side of the brain and delivered to the subthalmic nucleus, where it expresses GAD, which in turn catalyses the synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). The neurotransmitter acts to reduce the activity of neurons in the subthalmic nucleus, which are found to be increased in Parkinson's disease patients. At one year follow up, all 12 patients were found to demonstrate an average clinical improvement of 25%, as rated by the Unified Parkinson's Disease Rating Scale (UPDRS). Importantly, no adverse effects or substantial toxicity were reported.

In 1999 however, the field of gene therapy suffered a major setback with the first gene therapy related fatality attributed to an inflammatory reaction to an adenovirus vector

(Hollon 2000; Raper, Chirmule et al. 2003). This was the death of an 18 year old patient who received treatment for the liver disorder ornithine transcarboxylase deficiency (OTCD), an X-linked defect of the urea cycle in which nitrogen metabolism is affected, leading to a spectrum of neurological symptoms including seizures and mental retardation. He was administered a high dose of an E1, E4-deleted recombinant adenoviral vector and died 4 days later from multiple organ failure. 17 other subjects were recruited onto this trial, with this patient being the second to receive the highest dose (Raper, Yudkoff et al. 2002). In the other patients, clinical reactions to the administered vector were mild and transient, with none reporting the systemic inflammatory response experienced by Gelsinger. Several rules of conduct were found to have been broken by the lead researchers however, including the failure to report the death of monkeys given similar treatments, in the informed consent discussion, failure to immediately report severe side effects in two other patients and the inclusion of Gelsinger into the clinical trial with high ammonia levels (www.fda.gov).

In 2007, a 36 year old woman died during a phase I/II safety trial for the treatment of rheumatoid arthritis (Kaiser 2007). AAV vectors expressing a tumour necrosis factor α (TNFα) inhibitor were injected directly into the knee joint of the patient. After, the second injection, the woman developed an illness which resulted in her death 22 days later. The trial was immediately stopped, although the role of gene therapy in her death remains unclear. It is thought that she died as a result of a fungus (*Histoplasma capsulatum*) infection. Because she was also found to be positive for herpes simplex virus (HSV), it was reasoned that it was possible that HSV proteins had allowed for the replication of the AAV virus, which resulted in a weakening of her immune system. However, the recombinant DNA advisory committee (RAC) found very low levels of AAV vectors in non-target tissues, making the possibility of AAV playing a role in her death highly unlikely. Another possibility is that the gene encoded by the AAV vectors interacted with the drugs that she was taking for the treatment of her arthritis. Since the trial began in 2005, 127 subjects have been recruited onto the trial, with this case being the only reported adverse effect. Questions have been raised as to her involvement in the clinical trial. Her disease was not classified as life-threatening and it would seem that she was not well informed as to the potential clinical outcomes of a phase I/II safety trial. And so the role gene therapy played in her death remains

ambiguous, leading to further investigation of AAV vector safety (Kaiser 2007) and highlighting the importance of good trial design.

These failures can be offset with the positive results obtained from more successful trials. Despite these promising clinical trials however, no gene therapy vector has yet been licensed in the UK as a viable treatment. However, several phase III clinical trials for cancer gene therapies (pancreatic, prostate and renal) have commenced and so the goal of gene therapy as a therapeutic treatment is within sight.

In the UK there exists a gene therapy advisory committee (GTAC) to approve clinical trials of gene therapy products. This committee considers the potential benefits and risks of proposed clinical gene therapy trials, which must reach strict ethical criteria to be approved. Such advisory boards exist to limit the potential dangers associated with gene therapy as a clinical application.

1.3 Justification for gene therapy for cardiovascular disease

Cardiovascular diseases (CVD) remain the leading cause of mortality and morbidity in both men and women in the western population. The main forms of CVD are coronary heart disease (CHD) and stroke, but the term includes any diseases that involve the cardiovascular system. An estimated 2.6 million people have CVD in the UK, accounting for over 216,000 deaths in the UK in 2004. More than one in three people (37%) die from CVD (www.bhf.org.uk). Heart failure can be defined as a condition which leads to the heart being unable to meet the systemic demands for blood flow throughout the body. It can be as a result of any structural or functional disorder that impairs cardiac capabilities, and can be induced by a wide range of common diseases including hypertension, valvular insufficiencies and myocardial infarctions. The prognosis from heart failure is poor. A 50% death toll within 5 years is estimated for patients with mild to moderate heart failure, and 50% within 2 years for those with severe heart failure (Hobbs 2004). Cardiac hypertrophy is one of the heart's first responses to an abnormal increase in stress. During this remodelling process, cardiac myocytes will increase in length and width in order to thicken the wall of the heart to normalise ventricular wall tension. Hypertrophy can occur in either the right or the left ventricle, with left ventricular hypertrophy (LVH) having

the highest occurrence. Although hypertrophy can initially be considered a compensatory mechanism to myocardial stress, in the long-term this process can become pathological and thus predispose an individual to heart failure. The ventricle can become stiff leading to impaired filling and diastolic dysfunction.

Available pharmacological treatments for CVD and heart failure include prescription drugs, such as diuretics, lipid-lowering and antihypertensive therapies. Statins (otherwise known as 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG coA) reductase inhibitors), are a class of hypolipidemic agents used to lower low density lipoprotein (LDL) cholesterol levels. Statins have also been shown to have pleiotropic effects and have demonstrated anti-inflammatory (Liao 2002) and cardioprotective abilities (Schafer, Fraccarollo et al. 2005), as well as an association in the improvement of diastolic dysfunction (Fukuta, Sane et al. 2005). As such, the use of statins in reducing the incidence of major cardiac events in coronary artery disease is well established (Shanes, Minadeo et al. 2007). However, the use of statin therapy for patients with congestive heart failure remains controversial. Lower serum levels of cholesterol has been shown in some studies to be associated with a worse clinical prognosis (Rauchhaus, Clark et al. 2003). Antiplatelet agents (predominantly aspirin) are another class of drug used in the primary or secondary prevention of thrombotic cerebrovascular or cardiovascular disease. They interfere with platelet aggregation helping to prevent the formation of blood clots, and as such have been shown in randomised trials to reduce the risk of myocardial infarction, stroke and transient ischemic attack (TIA) (Grundy, Cleeman et al. 2004). Activation of platelet aggregation via pathways not blocked by antiplatelet agents can occur. Antiplatelet drugs are also often associated with hemorrhagic side effects, although the benefits are considered to outweigh the risks for most patients whose 10 year risk is $\leq$ 10% (Grundy, Brewer et al. 2004).

Coronary revascularisation operations may be required as CVD progresses and worsens. Coronary artery bypass surgery (CABG) grafts a blood vessel from the chest, leg or arm to the aorta and attaches it to the coronary artery at a place beyond the existing blockage so as to bypass a narrowed or blocked coronary artery. It is one of the most frequently performed surgeries with approximately 30,000 operations per annum being carried out in the UK (www.heartstats.org). CABG can improve the

previously restricted blood flow to the heart and with the ultimate goal of reducing the risk of future heart attacks. A randomised trial to compare surgically and medically treated patients with systolic dysfunction and ischemic heart disease showed that CABG-treated patients had a significantly lower mortality for up to 10 years (O'Connor, Velazquez et al. 2002). Vein graft failure is a significant clinical problem with 15-30% of all vein grafts failing within one year of surgery and 50% being fully occluded after 10 years (Mehta, Izzat et al. 1997; Tsui and Dashwood 2002). Thus several patients will require repeat surgeries or further reintervention in the future.

Percutaneous coronary interventions (PCI) encompass many non-surgical procedures, including balloon catheter angioplasty and stent placement, for the treatment of patients with coronary narrowing. Over 70,000 PCIs are carried out each year in the UK (www.heartstats.org). The main purpose of a PCI is to re-establish blood flow to the heart by diminution of the impeding blockage. During balloon catheter angioplasty, a cardiac catheter with a balloon around it is inserted into the narrow area of the coronary artery where it is inflated. This expansion presses obstructing plaques against the wall of the artery, augmenting the blood flow through the artery. Angioplasty may be followed by the intravascular implantation of a metal stent to prevent constriction of the artery. Stents are thrombogenic, requiring the use of anti-coagulants, and are also associated with higher risks of complications and a longer hospital stay than angioplasty alone. Coronary restenosis is another major problem that is associated with both PCI and vein grafting. Restenosis occurs in 30-50% of all angioplasty interventions and in 10-30% of patients receiving an intravascular stent (Weintraub 2007). Drug-eluting stents which are saturated with drugs that prevent vascular smooth muscle migration and proliferation have been shown to reduce restenosis after PCIs (Degertekin, Regar et al. 2003; Fajadet, Morice et al. 2005). However, there is a higher risk of late stent thrombosis and myocardial infarction in patients receiving drug-eluting stents in comparison to metal stents (Jensen, Maeng et al. 2007). These risks must be considered against the improved clinical outcomes to conclude whether the risk outweigh the benefits.

Despite advances and improvements in treatments, the incidence of CVD continues to increase worldwide. Additionally, many patients are not candidates for these traditional treatments and thus there is a requirement for new therapies to treat

conditions such as atherosclerosis, hypertension, vein graft failure and post-angioplasty restenosis. Gene therapy for the treatment of CVD is currently being optimised and evaluated.

1.4 Therapeutic genes for cardiovascular diseases

The end point of any vector development study is to express a gene that will exert a therapeutic effect, and recent advances in genomics and proteomics may help to achieve this. With the identification of genes involved in CVD and the assignment of function to genes, the potential to translate this information and identify potentially therapeutic genes is high. There are many potential genes to be studied which may have therapeutic benefit. Genes to be studied include those implicated in CVD. For the treatment of heart failure, genes such as the sarco-endoplasmic reticulum calcium ATPase pump (SERCA2a) may be targeted, whilst for the treatment of hypertension components of the RAS may be investigated. For the induction of therapeutic angiogenesis, angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) may be manipulated.

One of the most common groups of genes studied for use in cardiovascular gene therapy is the group of genes encoding growth factors, including VEGF and FGF. VEGF production is induced in response to a number of stimuli, such as hypoxia. Its activity can result in a revascularisation process, known as therapeutic angiogenesis (Josko, Gwozdz et al. 2000; Lee, Rentz et al. 2003), through the induction of endothelial cell proliferation. Being an angiogenic factor, and thus having the ability to induce the formation of new blood vessels from the existing vascular bed, makes VEGF an ideal gene to overexpress in the context of ischemic vascular disease. Direct injection of human VEGF cDNA into the muscles of patients with ischemic limbs lead to increased blood flow to the limbs, and to subsequent healing of ulcers and cessation of associated pain (Baumgartner, Pieczek et al. 1998; Shyu, Chang et al. 2003). However, some experiments have demonstrated that unregulated overexpression of pro-inflammatory and vascular permeabiliser VEGF can lead to detrimental effects, including hypotension and arthritis, and so an element of transcriptional control needs to be included. AAV vectors expressing the VEGF

transgene under the control of hypoxia response elements (HRE), induced gene expression in ischemic mouse hearts *in vivo* (Su, Arakawa-Hoyt et al. 2002).

The absence of heme oxygenase (HO)-1 has been implicated in the exacerbation of atherosclerosis, demonstrated by accelerated and more advanced atherosclerotic lesion formation in HO-1 deficient mice (Yet, Layne et al. 2003). Retroviral-mediated overexpression of HO-1 in the spontaneously hypertensive rat resulted in the attenuation of hypertension (Sabaawy, Zhang et al. 2001), whilst adenoviral-mediated HO-1 gene transfer prevented the development of atherosclerosis in apolipoprotein E (apoE) deficient mice (Juan, Lee et al. 2001). Adenoviral mediated HO-1 overexpression has also been shown to attenuate the remodelling response to experimental vascular injury (Tulis, Durante et al. 2001). The many advantageous effects of this gene make it an important novel target in the treatment of vascular disease.

The potential of gene therapy in the treatment of hypertension, a relatively poorly understood condition, has also been explored. Nitric oxide (NO) has been shown to play an important role in vascular smooth muscle relaxation, to dilate the vessel and increase blood flow. It has also been shown to act on cardiac muscle to decrease heart rate and contractility and is synthesised by nitric oxide synthase (NOS). Many vascular diseases are influenced by a reduction in NO bioavailability. Gene therapy approaches aim to increase NO bioavailability to improve vascular function. Direct injection of a plasmid carrying the human endothelial NO synthase (eNOS) fused to the CMV promoter significantly reduced systemic blood pressure in the spontaneously hypertensive rat (SHR) and was sustained for 5-6 weeks (Lin, Chao et al. 1997). *In vitro*, adenoviral-mediated expression of eNOS and iNOS had antiproliferative and antiangiogenic effects on porcine coronary artery smooth muscle cells (PCSMCs) (Sato, Nair et al. 2000), rat vascular smooth muscle cells (Kibbe, Li et al. 2000), human coronary artery smooth muscle cells (HCSMC) and on human umbilical vein endothelial cells (HUVEC) (Cooney, Hynes et al. 2006). NOS is just one of the many genes that have beneficial effects on endothelial function and on blood pressure. Other vasodilatory promoting genes include atrial natriuretic peptide (ANP), human kallikrien (HK) and bradykinin, and are being investigated for their role in the treatment of cardiovascular diseases.

1.5 Requirements of a gene delivery vector

A multitude of vector systems, both viral and non- viral, have been assessed as tools for delivery of genes into cells, all requiring a therapeutic gene product coupled with an efficient vector for successful transgene expression. It is unlikely that a generic vector would be suitable for use in all circumstances; gene expression is required in different target tissues for varying lengths of time for different conditions. All vectors will share inherent properties, allowing them to be selective and efficient at transducing their target cell or tissue. The safety profile of a vector is important and so much focus has been applied to developing vectors with low toxicity (Huang, Liu et al. 2007). To avoid eliciting host immune responses, a lack of immunogenicity is desirable and would also allow the re-administration of a vector if required. The induction of immune response is particularly a limiting factor for adenovirus vectors based on serotype 5 (Ad5), which have been shown to target dendritic cells and some monocytes mediated by a putative heparin-sensitive receptor recognized by a distinct segment of the Ad5 fiber, the shaft (Cheng, Gall et al. 2007). The removal of virulence genes in viral vectors may help to limit host defences (Morral, O'Neal et al. 1999; Barcia, Jimenez-Dalmaroni et al. 2007). Vectors capable of sustained transgene expression would avert the problems of vector re-administration; however some gene therapy application may only require transient transgene expression. Vector production must also be scalable resulting in high vector concentrations. To date, no one vector possesses all of these qualities, although many steps are being made to overcome these hurdles.

The different types of vectors being researched can broadly be divided into two categories, non-viral and viral vectors. The development of viral vectors seems more promising than that of non-viral vectors, as in general, non-viral vectors are very inefficient (Nishikawa and Huang 2001). Non-viral vectors have no mechanism with which to cross cell membranes or traffic the injected DNA into the nucleus of the host cell (Lechardeur, Sohn et al. 1999; Johnson-Saliba and Jans 2001). As a result of many viruses naturally replicating in the nucleus of their host, they have evolved highly specialised mechanisms to allow efficient nuclear translocation (Whittaker, Kann et al. 2000). Thus viral vectors will mediate higher levels of transgene expression. Nonetheless, higher efficiency comes at the price of a higher safety risk

than non viral vectors. Another important property to be considered is the vector's capacity for transgene insertion. In viral vectors this is determined by the amount of genome that can be removed whilst preserving vector infectivity, whilst non-viral vectors generally have a large capacity for transgenes. Each vector system has its own advantages and disadvantages, depending on its intended use.

For cardiovascular gene therapy, work has focused on the development of vectors with the ability to transduce either the cells of the vasculature (endothelial and smooth muscle cells) or of the myocardium. To increase specificity of vascular gene therapy vectors, methods of tropism alteration and incorporation of cell-specific promoters can be applied (Wickham 2000; Reynolds, Nicklin et al. 2001; Barnett, Tillman et al. 2002). There is a need to modify native virus tropism to improve efficiency of transduction of vascular cells particularly *in vivo*.

1.6 Ex vivo and in vivo gene delivery for CVD

Genes are delivered either through viral vectors, a non-viral vector or through direct delivery of naked DNA. These approaches are based on two major concepts; *ex vivo* and *in vivo* delivery. In *ex vivo* cell based gene therapy, autologous cells or tissue are harvested from a patient and incubated with the vector carrying the desired therapeutic gene. The genetically modified cells or tissue is then re-introduction into the patient. Re-introduced cells or tissue will express the transgene and usually at high levels. Due to the lack of effective pharmacological interventions, this method is being developed for gene therapy of vein graft failure during coronary artery bypass surgery (CABG). CABG surgery is performed on patients with significant atherosclerotic narrowing and blockages of the arteries; these arteries are bypassed by the grafting of arteries or veins from other parts of the body. CABG allows for the incubation of the graft vessel with a gene therapy vector prior to coronary grafting. Late vein graft failure is a common clinical problem (Campeau, Enjalbert et al. 1983; Davies and Hagen 1994) and occurs due to neointima formation and accelerated atherosclerosis; a process in which a role for matrix-degrading matrix metalloproteinases (MMPs) and neuronal nitric oxide synthase (nNOS), amongst others, has been implicated. Tissue inhibitor of metalloproteinase-3 (TIMP-3) has been shown to inhibit MMP activity and promote apoptosis thus inhibiting

progression of neointima formation associated with late vein graft failure (George, Lloyd et al. 2000). Adenovirus overexpressing TIMP-3 has been used in a pig model during bypass surgery, in which the autologous saphenous vein was interpositioned into the carotid artery, and was shown to significantly inhibit neointima formation in comparison with uninfected and control virus vein. Similarly, adenovirus mediated overexpression of nNOS was shown to induce beneficial effects on vein graft remodelling and improved endothelial function (West, Qian et al. 2001), demonstrating the potential of this technique. Transgene expression in non-target tissue is limited by this *ex vivo* method as well as unwanted immune responses by the removal of excess virus.

Another approach for *ex vivo* gene delivery has been in the treatment of familial hypercholesterolemia (FH), in which patients have a deficiency of low density lipoprotein receptors (LDLRs). For this approach, autologous hepatocytes were harvested and transduced with recombinant retroviruses expressing LDLR. The hepatocytes are genetically corrected *ex vivo* before being transplanted back into the patient. This technique has been validated in rabbit models of FH (Chowdhury, Grossman et al. 1991) and in patients (Grossman, Rader et al. 1995), both showing persistent and significantly reduced levels of LDL cholesterol. However, *ex vivo* approaches are limited to largely invasive surgical procedures and to tissues and cells that can easily be removed from the body and then re-implanted. Thus its clinical applications are severely limited. *In vivo* gene delivery may be able to help overcome this limitation, although it faces many challenges of its own.

For *in vivo* gene delivery the vector is either administered directly into diseased tissue within a patient (local delivery), or is systemically delivered and thus targeted to the site of action by the vector. Success lies in the ability of the delivery vector to transduce cells and utilise the host cells' machinery to produce the desirable transgene product. The route of administration has a major influence on the ability of the vector to transduce various cells and tissues. Local delivery may be used in the absence of suitable systemically deliverable vectors, where the direct injection of vector will ensure efficient transduction of target cells unattainable by systemic administration. Local *in vivo* delivery methods can either be through direct injection into the tissue of interest, or can utilise catheter-mediated gene transfer techniques (Sasano, Kikuchi et

al. 2007) or perfusion during cardiopulmonary bypass (Bridges, Burkman et al. 2002). Local delivery, either through catheters, direct injection or surgical procedures can avoid the need for the delivery vector to cross the endothelial barriers and will result in high levels of the vector in the target tissue (Schwarz, Speakman et al. 2000). Intramyocardial injection of rAAV2 vectors was used to achieve beneficial therapeutic effects in rat ischemia/reperfusion models and demonstrated highly selective transduction of myocardial tissue (Melo, Agrawal et al. 2002). Infusion-perfusion catheters have been used in the context of prevention of restenosis after coronary angioplasty. In this case, either adenovirus expressing human vascular endothelial growth factor 165 ($hVEGF_{165}$) or plasmid-liposome complexes containing the $hVEGF_{165}$ gene were delivered directly into the artery. However, in both groups there was no significant change in lumen diameter or in clinical restenosis rate compared to the control group (Hedman, Hartikainen et al. 2003). A surgical technique to improve the efficiency of gene delivery involves treating the heart with permeability agents *in vivo*. This involves simultaneously clamping all vessels to/from the heart and then the continuous retrograde perfusion of the heart through a catheter positioned in the aortic root (O'Donnell and Lewandowski 2005). This technique also allows for a washout phase to eliminate any excess virus, which ultimately will reduce the infection of peripheral tissues. Local delivery of vectors can result in leakage of transgene expression into other non-target tissues (Ikeda, Gu et al. 2002; Champion, Georgakopoulos et al. 2003).

Systemic delivery makes use of the bloodstream to deliver therapeutic genes and is used extensively for liver gene transfer. It is the ultimate goal for many gene therapy applications as it is, in concept, a simple and non-invasive route for delivering therapeutic genes. Many cells and tissues remain inaccessible to local administration. However, the challenge with this approach is that the body has evolved many highly specific techniques to remove foreign particles and pathogens from the bloodstream. Existing vectors for systemic gene transfer remain ineffective at delivering genes to the vasculature and to the myocardium, as a result of liver sequestering after vector administration. Sequestration in the liver is a major limitation of Ad vectors, which to date are mainly based on serotype 5 (Huard, Lochmuller et al. 1995; Mizuguchi, Koizumi et al. 2002). Ad5 virus is known to interact *in vitro* with CAR, however, it was soon discovered that ablating CAR binding alone did not change the hepatic

tropism of these vectors *in vivo* (Alemany and Curiel 2001; Nicol, Graham et al. 2004), and suggests the use of alternate receptor pathways *in vivo* by Ad5 vectors. This hepatic tropism limits the use of systemic routes of delivery to gene therapy for liver disorders or for the delivery of soluble factors. Advances in vector technology and development are helping to overcome this major barrier. Some AAV serotypes have been recently been shown to efficiently cross the blood vessel barrier and as such can be intravenously injected (Blankinship, Gregorevic et al. 2004; Pacak, Mah et al. 2006). The major limitation of the use of these vectors is that other non-cardiac organs may also be targeted. Transductional and transcriptional targeting strategies can be used to improve transgene expression and cell specificity. This is discussed in detail later (section 1.9).

1.7 Non-Viral Vectors

Non-viral vectors account for approximately 25% of the clinical trials currently in operation (www.wiley.co.uk/genmed/clinical). The simplest form of the vector is naked plasmid DNA encoding for the gene of interest and can be directly injected into the target tissue. Non-viral vector gene delivery is however highly inefficient with levels of transduction being significantly less than those achieved by viral vector gene delivery. Strategies to improve the delivery of this vector can be categorised into two general groups; (1) the association of the DNA with other molecules, and (2) the application of physical energy to aid cell entry through the cell membrane (Table 1.1). The major problems of non-viral vector delivery include the interactions of the vector-DNA complex with blood plasma proteins and non-target cells, and entrapment within endosomes from which the vector must escape. Once inside the target cell, the challenge of resisting non-specific cytoplasmic degradation and passage through the final physical barrier of the nuclear envelope into the nucleus must be faced (Johnson-Saliba and Jans 2001). Additionally, plasmid DNA that reaches the nucleus remains extrachromasomal and so is not usually replicated and is thus lost during break-down of the nuclear envelope at mitosis (Niidome and Huang 2002). Consequently, recent studies have focused on the development of specially designed vectors which have reduced affinity for intracellular proteins and cellular surfaces (Ogris, Brunner et al. 1999; Kursa, Walker et al. 2003) and on mimicking the properties of viruses that will allow the non-viral vector to be maintained and replicate in the nucleus of target cells.

Method of gene transfer		Advantages	Disadvantages
Physical	Hydrodynamic injection	Potent gene transfer in internal organs	Gene transfer mostly restricted to the liver
	Bioballistic (gene gun)	Large capacity for DNA (>20 kb) High transfection efficiency	Shallow penetration of DNA into the tissue Short duration of gene transfer Dependent on cell line used
	Ultrasound	Low invasiveness Non-toxic	Relatively short duration of gene expression
Chemical	Liposomes	Large capacity for DNA (>20 kb) Lack of immunogenicity Broad tropism	Low transfer efficiency in comparison to viral vectors Poor efficiency in transduction of non-dividing cells
	Polycation DNA complexes	Safe *in vivo* High transduction efficiency *in vitro*	Instability Cleared rapidly from blood stream Non-specific interactions with other proteins
	Peptide DNA complexes	Low toxicity Low immunogenicity	Conjugation reactions may reduce biological activities of the proteins and peptides

Table 1.1 Characteristics of non-viral gene delivery techniques.

As plasmids contain no proteins to interact with cellular receptors, physical methods of gene delivery can be applied to bring the vector into closer proximity with the cell membrane or to temporarily disrupt the cell membrane making it permeable to the DNA, thus allowing access into the cell. Potentially, the use of non-viral vectors offers several advantages over the use of viral vectors including ease of mass-production, low theoretical risk of insertional mutagenesis, lessened immunogenicity, and a lower risk of unwanted transgene expression in tissues other than those targeted. However, the clinical applications of non-viral vectors remain impeded by their low efficiency of transfection and transient way of expression of the introduced genes. Producing sustained gene expression and potentiating the efficiency of delivery remains a goal of non-viral gene therapy applications.

1.8 Viral Vectors

Viruses are pathogenic agents and the etiological causes of disease. Viruses infect cells and take over host cellular machinery to preferentially express their own viral genes. They have evolved highly specialised mechanisms to enable them to insert their genomes into target cells, making viruses an ideal candidate to deliver therapeutic genes to mammalian cells. In a direct comparison of gene transfer vectors for myocardial gene transfer, recombinant (E1-/E3-) adenovirus, recombinant adeno-associated virus and recombinant (ICP27-) herpes simplex virus all exhibited robust transgene expression, whilst uncomplexed and complexed naked DNA displayed very limited expression (Wright, Wightman et al. 2001). The efficiency of viral vectors can be attributed to the viral proteins that engage with cell surface receptors and in the trafficking of the genome to the nucleus (Roelvink, Mi Lee et al. 1999; Ding, Zhang et al. 2005). However, low level expression of viral genes can often evoke an adaptive immune response, and as such the host would destroy the virus and any therapeutic DNA it was carrying (McConnell and Imperiale 2004). Ad vectors in particular evoke strong immune responses and upon administration can activate an innate immune response mediated by the viral particle itself (McConnell and Imperiale 2004). This type of immune response is not specific and is aimed at clearing the body of foreign particles, being the first line of defence. After liver gene transfer, rapid clearance of the vector by cellular elements of the innate immune response involves Kupffer cells (Worgall, Wolff et al. 1997), the activation of the classical arm of the complement

pathway (Cichon, Boeckh-Herwig et al. 2001) and an inflammatory response. This ultimately results in the induction of the adaptive cellular response which activates cytotoxic T lymphocytes (Muruve 2004). B cells are then activated during the humoral response, which can result in the production of neutralising antibodies and so eliminate the option of vector re-administration. By removing genes necessary for viral replication to provide space in which to insert foreign genes of interest, viruses can be manipulated to express these foreign genes in any cells that the virus transduces. This would also help to minimise host immune responses. These recombinant vectors are thus replication deficient, and in order to produce such vectors, the replication genes must be provided in *trans*, either integrated into the genome of the packaging cell line or on a plasmid.

In principle, any virus can be used as a vector. DNA viruses were the first to be developed for such purposes, due to the ease of genetic manipulation of the viral genome or the use of homologous recombination to insert a gene of interest into the vector. The discovery of reverse transcriptase provided the means to produce complementary DNA (cDNA) from mRNA, which in turn allowed cDNA cloning and hence the use of RNA viruses as vectors. There are five main classes of clinically applicable viral vectors being studied for cardiovascular applications; retroviruses, lentiviruses, herpes simplex viruses (HSV), adenoviruses (Ad) and adeno-associated viruses (AAV), a summary of which can be seen in Table 1.2. These five vector classes can be further subcategorised according to whether the vector genome integrates into the host chromosome or exists extra-chromosomally (Thomas, Ehrhardt et al. 2003). Integrating vectors are associated with an increased risk of oncogenesis (Hacein-Bey-Abina, von Kalle et al. 2003), although careful engineering can be applied to minimise these risks. For example, the engineering of vectors that integrate into a predetermined site could allow long-term transgene expression whilst preventing the detrimental effects through inappropriate integration (Kay, Glorioso et al. 2001). Since each vector system has its own unique set of properties, one vector may be preferential above another in a particular setting and will determine its range of uses in gene therapy.

Vector	Ability to Integrate	Transgene Capacity	Tropism	Immune Response Activation	Longevity of transgene expression	Reference
Retrovirus	Yes	9 kb	Dividing cells only	Minimal	Yes	(Kay, Glorioso et al. 2001)
Lentivirus	Yes	9 kb	Dividing and non-dividing cells. Ideal for endothelial cells.	Minimal	Yes	(Zennou, Petit et al. 2000; Dishart, Denby et al. 2003)
Herpes Simplex Virus-1	No	25 kb	Dividing and non-dividing cells. Natural tropism for neuronal cells.	Minimal	Yes	(Latchman 2001)
Adenovirus (Ad)	No	36 kb	Dividing and non-dividing cells.	Yes	Transient	(McConnell and Imperiale 2004)
Adeno-associated Virus (AAV)	Yes	4.6 kb	Dividing and non-dividing cells.	Minimal	Up to 1.5 years	(Hermonat, Quirk et al. 1997; Xiao, Chirmule et al. 1999)

Table 1.2 Characteristics of viral vectors for use in gene therapy.

1.8.1 Retrovirus

Retroviruses were the first viral vectors to be used in human gene therapy (Nabel, Plautz et al. 1990) and approximately 25% of the world's gene therapy clinical trials use retroviruses as their platform vector (www.wiley.co.uk/genmed/clinical). Retroviruses can be further subdivided into oncoretroviruses, lentiviruses and spumaviruses, all of which are being developed for gene therapy applications to varying extents. Retroviruses are small enveloped RNA viruses, which replicate via an integrated DNA intermediate by the actions of the enzyme reverse transcriptase. The viral genome is approximately 10 kb, comprising of at least three genes: *gag* (group specific antigens), *pol* (reverse transcriptase) and *env* (the viral envelope protein). These viral genes are flanked by long terminal repeats (LTRs) which are required for integration into the host genome and control viral gene expression. The genome also contains a packaging sequence that allows it to be distinguished from other RNA in the host cell (Verma and Somia 1997).

Retroviral vectors have all their viral genes removed and replaced with the transgene of interest, and thus rendering them replication-incompetent (Young, Searle et al. 2006). Not only does this help to prevent the initiation of an immune response against viral gene products, the removal of viral genes also functions to improve the safety of these vectors as it will significantly reduce the possibility of recombination with wild type retroviruses (Dull, Zufferey et al. 1998). Despite their wide use as gene delivery vectors, the small genome of retroviruses allows for only 9 kb of foreign sequence to be inserted. Production of high-titre preparations required for gene therapy applications is problematic. Retroviruses are associated with low efficiency gene transfer owing to their inability to deliver genes to non-dividing cells (Miller, Adam et al. 1990). Their need to infect replicating cells could be advantageous in the targeting of rapidly replicating cells for use in cancer therapy. However, their utility as gene delivery vectors, in particularly for vascular applications is severely limited as they are not able to infect normal vascular cells, as vascular endothelial and smooth muscle cells have low mitotic rates (Gordon, Reidy et al. 1990). These inefficiencies have led to the development of lentiviral vectors which are capable of infecting both dividing and non-dividing quiescent cells (Lewis, Hensel et al. 1992; Tsui, Kelly et al. 2002; Dishart, Denby et al. 2003).

The genome of retroviruses is capable of integration into the host's genome through the actions of the virally encoded integrase enzyme, and will subsequently replicate as part of the cell's DNA, leading to the potential for long-term expression of a transgene. However, this integration is not site-specific and subsequently this vector has many safety concerns associated with it. Whilst genome integration is not site-specific, it has been shown to take place recurrently in gene rich areas, particularly near the start of transcriptional units (Wu, Li et al. 2003). Random insertion of an LTR sequence adjacent to a cellular proto-oncogene can lead to inappropriate expression of a protein involved in cellular regulation. Random insertional mutagenesis could also disrupt a tumour suppressor gene potentially leading to dysregulation and a malignancy. The safety concerns associated with the use of retroviral vectors has been demonstrated in X-linked severe combined immunodeficiency (X-SCID) human gene therapy trials. In 2000, a clinical trial carried out in France to treat children with X-SCID, illustrated the oncogenic potential of retroviral vectors (Cavazzana-Calvo, Hacein-Bey et al. 2000). This study was based on the use of complementary DNA containing a defective gamma Moloney retrovirus-derived vector and *ex vivo* transfer of the *γc* gene into CD34+ cells. After 10 months the therapy was found to provide sustained full correction of disease phenotype demonstrating the unique potential of gene therapy. However, by 2003, two out of the ten patients had developed a serious adverse complication consisting of uncontrolled leukaemia-like clonal lymphocyte proliferation (Hacein-Bey-Abina, von Kalle et al. 2003), with a third case of leukaemia-like illness being reported in 2005 (Couzin and Kaiser 2005). Two of the three patients were found to have retrovirus integration within or within close proximity to the LMO2 proto-oncogene promoter, which is associated with childhood leukaemia. This integration resulted in the inappropriate upregulation of the proto-oncogene and proved fatal in one of the patients (Hacein-Bey-Abina, Von Kalle et al. 2003). However, the beneficial outcomes in the remaining patients are not to be overlooked. To date, 17 out of 20 patients in both the Paris and London clinical trials have had their immune system restored and has remained functional for over 7 years (Cavazzana-Calvo and Fischer 2007).

1.8.2 Lentivirus

Lentiviruses are a subclass of retroviruses that are often used in gene therapy. In particularly, they are being studied and developed for the treatment of neurodegenerative disorders due to their ability to efficiently transduce cells of the nervous system (Mitrophanous, Yoon et al. 1999) (Wong, Azzouz et al. 2004). The lentiviruses used are usually derived from human immunodeficiency virus-1 (HIV-1) and so raise many clinical safety concerns. HIV can integrate into a potentially pro-oncogenic site or result in germline alteration. In order to address this issue and to improve the biosafety of these vectors, significant modification to the HIV-1 genome can be made (Kim, Mitrophanous et al. 1998). Deletion of accessory genes *tat, vif, vpr, vpu* and *nef* led to the production of minimal vectors that contain only genes necessary for replication and packaging, thus minimising deleterious effects (Kim, Mitrophanous et al. 1998). Development of non-human lentiviral based systems, including simian (Fischer-Lougheed, Tarantal et al. 2007), feline (Browning, Schmidt et al. 2001; Lin, Noel et al. 2004) and bovine immunodeficiency viruses (Takahashi, Luo et al. 2002; Molina, Ye et al. 2004), has also been given attention in order to increase the safety profile of these vectors. However, potentially, this could give rise to new mutant strains of viruses capable of infecting and being transmitted between both human and animals.

Lentiviruses have more complex genomes than other retroviruses, containing an additional 6 proteins. Lentiviruses have a relatively large packaging capacity of up to 8 kb and an ability to infect a wide range of cells. They are also minimally immunogenic having been shown to sustain gene expression for several months (Zhang, La Russa et al. 2002) without detectable pathology (Naldini, Blomer et al. 1996; Azzouz, Ralph et al. 2004; Abordo-Adesida, Follenzi et al. 2005). Gene transfer through lentiviruses is relatively stable as the transgene integrates into the host genome and is copied along with the host genome every time the cell divides. One of the most appealing features of these vectors is that unlike other retroviruses, lentiviruses can infect non-dividing cells, being able to enter the nucleus without mitosis (Uchida, Sutton et al. 1998; Zennou, Petit et al. 2000). This ability makes these vectors ideal for targeting cell types for which previous gene therapy methods could not be used. For example they are ideal for targeting the endothelium, which is

largely comprised of non-dividing cells. Lentivirus transduction of both primary human saphenous vein endothelial cells and smooth muscle cells was shown to be efficient and without toxicity (Dishart, Denby et al. 2003), but there are relatively few studies to date. Lentivirus-based vectors have been also been shown to be successful at transducing adult cardiomyocytes of a transplanted heart (Zhao, Pettigrew et al. 2002), and the hearts of SHR in a study of cardiac physiology (Diez-Freire, Vazquez et al. 2006).

Recently, a new generation of lentiviral vectors have been engineered and has enormous potential. These are in the form of non-integrating lentiviral vectors which can be considered much safer than the previous vectors. By introducing mutations into highly conserved acidic residues in the viral integrase gene, catalytic site or chromosome binding site, vectors can be rendered integration defective without interrupting viral DNA synthesis or accumulation in the nucleus (Leavitt, Robles et al. 1996; Engelman 1999; Apolonia, Waddington et al. 2007). Efficient sustained transgene expression *in vivo* is attainable with non-integrating lentiviral vectors as has been demonstrated in muscle (Apolonia, Waddington et al. 2007) and in rat ocular and brain tissue at levels high enough to improve retinal degeneration in an appropriate disease model (Yanez-Munoz, Balaggan et al. 2006).

1.8.3 Herpes simplex virus (HSV)

HSV type 1 is an enveloped double-stranded DNA virus containing an icosahedral-shaped capsid surrounded by a layer of proteins referred to as tegument. It has a relatively large genome of 150 kb, which facilitates large foreign DNA inserts of up to 30-40 kb (Latchman 2001). HSV is able to infect a broad range of cell types including non-dividing cells. Natural viral infection can take the form of a cycle of lytic replication or can enter a latent state in which the viral genome persists without the expression of any viral proteins, possibly for the life of the host. Latently infected neurons function normally and do not illicit an immune response (Jacobs, Breakefield et al. 1999). HSV-1 has many key features making it a highly desirable vector for gene delivery. Firstly, it has a large transgene capacity which is provided by deletion of genes superfluous for viral replication with as much as 30kb of the HSV genome being available for deletion. However, because its genome does not integrate, HSV

vectors are unlikely to be suitable for the treatment of conditions requiring long-term gene expression. Due to its natural tropism for neuronal cells it has become a promising vector for the treatment of neurological disorders such as Parkinson's disease (Burton, Glorioso et al. 2003) and cisplatin neuropathy (Chattopadhyay, Goss et al. 2004). HSV vectors have also emerged as promising vectors in cancer therapies in the form of replication-selective oncolytic vectors (Kirn, Martuza et al. 2001; Liu, Robinson et al. 2003; Han, Assenberg et al. 2007). These vectors fail to replicate efficiently in healthy cells and will replicate in cancer cells only, destroying them through oncolysis.

1.8.4 Adenovirus

Adenoviruses are non enveloped dsDNA viruses with an icosahedral capsid consisting of 3 main structural proteins, hexon, fiber and penton base and several minor capsid proteins. Their genomes range in size from 26-45kb. Adenoviruses were first isolated from tonsils and adenoid tissue (Rowe, Huebner et al. 1953) and are infectious human viruses, which often cause mild infection of the gastrointestinal and upper respiratory tract, and can also cause ophthalmological and neurological manifestations. Most adenoviral infections are self limiting being efficiently counteracted by the host's immune system. Deletion of the virulence genes during vector production may help to reduce the pathogenesis of these viruses.

Adenoviral vectors, most commonly adenovirus serotype 5 (Ad5) and serotype 2 (Ad2), are a popular choice in gene therapy and such status has lead to much data becoming widely available. As such, adenovirus is a well characterised virus that can be easily genetically altered and grown to high titres. They have a high capacity for the insertion of foreign DNA allowing up to 36 kb (helper-dependent Ads) to be accommodated. They were initially deemed promising vectors for cardiovascular gene therapy applications as they were shown to transduce human vascular cells *in vitro* (Lemarchand, Jaffe et al. 1992) and *in vivo* (Lemarchand, Jones et al. 1993; French, Mazur et al. 1994). Adenoviral vectors exhibit a tropism for many human cells and can infect quiescent as well as dividing cells (Berkner 1988). This is an important characteristic as it is known that vascular endothelial and smooth muscle cells have low mitotic rates, even in diseased states (Gordon, Reidy et al. 1990). They are

considered relatively safe vectors as adenovirus replicates episomally thus reducing the risk of random integration into the host genome, which can potentially result in the disruption of tumour suppressor genes or in the activation of proto-oncogenes, both of which can result in oncogenesis. However, because Ad vectors are non-integrating, it means that their genomes are lost in proliferating cells, and so transgene expression will be transient, although this may be advantageous in certain clinical applications. Transient gene expression coupled with hepatic tropism is a major limiting factor for adenoviral vectors and has lead to their use in niche areas such as vein grafting, where gene transfer can be carried out *ex vivo* (George, Lloyd et al. 2000; Turunen, Puhakka et al. 2006).

The major inadequacy of adenoviral vectors is their lack of ability to efficiently "hide" from the host immune system. Many individuals will produce neutralizing antibody and memory T-cells directed at Ad proteins after exposure to the vectors. This is a result of the expression of viral genes, which trigger a cascade of humoral and innate immune responses (Muruve 2004). This is a significant problem as gene expression is consequently short-lived (Wen, Schneider et al. 2000) and re-administration of the vector is less effective (Yang, Li et al. 1995). In view of this, current studies focus on strategies to eliminate host immune responses, which will allow persistent transgene expression (Schiedner, Morral et al. 1998; Morral, O'Neal et al. 1999), and also on engineering vectors with increased transduction of cardiovascular cells. This can be achieved in several ways, one of which involves the abolition of the natural tropism of the virus and subsequently endowing it with a new tropism for the target cell type (Dmitriev, Krasnykh et al. 1998; Biermann, Volpers et al. 2001; Haviv, Blackwell et al. 2002).

1.8.4.1 Ad Vector Development

In order to reduce the immunogenicity of Ad vectors and to create genome space for the insertion of new genetic material, Ad has been altered in several ways to remove unnecessary parts of the genome (Figure 1.2). Expression of adenovirus proteins occurs in phases – early and late. The adenovirus genome contains five early transcription units (E1A, E1B, E2, E3, E4), two early delayed (intermediate) transcription units and five late units (L1–L5), and encodes over 70 gene products (Mizuguchi, Kay et al. 2001). The genome is flanked by inverted terminal repeats

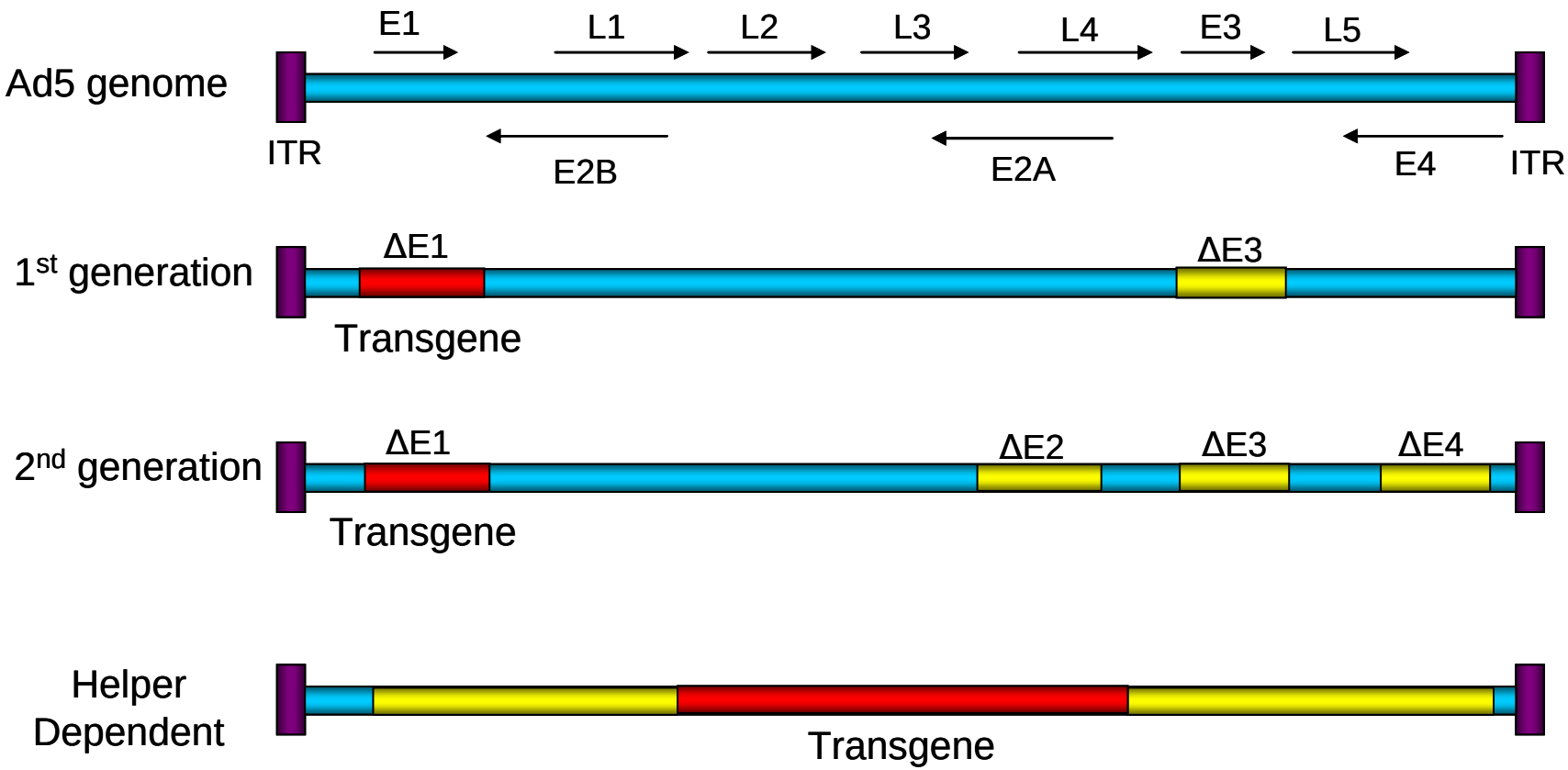

Figure 1.2. Ad5 vector development.

Adenovirus 5 genome and maps of 1st, 2nd and 3rd generation adenoviral vectors showing regions of the genome deleted to facilitate transgene insertion. (Adapted from (Alba, Bosch et al. 2005)

(ITRs) of 100-140 bp in size that serve as replication origins. Early genes (E1A and E1B) are involved in gene expression regulation and activation of them leads to the expression of viral late genes (involved in the expression of structural proteins) and ultimately in the production of infectious viral particles. The foreign gene can be inserted into the region occupied by either E1 or E3 genes with one or both being deleted in the vector construct. In the first generation Ad vector, the E1 (E1A and E1B) gene is replaced by the gene of interest and the resultant defective virus is propagated in cell lines, such as 293 cells (Graham, Smiley et al. 1977), that provide the early gene products in *trans*. The progeny virus cannot replicate in normal cells and upon introduction into the host, they will infect cells and express the foreign gene but no progeny virus will be produced. As the E3 region of the genome is dispensable in viral replication, many first generation vectors will also have all or part of the E3 region deleted. Despite these deletions, first generation vectors still express wild-type late viral genes at low levels and triggers a cytotoxic T lymphocyte (CTL) immune response (Yang, Nunes et al. 1994) ultimately resulting in a short duration of transgene expression.

Second generation Ad vectors also have the E2 and/or E4 regions deleted from their genomes in addition to the E1±E3 deletion. However, local delivery of second generation vectors were not found to reduce inflammation of humoral immune response to adenovirus in rabbit models in comparison to first generation vectors, and most disappointingly did not increase longevity of transgene expression (Wen, Schneider et al. 2000).

Third generation vectors, called helper-virus dependent or gutless vectors, have essential regions of the viral genome (L1, L2, VA and TP) deleted and rely on the provision of essential viral functions from a helper virus. The gutless adenovirus only keeps the two ITRs and the packaging signal from the wild-type adenovirus required for DNA replication and packaging (Mitani, Graham et al. 1995; Kochanek, Clemens et al. 1996; Parks, Chen et al. 1996; Ng, Beauchamp et al. 2001). By deleting most of the viral genome it is possible to accommodate up to 37 kb of insert DNA into defective adenoviral vectors. *In vivo* studies have shown substantially longer transgene expression with helper-dependent vectors (Schiedner, Morral et al. 1998; Morral, O'Neal et al. 1999) sustained up to a year in 2 baboons. However, an innate

immune response is still activated against these adenoviral vectors (Muruve 2004). Recently, however, Barcia et al demonstrated that helper-dependent Ad vectors mediated sustained transgene expression for up to one year in the brains of mice pre-immunised against adenovirus (Barcia, Jimenez-Dalmaroni et al. 2007). This highlights the potential of these vectors in the treatment of chronic diseases, as the immune system was unable to inhibit transgene expression.

1.8.4.2 Vector capsid engineering

There are more than 50 different serotypes of human adenovirus, classified into six sub-groups (A-F) based on biochemical and immunological properties. These viruses can infect different cell types through the utilisation of different primary cellular receptors and thus have a wide tissue tropism range. Most adenoviruses, except subgroup B and the short fiber of subgroup F use the coxsackie virus and adenovirus receptor (CAR) (Bergelson, Cunningham et al. 1997; Tomko, Xu et al. 1997). Ad5, which belongs to subgroup C, is the most extensively studied of all the serotypes. The two-step mechanism of Ad5 infection is well characterised, making it possible to re-engineer it to alter its tropism. Ad5 virus is known to interact *in vitro* with CAR by means of the knob domain of the capsid fiber, bringing the capsid into close proximity with integrins. After attachment, the RGD motif in the penton base at the N-terminus of the fiber interacts with co-receptors $\alpha_v\beta_3$/ $\alpha_v\beta_5$ integrins (Wickham, Mathias et al. 1993). Adenovirus is then internalised by receptor-mediated endocytosis and released by endosomal acidification in fiber-free form to the cytosol before trafficking to the nucleus. Ad5 can transduce endothelial cells (Lemarchand, Jaffe et al. 1992), coronary arteries (French, Mazur et al. 1994), the heart (Palomeque, Chemaly et al. 2007) and at lower efficiency vascular smooth muscle cells (SMC) (Ohno, Gordon et al. 1994). This is reflective of the distribution of CAR expression, with high CAR expression leading to high transduction efficiency. Indeed, after systemic injection in the rat and mouse models, Ad5 virions preferentially accumulate in the liver and spleen (Huard, Lochmuller et al. 1995; Koeberl, Alexander et al. 1997). This highlights the need to substantially alter Ad5 tropism to retarget it to alternative sites, for example the brain, kidney and heart vasculature, unless local delivery is possible.

Genetic strategies to alter the tropism of adenoviruses can either focus on pseudotyping the Ad5 fiber with that of another serotype, or on ablating receptor binding. Native hepatic tropism can be altered by mutating the virus in areas integral to cellular receptor binding. The identification of the residues in the knob involved in CAR binding (Kirby, Davison et al. 1999) has allowed the production of detargeted Ad5 by mutation of these residues (Jakubczak, Rollence et al. 2001). As proof of concept, a mutated putative heparin binding domain reduces Ad liver accumulation 15-fold, whilst combining this mutation with one that ablates CAR binding reduces liver accumulation 100-fold (Huang, Sexton et al. 2003). Thus mutation of the fiber of Ad can lead to the ablation of transduction of non-endothelial cells.

1.8.4.3 Adenoviral retargeting by pseudotyping

The adenovirus fiber protein mediates primary binding of adenovirus to its receptor, and so one approach of vector retargeting is the use of chimeric vectors that incorporate the entire fiber or part of the fiber from a different serotype in place of its own. This could potentially ablate the virus's natural tropism by removal of both the CAR- and heparan sulphate proteoglycan (HSPG)-binding sites and bestow a new tropism upon the vector. Several adenovirus serotypes have been shown to have increased transduction of specific tissues. Proof of concept of chimeric vectors was first shown in 1996 with the production of a functional adenoviral vector in which the fiber was composed of the tail and shaft domains of adenovirus serotype 5 and the knob domain of serotype 3 (Krasnykh, Mikheeva et al. 1996). Alterations in adenoviral tropism were achieved through primary binding via the Ad3 receptor with subsequent internalisation steps achieved via domains of the penton base of Ad5.

Following systemic Ad delivery, Ad5 vectors pseudotyped with serotype 37 and 19p fibers have been shown to lack a native hepatic tropism (Denby, Work et al. 2004) and as such can be considered suitable platform vectors for retargeting. In comparison to non-modified Ad5 vectors, Ad19p and Ad37 pseudotyped vectors lacked tropism for mouse, rat, and human hepatocytes *in vitro* and demonstrated greatly reduced transduction of liver after systemic injection into rats (Denby, Work et al. 2004). Further genetic modifications can allow the development of targeted and thus more efficient vectors. Isolated targeting peptides can be genetically incorporated into the HI loop of the fiber of Ad19p between amino acids 331 and 332 (Figure 1.3). Kidney

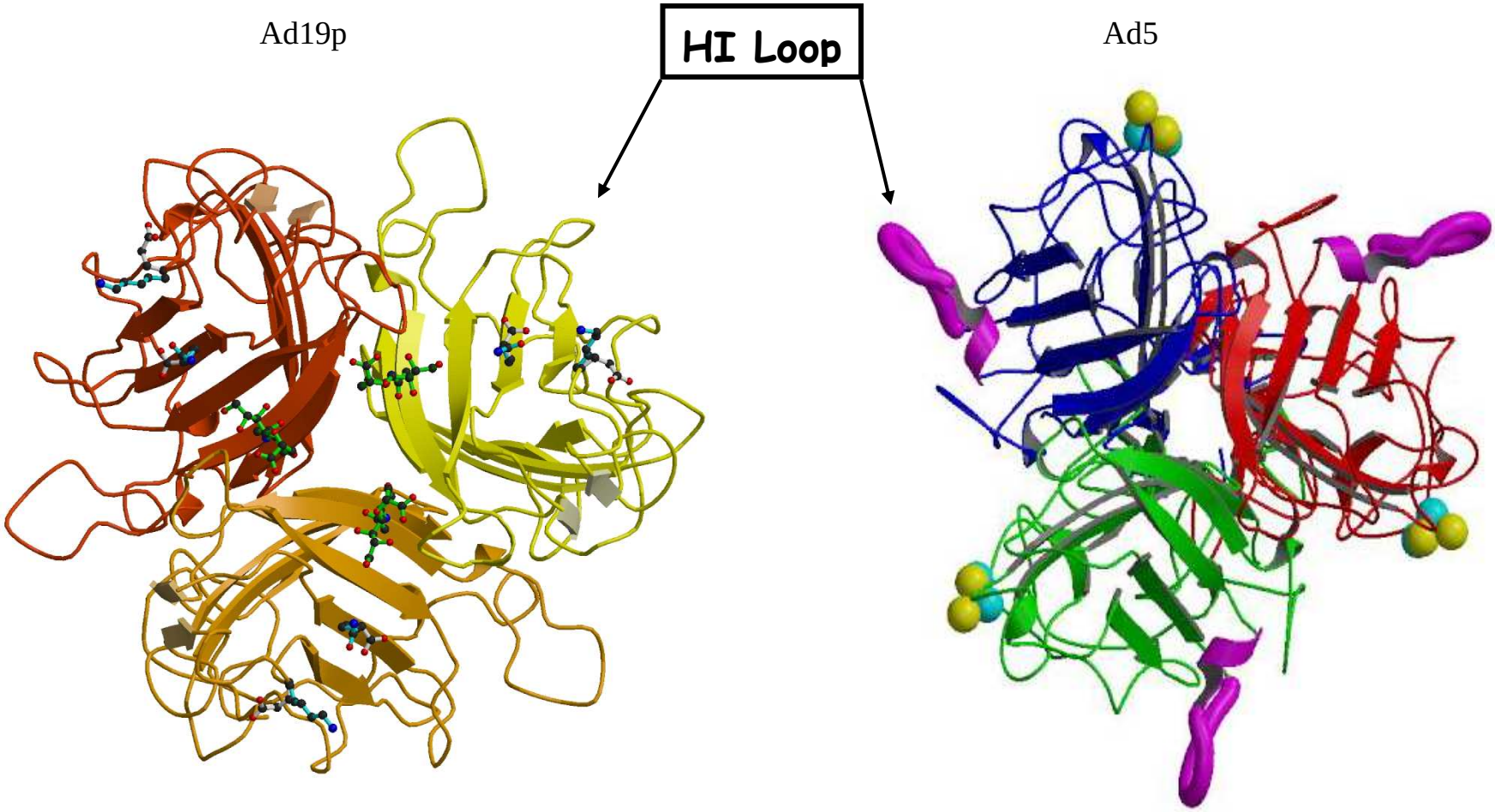

Figure 1.3 Crystal structures of Ad5 and Ad19p fiber heads

Showing HI loop for insertion of targeting peptides into the Ad 5 and Ad19p capsid. Ad5 crystal structure provided by Vijay Reddy, Scripps Research Institute. Ad19p structure modified from (Burmeister, Guilligay et al. 2004).

targeting peptides HTTHREP and HITSLLS, which were identified through *in vivo* phage display, were incorporated into Ad19p-pseudotyped vectors. These peptide-modified vectors were shown *in vitro* and *in vivo*, after systemic administration, to display a significant increase in selective renal targeting with higher levels of transduction than the unmodified Ad19p vectors (Denby, Work et al. 2007).

1.8.4.4 Non-genetic targeting

A simple way of altering vector tropism without the need to genetically modify the vector genome or capsid is the coating of the viral particle with a bispecific antibody. One domain of the bispecific molecule binds to the virus capsid whilst the other domain binds to a novel receptor thus acting as a molecular bridge. This concept has been used *in vitro* to enhance Ad-mediated transduction of human umbilical vascular endothelial cells (Nettelbeck, Miller et al. 2001), and *in vivo* to redirect Ad vectors to a new cellular receptor after systemic delivery (Printz, Gonzalez et al. 2000; Reynolds, Zinn et al. 2000). Although the addition of a protein adapter enhances the affinity of Ad vectors for their targets, it also increases the difficulty of crossing the barrier from laboratory to clinic as there are more components to be considered and reproduced without batch variation.

1.8.4.5 Retargeting detargeted vectors by ligand insertion

Modification of the fiber knob is an appealing option to surmount the restrictions of the CAR binding dependent nature of adenovirus infection. The insertion of targeting peptides into the fiber gene of Ad5 can provide new tropism to detargeted vectors. The exposed HI loop (Figure 1.3) has been identified as a preferred insertion site for peptides (Dmitriev, Krasnykh et al. 1998; Krasnykh, Dmitriev et al. 1998) and this occurs without detriment to virion assembly or fiber trimerisation. A restriction site has been incorporated into the HI loop sequence of the fiber gene of the detargeted vector AdKO1 (Nicklin, Von Seggern et al. 2001). Into this restriction site peptides can be inserted for exposure on the outside of the virion. As the fiber is present at a frequency of 36 copies per virion, the vector can display the targeting peptide a maximum of 36 times. Foreign peptides have also been successfully incorporated in the hypervariable region 5 surface loop of the hexon of Ad vectors (Vigne, Mahfouz et al. 1999). In this region, peptides can be displayed at a copy number of 720. However, in a direct comparison of peptide modified fiber and hexon vectors, hexon-

mediated targeting failed to change the tropism of the vectors (Campos and Barry 2006).

Recent work has shown the application of the phage display technology to identify sequences with desired biological properties, and subsequently introduced these sequences in the re-targeting site of the vector (Engelstadter, Bobkova et al. 2000; Grifman, Trepel et al. 2001; Nicklin, Buening et al. 2001). One potential disadvantage of these small targeting peptides is their often weak binding affinity for their targets.

The concept of phage display of exogenous peptides was first conceived in 1985, and is simply the display of peptides or proteins on the surface of bacteriophage. A foreign gene fragment was inserted into the gene encoding one of the coat proteins (minor capsid protein pIII gene) of filamentous M13 phage creating a fusion protein. The fusion protein was found to be incorporated into the virion, retaining infectivity and displaying the foreign amino acids on the fully immunologically accessible phage surface (Smith 1985). The technology of phage display has since been developed and is now used in a wide range of applications including the rapid isolation and identification of novel peptides with the ability to bind to defined targets molecules *in vitro* or *in vivo* (Johnsson and Ge 1999). For use in cardiovascular applications, phage display could potentially identify ligands which are specific for the vasculature. In other techniques, the choice of ligand to be inserted relies on knowledge of peptides and their affinity for a target receptor. Phage display allows identification of cell-selective peptides without prior knowledge of the target receptor. Peptide libraries are a heterogeneous mixture of phage clones constructed from phage into which random oligonucleotides have been inserted, facilitating the high diversity of phage libraries which can contain more than a billion different peptide sequences (Scott and Smith 1990). This allows the fast screening of an enormous number of peptide sequences. Highly efficient and selective peptides for diverse cell types can be isolated by affinity screening the phage library against immobilized proteins of interest in a process called biopanning. Successive rounds of biopanning enrich the pool of phage with clones that specifically bind the target.

Targeting peptides can be identified through *in vitro* and *in vivo* techniques. The distinct disadvantage of using *in vitro* biopanning is that the question remains as to

whether the ligands isolated *in vitro* will display the same specificity *in vivo*. *In vivo* phage display offers many advantages over *in vitro* biopanning. It can overcome the problems of cells losing their tissue-specific nature when removed from an organ. Phage libraries can be directly introduced into live animals, in order to select for peptide sequences which confer the ability to home to selected tissues. However, targeting peptides identified in animal models may not always be applicable and achieve the same targeting or level of targeting in humans, as they have been shown to have a higher level of complexity. In 2002, the first *in vivo* screening of a peptide library in a patient was carried out (Arap, Kolonin et al. 2002). Isolated motifs from tissue biopsies showed high similarity to ligands for cell-surface proteins of the human vasculature. This method has since been used in stage IV cancer patients to identify tumour-targeting ligands (Krag, Shukla et al. 2006). Phage libraries were administered intravenously and tumours were subsequently excised 30 minutes later, and tumour homing phage recovered. This study displays how this method can be directly applicable in a clinical setting. A variation on this approach is the performance of biopanning on animals bearing human tissue xenografts (George, Lee et al. 2003). This also allows the isolation of peptides that are relevant to the human vasculature.

1.8.5 Adeno-associated virus (AAV)

AAV vectors have developed rapidly over the past decade and have become promising vectors for several genres of gene therapy. Due to their unique properties and broad tissue tropisms, rAAV vectors have been investigated for a wide range of applications including haemophilia (Kay, Manno et al. 2000; Jiang, Couto et al. 2006), cystic fibrosis (Wagner, Messner et al. 1999; Moss, Rodman et al. 2004), Duchenne's muscular dystrophy (Gregorevic, Blankinship et al. 2004; Wang, Kuhr et al. 2007) and rheumatoid arthritis (Goater, Muller et al. 2000). The AAV2 genome was the first serotype to be cloned into bacterial plasmids in 1982 (Samulski, Berns et al. 1982), and since then AAV vectors have maintained their position as strong candidates for gene therapy. As a result of poor uptake of Ad into vascular cells and the efficient performance of rAAV in other disease models, rAAV vectors have been developed for cardiovascular gene delivery. The potential of these vectors in cardiovascular gene therapy was first shown through rAAV-mediated expression of

the cytoprotective gene HO-1 in rat myocardium (Melo, Agrawal et al. 2002). The safety and efficiency of these vectors was further proven through rAAV-mediated myocardial gene transfer in mice. Transgene expression was observed one year post-infusion with no significant inflammatory response or adverse affects on LV systolic function, as assessed by echocardiography (Woo, Zhang et al. 2005). AAV vectors are thus minimally pathogenic and also possess the ability to mediate long-term transgene expression through stable integration targeted to a specific locus in the host genome. In a study by Xiao et al (Xiao, Li et al. 1996), the introduction of recombinant AAV vectors expressing the *lacZ* gene into the muscles of immunocompetent mice resulted in persistent gene expression for more than 1.5 years (Xiao, Li et al. 1996). Thus AAV vectors could prove useful in clinical situations where prolonged expression of the transgene expression is required. Stable transgene expression is a prerequisite for vectors to treat inherited disorders and would be desirable in the treatment of many acquired cardiovascular diseases which progressively worsen overtime. However, the progress of AAV vectors has been hampered by their poor transduction of a range of target tissues.

Long-term transgene expression is facilitated by the fact that recombinant AAV vectors evoke little innate immune response with only transient infiltration of neutrophils and chemokines (Zaiss, Liu et al. 2002). Immune response against the virus appears to be restricted to the generation of antibodies specific for the viral capsid protein (Bessis, GarciaCozar et al. 2004). AAV transfer into muscle fibers *in vivo* was found to activate no cellular or humoral response to transgenic products (Jooss, Yang et al. 1998). This is in stark contrast to similar experiments with adenoviral vectors in which a T-cell mediated response to transgenic and viral products were found after intramuscular injection, and led to the loss of transgene expression and destruction of muscle fibers (Yang, Su et al. 1996). Further investigation revealed that unlike Ad vectors, AAV vectors were inefficient transducers of antigen presenting cells (APCs) such as macrophages and dendritic cells, which are believed to be necessary in the production of cellular immune responses (Jooss, Yang et al. 1998). And so AAV vectors may be capable of evading the immune system, making them ideal gene therapy candidates. However, recently the duration of transgene expression in the liver mediated by rAAV2 vectors was found to be limited to 8 weeks (Manno, Pierce et al. 2006). Upon further

investigation, it was suggested that transduced hepatocytes were destroyed by the activation of T-cells against the capsid of rAAV2 (Manno, Pierce et al. 2006; Vandenberghe, Wang et al. 2006). Direct comparison of T-cell responses activated against the capsids of rAAV serotypes 2, 7 and 8 revealed little evidence of T-cell activation against rAAV7 and 8 and postulated a potential role for heparin binding in directing immune response against the capsid proteins (Vandenberghe, Wang et al. 2006). Thus utilisation of alternative serotypes that do not use HSPG as their receptor for cell entry may help to avoid this limitation.

In AAV vectors, the viral DNA, except the ITRs, has been eliminated making room for foreign DNA to be inserted. This adds a safety feature that will reduce any host immune response directed at viral gene expression and also eliminate the possibility of the generation of replication competent pseudo wild type AAV. Gene transfer vectors based on AAV serotype 2 have been extensively researched and these are the most characterised and predominantly used of the AAV vectors. Other serotypes that have recently been identified are presently under investigation and demonstrate a vast potential for cardiovascular gene therapy. Importantly, no serotype is the causative agent of any human pathology providing these vectors with a good safety profile.

One important safety concern with AAV vectors to be considered is the potential for AAV-mediated germ-line transmission. Intramyocardial injection of AAV vectors expressing *lacZ* into Sprague–Dawley rats resulted in the detection of *lacZ* expression and β-galactosidase activity in the testes at 6 months post-infusion (Pachori, Melo et al. 2004). In a similar study, Arruda et al found that whilst vector DNA could be detected in the gonad of rat, mouse, rabbit and dog, no AAV vector sequences could be detected in the semen (Arruda, Fields et al. 2001).

Another major safety concern lies amongst reports of high incidences of hepatic carcinomas after rAAV vector infusion into mice (Donsante, Vogler et al. 2001). Hepatocellular carcinomas that developed in these mice were subsequently found to contain AAV vector proviruses at a specific chromosomal locus (Donsante, Miller et al. 2007), implicating insertional mutagenesis by AAV vectors as a causative factor. These findings raise questions of rAAV vector safety.

1.8.5.1 AAV biology

Adeno-associated viruses (AAV) are small 4.7-kb linear single-stranded DNA non-enveloped viruses. Their genomes are organised in similar ways, being extremely simple in composition and containing only two large open reading frames (ORFs) flanked by ITRs of approximately 145 bp (Figure 1.4). The ITRs are *cis*-acting elements that form a hairpin secondary structure that is required for viral genome replication and packaging. The two ORFs encode 2 genes, rep (replication) and cap (capsid), which are respectively involved in gene expression regulation and structure. Four multifunctional rep isoforms with molecular masses of 78, 68, 52, and 40 kDa are encoded by the 5' ORF and are transcribed from two different promoters. The rep proteins are involved in specific DNA-binding, helicase and site-specific endonuclease and modulation of transcription of viral genome promoters. The 3' ORF encodes 3 capsid proteins (VP1, VP2 & VP3) through alternate splicing of the cap gene, and are expressed at a molar ratio of 1:1:18 (Opie, Warrington et al. 2003). A novel and weak transcription initiation start codon (ACG) is used for VP2, resulting in reduced translation of the protein (Becerra, Koczot et al. 1988). All 3 proteins use the same stop codon, and so VP2 and VP3 are essentially successive amino-terminal truncated forms of VP1. The three proteins interact together to form a capsid with icosahedral symmetry. VP1 protein carries a phospholipase A2 (PLA2) motif, thought to be involved in initiation of early gene expression and in nuclear translocation – mutations in this area of the gene result in virions with reduced infectivity (Girod, Wobus et al. 2002). VP2 is of unknown function and is non-essential in virion production (Warrington, Gorbatyuk et al. 2004). The third viral protein (VP3) contains areas of the capsid that are important in cell-receptor binding. When used as gene delivery vectors, the rep & cap genes, which make up 96% of the genome, are replaced by the transgene. Recombinant vectors are produced by supplying these deleted genes in *trans*. The resultant vectors are structurally simple and less likely to evoke a host immune response. The small size of the AAV virion is responsible for the limited DNA packaging capacity and is a major disadvantage of AAV vectors. Transgenes can be packaged as long as they are not significantly larger (119% maximum capacity) or smaller than the wild-type genome (Hermonat, Quirk et al. 1997). Outwith these limits, resultant vectors are severely defective for producing infectious virions. One method to overcome this limitation is the trans-splicing of larger genes between two independent AAV vectors that will be co-administered to

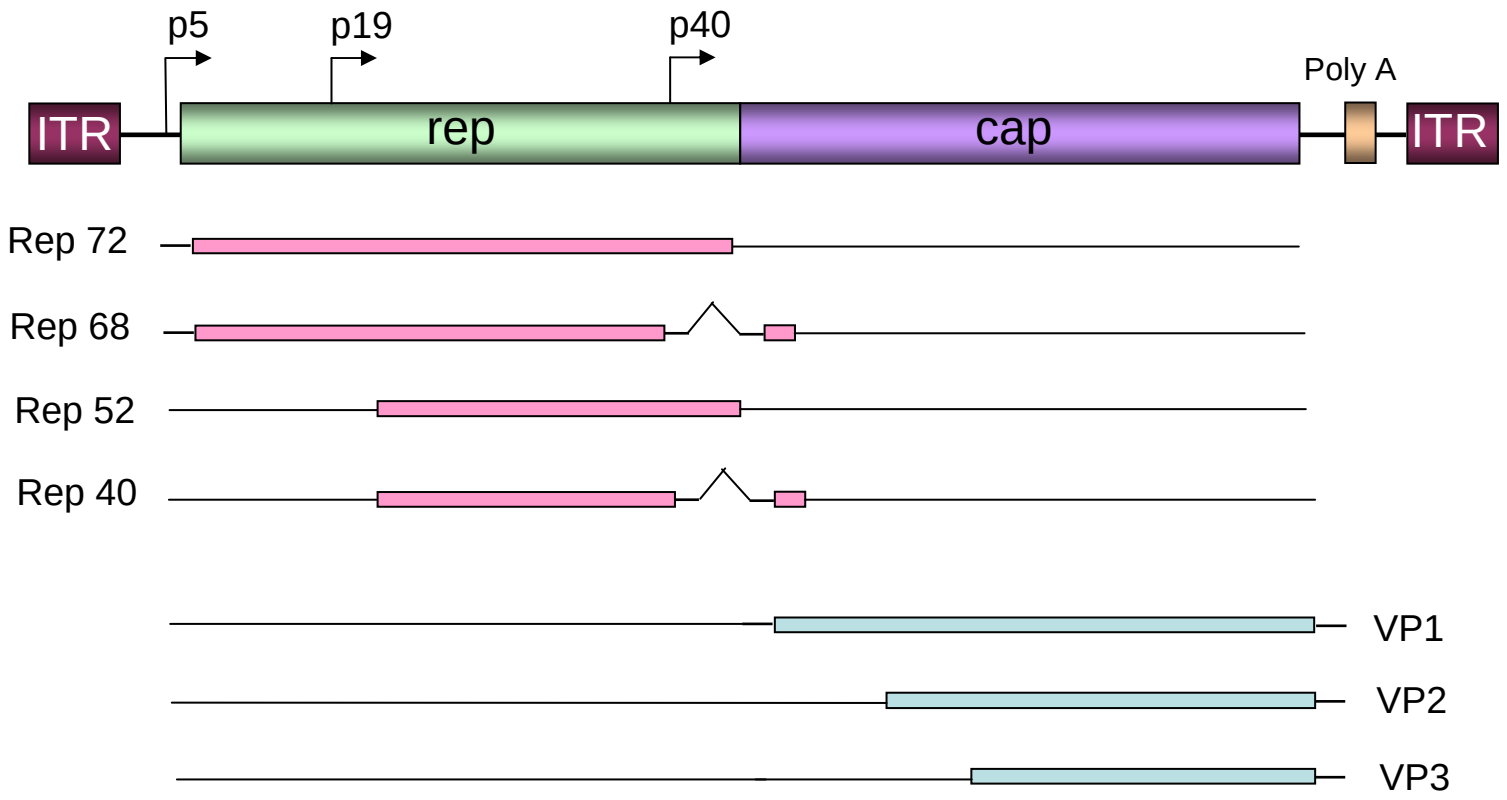

Figure 1.4 Genome organisation of AAV.

The AAV genome is a 4.7 kb single-stranded linear DNA genome and is made up of 2 genes, rep and cap, with two flanking ITRs. Three different promoters drive transcription: P5, P19 and P40. 4 transcripts encode non-structural genes (Rep72, 68, 52 and 40) and 3 transcripts encode structural proteins (VP1,2 and 3).

the same target tissue (Yan, Zhang et al. 2000). This technique utilises the ability of AAV genomes to combine, although results in lower transgene expression as a result of the complexity of the system. However, further development may help to increase the utility of AAV vectors allowing them to appeal to a wider range of applications.

1.8.5.2 AAV replication

AAVs belong to the *Parvoviridae* family and are ascribed the genus *Dependovirus*, so called as they are helper-dependent viruses with a bi-phasic life cycle. They cannot replicate autonomously, instead requiring co-infection with an unrelated virus, such as Ad or HSV, in order to complete its life cycle. In the absence of co-infection, AAV can undergo latent infection as an episome or may integrate its viral DNA into the host genome (Cheung, Hoggan et al. 1980) in human chromosome 19 by site- specific recombination directed by the viral *rep* function (Kotin, Menninger et al. 1991). It is important to note that recombinant AAV vectors lack the integration function as their viral rep genes have been removed. AAV genomes can be excised from the host genome in the presence of helper factors and can lead to a productive infection cycle (Berns, Pinkerton et al. 1975). AAV is attractive for gene therapy applications as a gene of interest can persist in the host cell genome for long periods.

Advances in AAV vector production have eliminated the need for helper adenovirus infection (Xiao, Li et al. 1998). Instead, to be packaged into functional vectors, genomes must be provided with all rep, cap and helper functions *in trans* on exogenous plasmids (Grimm, Kern et al. 1998; Xiao, Li et al. 1998). The production of recombinant vectors takes advantage of the ability of viral genes to accomplish their role in the replication of viral DNA and in the packaging of mature virions even when provided to the host cell *in trans* on exogenous plasmids. Minimum regions in helper adenovirus that mediate replication of AAV vector are E1, E2A, E4 and VA (Matsushita, Elliger et al. 1998). The 293T cell line, of human kidney embryonic cells, encodes the E1 region of the Ad5 genome. Thus when a plasmid that encodes the E2A, E4 and VA regions (Ad-helper plasmid) together with a plasmid that encodes the genome of the AAV vector (vector plasmid) and a plasmid with the rep and cap genes are transfected into 293T cells, AAV vector is produced as efficiently as when infection by wild type Ad is used (Grimm, Kern et al. 1998; Grimm and Kleinschmidt 1999). This is a triple transfection protocol, but has been refined into a

two plasmid co-transfection protocol and produces high titre recombinant AAV vectors (Grimm, Kay et al. 2003). It also eliminates the feasible potential of helper virus contamination.

Much is still to be learned about the cellular mechanisms controlling AAV infection. There are several common stages for replication of all AAV vectors that must be carried out for successful transgene expression. The first step in infection is the attachment of the vector to the cell surface receptor, and in the case of AAV vectors will require the use of co-receptors to assist in internalisation. The virus must then be internalised into the cell by the process of receptor-mediated endocytosis from clathrin-coated pits. During this procedure, the cell membrane folds in on itself and eventually results in the formation of cytoplasmic vesicles in which the virus is temporarily contained. The vector is subsequently trafficked from early endosomes to late endosomal compartments (Douar, Poulard et al. 2001). It must then escape the endosome to be released into the cytosol, where nuclear translocation must then take place. Endosomal processing is thought to be an essential step for AAVs, exemplified by the fact that AAV2 directly injected into the cytosol fails to reach the nucleus (Ding, Zhang et al. 2005). It is not clear where in the cytosol the virus is released, but the co-localisation of fluorescein isothiocyanate (FITC)-labelled transferrin and Cy3-AAV within intracellular vesicles near the nucleus is suggestive of a release site at or near the nuclear membrane pores (Sanlioglu, Benson et al. 2000). After release from the endosome, that may occur by the weak acidification of the vesicle, AAV rapidly trafficks to the nucleus and accumulates in the perinuclear region (Bartlett, Wilcher et al. 2000). Trafficking to the nucleus may involve the use of functional microtubules and microfilaments, as visualised by the use of Cy3-labeled rAAV (Sanlioglu, Benson et al. 2000). For AAVs, the process of nuclear translocation was initially thought to occur through the virus slowly penetrating the nuclear pore complex (NPC) into the nucleus, with the majority of the virus remaining in perinuclear compartments (Bartlett, Wilcher et al. 2000). However, entry into the nucleus has since been shown to occur independently of the NPC through the use of agents that block NPC function (Hansen, Qing et al. 2001). It is unknown whether viral uncoating to release the genome occurs within or outwith the nucleus. However, capsid proteins (Bartlett, Wilcher et al. 2000; Sanlioglu, Benson et al. 2000) and the necessary machinery for virion uncoating (Hansen, Qing et al. 2001) have been identified within the nucleus,

suggesting that nuclear virion uncoating may be a reality, although direct evidence is lacking. It is known that the single-stranded DNA genome is converted to double-stranded DNA within the nucleus and is then the template for transcription. Genome conversion is also a rate-limiting step that is enhanced by helper-virus co-infection (Ferrari, Samulski et al. 1996). It is after entry into the host cell nucleus that the virus can either establish a lytic or lysogenic life cycle and it is the presence or absence of helper virus that will determine this. The efficiency of all these steps of replication will determine the overall efficiency of the vector.

1.8.5.3 AAV serotypes and receptors

To date, over 100 AAV genetic variants have been isolated (Gao, Vandenberghe et al. 2004). Eleven known serotypes of AAV have been identified, all displaying a variety of tissue tropisms and receptor-binding characteristics (Table 1.3) and sharing different levels of sequence homology. It is thought that many more serotypes and isolates are yet to be identified as they are believed to be widely dispersed throughout multiple tissues of non-human primate species (Gao, Alvira et al. 2002; Gao, Vandenberghe et al. 2004). AAV1 – 4 and 6 were originally isolated as contaminants of adenovirus stocks. Although isolated from a simian adenovirus type 15 (SV15) stock, AAV1 is considered to be of unknown origin. AAV2 and 3 were later isolated from human infants and AAV4 was later isolated from a culture of rhesus monkey kidney cells. AAV5 was isolated from a human penile condylomatous lesion (Bantel-Schaal and zur Hausen 1984), and is one of the most divergent of the AAV serotypes (Chiorini, Afione et al. 1999). AAV6 is thought to have arisen from homologous recombination between AAV1 and AAV2 (Xiao, Chirmule et al. 1999). AAV7 and AAV8 were both isolated from rhesus monkey by PCR techniques (Gao, Alvira et al. 2002; Gao, Vandenberghe et al. 2004), whilst AAV9 was isolated from human tissues by similar methods. AAV10 and 11 were identified in the tissues of non-human primate cynomolgus monkey and are capable of infecting both human and monkey cells (Mori, Wang et al. 2004). The sequence identities among the different serotypes are high with a general homology in nucleotide sequence of approximately 80%. The greatest divergence in sequence can be observed in the capsid proteins, especially in regions thought to lie on the utmost exterior of the virion (Gao, Alvira et al. 2003). This may account for the differing natural tropisms of these viruses. The pattern of transgene expression has been demonstrated to be affected by the serotype of AAV

Serotype	Tropism	Receptor
AAV1	Skeletal muscle (Hauck and Xiao 2003) cardiac tissue (Palomeque, Chemaly et al. 2007)	α2-3 linked or α2-6 linked sialic acid
AAV2	Broad tropism – muscle, brain, retina, liver, lung.	HSPG, αVβ5 integrin, fibroblast or hepatocyte growth factor receptors, 37/67-kDa laminin receptor
AAV3	Cochlear inner hair cells (Liu, Okada et al. 2005)	heparin, heparan sulphate, and FGFR-1, 37/67-kDa laminin receptor
AAV4	Ependymal cells (Davidson, Stein et al. 2000)	α2-3 O-linked sialic acid
AAV5	Neurons (Alisky, Hughes et al. 2000), dendritic cells (Xin, Mizukami et al. 2006)	PDGFR, α2-3 N-linked sialic acid
AAV6	Skeletal muscle, cardiac tissue (Blankinship, Gregorevic et al. 2004)	α2-3 linked or α2-6 linked sialic acid
AAV7	Skeletal muscle (Gao, Alvira et al. 2002)	Unknown
AAV8	Liver (Gao, Alvira et al. 2002)	37/67-kDa laminin receptor
AAV9	Liver, skeletal muscle, cardiac tissue (Pacak, Mah et al. 2006)	37/67-kDa laminin receptor
AAV10	Liver, heart, skeletal muscle, lung, kidney, uterus (Mori, Wang et al. 2004)	Unknown
AAV11	Muscle, kidney, spleen, lung, heart, stomach (Mori, Wang et al. 2004)	Unknown

Table 1.3 AAV serotypes and their varying tropisms and receptors.

(Rabinowitz, Rolling et al. 2002), which may be due, in part, to viral receptor distribution, as receptor binding is the primary step in viral infection. The discrepancies in tissue tropisms between serotypes are likely as a result of different mechanisms of uptake into a target T-cell. In order to comprehend the differences in transduction efficiencies of the different serotypes, it is important to understand the full mechanism of the initial AAV binding and infection steps.

It is known that AAV2 has a wide host range and utilises HSPG as an attachment receptor (Summerford and Samulski 1998), and at least three different co-receptors including αvβ5 integrin (Summerford, Bartlett et al. 1999), and the fibroblast or hepatocyte growth factor receptors (Qing, Mah et al. 1999). The structure of AAV2 has now been determined to 3-Å resolution by X-ray crystallography (Xie, Bu et al. 2002), and support the results of insertional mutagenesis studies that identified residues that mediate binding of AAV2 to HSPG. AAV3 has been shown to bind to heparin, heparan sulphate, and FGFR-1, making its array of receptors similar to those of AAV2 (Blackburn, Steadman et al. 2006). Competition assays identified that closely related serotypes AAV1 and AAV6 use either α2-3 linked or α2-6 linked sialic acid as primary receptors when transducing numerous cell types (Wu, Miller et al. 2006). Platelet derived growth factor receptor (PDGFR) has been identified as a co-receptor for AAV5, with the *in vivo* tropism of AAV5 correlating with the distribution of PDGFR (Di Pasquale, Davidson et al. 2003). AAV5 also requires α2-3 sialic acid for binding and transduction (Walters, Yi et al. 2001). AAV4 shares the requirement of AAV5 for sialic acid, however the difference between these two vectors lies in linkage specificity; AAV4 requires O-linked sialic acid, whereas AAV5 requires N-linked sialic acid, offering an explanation for the difference in tropisms (Kaludov, Brown et al. 2001). A 2-yeast hybrid screen with subsequent functional studies revealed the 37/67-kDa laminin receptor (LamR) as important in binding and transduction of AAV8 (Akache, Grimm et al. 2006). It was also shown to be important in the binding of AAV2, -3 and -9. AAV10 and -11 have not yet been fully characterised.

Until recently, most gene therapy applications have employed rAAV2 based vectors. However, AAV2 vectors have been disappointing in the area of cardiovascular gene therapy demonstrating inefficiency in transduction of both myocardial cells and

endothelial cells. Their broad tropism also limits their use for systemic administration. Direct comparison of Ad5 and AAV2 for transduction of vascular cells has revealed the poor tropism of AAV2 for endothelial cells (Dishart, Denby et al. 2003). This difference in tropism between vectors can be attributed to vector size and the availability of co-receptors for each vector on endothelial and smooth muscle cells. Although no AAV serotype appears more efficient than AAV2 in transduction of the vascular endothelium, other endothelial cells have been transduced by alternate serotypes. AAV6 based vectors demonstrated a higher transduction efficiency of airway epithelia than AAV2 (Halbert, Allen et al. 2001), illustrating the potential of exploiting naturally occurring serotypes. The preferential transduction of AAV6 over AAV2 was facilitated by these two serotypes binding to different receptors. Membrane-associated HSPG has been identified as the viral receptor for AAV2 (Summerford and Samulski 1998) and marked deposits of HSPGs has been identified in the extracellular matrix of endothelial cells (Pajusola, Gruchala et al. 2002). Therefore, the matrix-associated receptors may be competing for virus binding and consequently reducing transduction of endothelial cells and could offer a potential explanation for the low infectivity of endothelial cells by AAV2. It was found that heparin does not inhibit AAV6 as it does AAV2, and in a transduction assay, the two viruses did not compete with one another suggesting different receptors for these serotypes (Halbert, Allen et al. 2001). Additionally, the transduction of vascular endothelial cells has been shown to be inefficient with AAV2 vectors resulting in virion degradation by the proteasome during the trafficking process (Nicklin, Von Seggern et al. 2001).

Thus alternate serotypes with naturally occurring differences in tropism can be exploited as potential gene therapy vectors to see if they offer an enhanced tropism for cardiovascular tissues. AAV serotypes 1 and 6 have shown preferential transduction of the skeletal musculature.

1.8.5.4 AAV transcapsidation

Recombinant AAV vectors are based on the AAV2 genome onto which the capsid proteins from a different serotype have been pseudotyped. Capsid proteins from most serotypes have been successfully cross-packaged with ITRs from AAV2. Pseudotyping is considered a safer alternative than evaluating native wild type

serotypes as much information for AAV2 has already been generated. Several studies have been carried out to compare the transduction efficiencies of the ever increasing array of alternate serotype AAV vectors. In a study by Du et al (Du, Kido et al. 2004), the capacity of AAV serotypes 1-5 for *in vitro* myocardial transduction was tested (Du, Kido et al. 2004). This study demonstrated both the differing capacities of the alternative serotypes, and identified AAV1 as having the highest enhanced ability to transduce adult human cardiomyocytes. In another study that compared the efficiency of recombinant vectors of eight different serotypes in transducing rat myocardium *in vivo*, AAV1, 6 and 8 demonstrated the highest efficiency in transducing rat hearts *in vivo* (Palomeque, Chemaly et al. 2007). It is difficult to compare between AAV serotype studies as no standard for titering AAV has been set up, and different routes of administration and different aged animals have been used. However, general trends can be observed, demonstrating that AAV serotypes 1, 6, 8 and 9 show higher levels of cardiac transduction than other serotypes.

Another advantage of new AAV vector serotypes could be the evasion of host humoral immune responses directed against AAV2. The prevalence of existing neutralising antibodies due to prior exposure amongst the human population (approximately 80% of the population) may render them immune to the vector and contribute to the limited gene transfer abilities of AAV2. In a study by Chirmule et al, virtually all patients studied were found to have Ig to AAV2, although only 32% of the cohort was found to possess virus neutralising antibodies (Chirmule, Propert et al. 1999). The effect of this on transgene expression is still to be established. It has been established that the presence of neutralising antibodies to wild type AAV2 did not inhibit or interfere with rAAV5-mediated transduction of the brain in rats; it did however prevent AAV2 mediated transduction (Peden, Burger et al. 2004).

1.8.5.5 Retargeting AAV vectors

Although several serotypes of AAV have been identified, there are still several cell types that remain non-permissive to AAV infection. Retargeting vectors may help to encompass these non-permissive cells into AAVs vast repertoire, and may help to improve the efficiency of transduction of cells already permissive to infection. Retargeting of AAV vectors has mainly been applied to AAV2 vectors, and has been achieved *in vitro* through two main strategies. These are (1) the use of bi-functional

antibodies (Bartlett, Kleinschmidt et al. 1999) and (2) the genetic modification of the capsid through the insertion of targeting peptides (Wu, Xiao et al. 2000). Vector binding can be enhanced by the use of bi-specific antibodies. During this process, one arm of the antibody will bind to the surface of the cell of interest, and the other arm to the AAV capsid structure. Bartlett et al (Bartlett, Kleinschmidt et al. 1999) achieved AAV2 mediated transduction of non-permissive human megakaryocytic cells through the interaction of a bispecific F $(ab)_2$ antibody with both the cell surface receptor $\alpha_{IIb}\beta3$, and the viral capsid. This ultimately facilitated the binding and internalisation of the vector via an alternative receptor and represents the potential to improve the binding and transduction profile of AAV2. This technique has also been used to redirect AAV binding by insertion of an immunoglobulin binding domain in order to couple it to various antibodies to mediate altered receptor binding (Ried, Girod et al. 2002). However, this technique relies on a very stable interaction between the antibody and the vector.

The AAV capsid protein is important in the initial stages of viral infection as it is the element that will primarily interact with the cell surface receptor. The capsid protein essentially determines the tissue tropisms of the virus through its selective interactions. Short peptide sequences can be cloned into the capsid gene with the aim of changing or expanding the vector tropism and can even be used to disrupt the native tropism. Targeting peptides may be derived from phage-display as previously described. To be successful, the peptide insertion should have minimal effects on subsequent vector assembly, packaging and infectivity. Several suitable sites for insertion of targeting peptides into the AAV2 capsid have been identified and evaluated for tolerance to insertions and mutations; peptides may be inserted at the optimal position of 587 in the AAV2 capsid in order to be displayed on the surface of the virion (Girod, Ried et al. 1999; Wu, Xiao et al. 2000). This insertion site was identified before the crystal structure of AAV2 was known and so sequence alignment studies of AAV2 VP1 protein with homologous protein of canine parvovirus (CPV) (for which the crystal structure was known) and other related parvoviruses were carried out. Based on these alignments, 6 putative surface exposed capsid sites were identified that were highly variable amongst the parvoviruses and were also found to tolerate a 14-amino-acid targeting peptide insertion, leading to successful virion packaging (Girod, Ried et al. 1999). Out of the six sites in the AAV2 capsid protein -

residues 261, 381, 447, 534, 573 and 587, residue 587 was identified as the optimal site for insertion (Girod, Ried et al. 1999).

Genetic incorporation of peptides into the AAV capsid has been used to enhance transduction of human endothelial cells (Nicklin, Buening et al. 2001) and to alter tropism toward cells expressing the CD13 receptor (Grifman, Trepel et al. 2001) and human luteinizing receptor (LH-R) (Shi, Arnold et al. 2001).

A variant of this technique is the use of AAV libraries, which is similar in concept to the use of phage libraries. In this approach, a random peptide is inserted into the AAV2 capsid sequence in a position that allows it to be displayed on the surface of the virion and at the same time ablating HSPG binding. Each viral particle in the library will display a different peptide. Chimeric capsid AAV libraries can then be screened to identify vectors that exclusively transduce a particular target cell or tissue type. This technique was first developed by Müller et al (Muller, Kaul et al. 2003), who used the AAV library to identify vectors that could transduce human coronary artery endothelial cells more readily than non-endothelial control cells. This approach was used by others to identify AAV vectors containing peptides that efficiently transduce acute myeloid leukaemia cell lines (Perabo, Buning et al. 2003), a cell type that no other vectors have been found to efficiently transduce. AAV libraries allow the selection of vectors with targeting peptides that have been identified whilst already in the AAV2 capsid. This eliminates the possibility of the targeting peptide loosing its specificity when incorporated into the vector.

1.9 Transcriptional Control

Vector targeting can now be achieved at the level of transgene regulation. The incorporation of tissue-specific promoters into a vector addresses both vector efficiency problems and safety concerns. Tissue specific promoters allow transcriptional control, in addition to that of selective transduction by the vector. They allow greater tissue specificity and control of gene expression in non-target tissue, although they will not prevent the uptake of the vector into non-target tissues. This minimizes the risk associated with the transfer of potentially dangerous genes into other tissues and also increases the concentration of the therapeutic gene product

delivered to the target tissue, maximizing the effect of the therapy and requiring lower doses of the vector. The most commonly used promoters in viral gene therapy are derived from cytomegalovirus (CMV) and Rous sarcoma virus (RSV). As these are constitutively active and unregulated in a broad range of cell types, these promoters are not considered optimal for tissue-specific transgene expression. High-level gene expression in all cell types transduced could have serious clinical implications. Viral promoters are generally more studied and widely used than human promoters but have the distinct disadvantage of being silenced by an immune response, particularly by the cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) (Qin, Ding et al. 1997).

A range of cellular promoters have been developed for a wide range of specific tissues. In many instances these tissue-specific promoters suffer from a lack of activity, specificity or both. Nevertheless, transcriptional targeting of HSV-1 vectors was achieved by the insertion of albumin enhancer/promoter-infected-cell polypeptide 4 (ICP4) transgene into the TK gene of mutant HSV-1 d120, deleted for both copies of the ICP4 gene. Expression only occurred in albumin expressing cells, which are uniquely in the liver (Miyatake et al 1997)**.** Endothelial cell (EC)-specific promoters have been investigated for use in vascular gene therapy. EC promoters, *fms*-like tyrosine kinase-1 (FLT-1) and intercellular adhesion molecule-2 (ICAM-2), have been shown to drive transgene expression from adenoviral vectors at levels comparable to the CMV promoter *in vitro* in endothelial cells (Nicklin, Reynolds et al. 2001). *In vivo*, leaky transgene expression was detectable from the ICAM-2 promoter but not from the FLT-1 promoter, demonstrating the potential of this promoter for use in gene therapy applications.

Cardiac promoters regulate the expression of myocardial proteins. Myosin heavy chain (MHC) and myosin light chain 2v (MLC-2v) have both been explored as cardiac-specific promoters to transcriptionally target gene expression to the heart. α-MHC is a myocyte-specific promoter and has been used to achieve cardiac-specific expression of the reporter gene luciferase after direct injection into the left ventricular cavity of mice (Champion, Georgakopoulos et al. 2003). This promoter was shown to work in both Ad and AAV vectors. MLC-2v is a major component of cardiac and striated skeletal muscles and is the ventricular form of myosin light chain. It is

important during embryogenesis in the development of the heart and alterations in expression result in cardiac defects. MLC-2v is thus highly specific for heart and the MLC-2v promoter can be used to mediate cardiac-specific transgene expression, and is the best characterized promoter for this purpose (Small and Krieg 2004). A series of MLC-2v fusion genes were constructed and transfected into primary neonatal rat myocardial cells and a non-myocardial cell line (CV-l), demonstrating that 250 bp of the MLC-2 5" flanking region was sufficient to confer cardiac specific expression (Henderson, Spencer et al. 1989). A fusion of 1.5 kb of the MLC-2v promoter and the CMV immediate early enhancer demonstrated a 50-fold increase in myocardial transgene expression levels in mice in comparison to the same vectors under the control of the CMV promoter alone (Muller, Leuchs et al. 2006). During *in vitro* experiments in cardiomyocytes (H9C2), an MLC2v promoter inserted into an AAV vector drove gene expression at levels comparable to CMV promoter. Two versions of the MLC2v promoter (1.7 kb and 281 bp versions) were then packaged into rAAV2 vectors. After intravenous injection into neonatal rat and mouse, promoter mediated cardiac-specific reported gene expression was detected from both forms of the promoter (Phillips, Tang et al. 2002). A 250bp fragment of the MLC-2v promoter inserted into Ad vectors significantly reduced expression levels *in vivo* in the liver, lung and kidney in comparison to Ad vectors under the control of the CMV promoter (Boecker, Bernecker et al. 2004).

The combination of a cardiac-specific promoter and the use of a target viral vector may lead to the production of a vector that is capable of switching on therapeutic genes in the myocardium.

Another method of achieving target cell transcriptional control is with the use of microRNAs (miRNAs). These have recently been utilised as a way of preventing the immune system from rejecting newly delivered genes. MicroRNAs (miRNAs) are small non-coding RNA molecules that function to downregulate gene expression. A new gene transfer system that exploits the endogenous miRNA machinery for transgene regulation was used to investigate the efficiency of miRNAs in turning off transgene expression in non-target tissues and cells. Mice were challenged with lentivirus encoding target sequences of endogenous miRNAs (Brown, Venneri et al. 2006). Expression profiles differed significantly from vector biodistribution as

determined by Q-PCR. The target sequences were effectively suppressed in haematopoietic lineages, but maintained in non-haematopoietic cells. Prevention of expression in haematopoietic cells was greater than that achieved with the hepatocyte-specific albumin promoter. In this system, the incorporation of miRNA regulation into a vector was shown to provide a level of control over transgene expression, showing the potential of microRNAs on gene regulation (Brown, Venneri et al. 2006).

1.10 Renin Angiotensin System

The renin angiotensin system (RAS) was first recognised in 1898 with the discovery of renin by Tigerstedt and Bergman (Tigerstedt and Bergman 1898). It has been studied for more than a century, yet continually new discoveries are being made regarding the importance of the RAS in the pathophysiology of cardiovascular and renal disease. In the last few years, new peptides, receptors and enzymes have been found, additional functions have been assigned to existing components of the RAS and alternative pathways of angiotensin (Ang) II generation have been found. Recent studies have added to the knowledge of the ever expanding RAS in both normal and diseased states. It is now known that there are two RAS systems; the classic systemically acting system and a more recently identified and highly complex tissue-localized system. Classically, the RAS can be described as an endocrine system that is a central regulator of cardiovascular, renal and adrenal physiology and haemastasis. It is a circulating co-ordinated hormonal cascade that is activated by a loss in blood volume or in response to a change in arterial pressure. Many of the components of the RAS have opposing functions helping to serve the main aim of the RAS in maintaining a strict homeostatic balance. This pathway is of major clinical importance. Overactivity of the RAS is associated with the pathophysiology of hypertension and progression of heart failure, and it thus a major target for therapy. Inhibition of components of the RAS, such as angiotensin converting enzyme (ACE), has been shown to markedly decrease blood pressure in hypertensives and exert beneficial effects on cardiac function and survival in cardiovascular and renal disease patients. Accordingly, knockout mice of components of the RAS, including ACE (Krege, John et al. 1995), renin (Yanai, Saito et al. 2000) and angiotensin receptor 1a homologue (AT_{1A}) (Oliverio, Best et al. 1997) have been generated and all display hypotensive tendencies with decreased blood pressure and compromised kidney

function compared to controls, indicating that these are key peptides in the control of this system. ACE inhibitors are used to treat hypertension worldwide, showing the enormous potential in modulating and controlling this system.

At the beginning of this coordinated cascade lies angiotensinogen, which is a circulating precursor protein produced by the liver (Figure 1.5). It provides the majority of systemic circulating angiotensin peptides, although it is also present in other tissues including the heart, vasculature, kidneys and adipose tissue (Carey and Siragy 2003). Angiotensinogen plays a role in human blood pressure and overactivity of the gene is associated with the development of hypertension (Menard, el Amrani et al. 1991; Jeunemaitre, Soubrier et al. 1992). Delivery of rAAV vectors expressing the angiotensinogen antisense gene led to a delayed onset of hypertension and to the significant attenuation of hypertension in adulthood in the SHR (Kimura, Mohuczy et al. 2001).

In response to volume and renal perfusion pressure changes detected by the juxtaglomerular apparatus as the blood enters the kidneys, the glycoprotein enzyme renin is secreted by the juxtaglomerular cells at the renal afferent arterioles. Renin cleaves the Leu-Val peptide bond at the N-terminus of angiotensinogen thus creating the decapeptide angiotensin I (Ang I). The cascade continues with physiologically inactive Ang I then being catalyzed to biologically active octapeptide angiotensin II (Ang II) by the cleavage of a dipeptide at the C-terminus (His-Leu) by endothelium-bound angiotensin converting enzyme (ACE) (Skeggs, Kahn et al. 1956). ACE is a glycoprotein that possesses two active carboxy-terminal sites and is also responsible for metabolising the vasodilator bradykinin (Yang, Erdos et al. 1970). It therefore increases the levels of the potent vasoconstrictor Ang II whilst limiting the presence of vasodilators. Angiotensin II is cleaved further by a variety of enzymes to produce the bioactive angiotensin fragments angiotensin III (Ang 2–8), angiotensin IV (Ang 3–8) and angiotensin-(1–7).

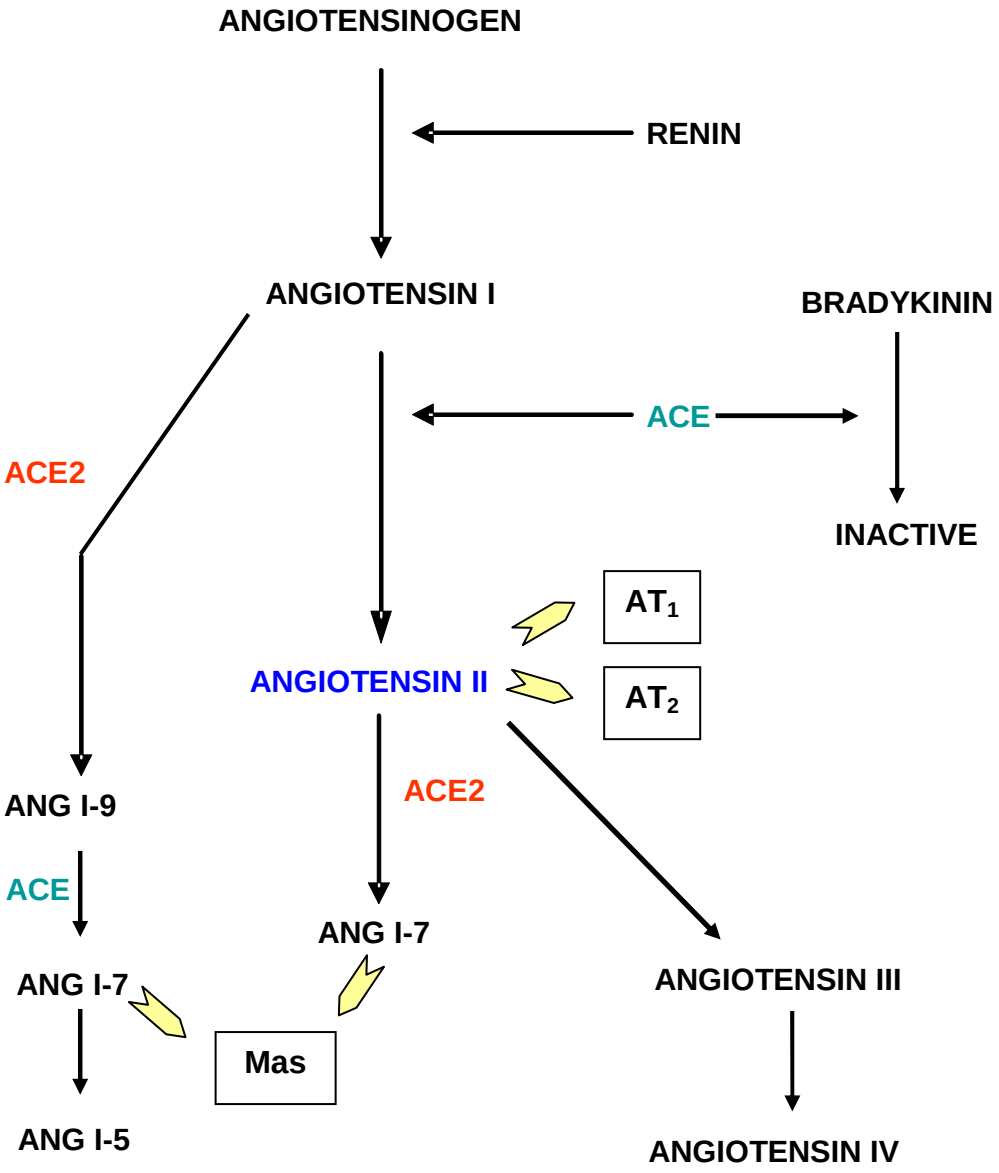

Figure 1.5 Overview of the renin angiotensin system.

Depiction of the RAS pathway, demonstrating how the peptide components interact with one another. AT_1, angiotensin type 1 receptor; AT_2, angiotensin type 2 receptor.

1.10.1 Cardiac RAS

The traditional concept of the RAS has had to be expanded to incorporate the increasing clinical and experimental evidence that strongly supports the existence of a local functioning cardiac RAS. It has been shown in recent years that many RAS components are present in different tissues, including the brain, kidney, heart and the vasculature, that could not be explained by the endocrine system (Paul, Poyan Mehr et al. 2006). Also, it was reasoned that a local RAS must exist due to the fact that inhibitors of the components of the RAS have beneficial effects beyond those on blood pressure. Thus it is accepted that the RAS is not just an endocrine system, but also an autocrine/paracrine system. Every component of the RAS has been identified in cardiac and vascular myocytes and fibroblasts. Generation of Ang I and Ang II is not restricted to the systemic circulation; production also takes place in the vasculature and other tissues.

1.10.2 Angiotensin II (Ang II)

Ang II is the principal physiological effector molecule in this pathway and exerts a plethora of effects throughout the body, being a potent vasoconstrictor. Its role in cardiovascular disease is well documented and its actions can result in cardiovascular damage. Ang II has a very short half life and is degraded within seconds into fragments, mainly des-aspartyl-Ang III (Ang III), Ang 1–7, and Ang IV. Ang II is transported to peripheral tissues and its actions are mediated through two G-protein-coupled receptors, angiotensin type 1 (AT_1) and angiotensin type 2 (AT_2) receptors (de Gasparo, Catt et al. 2000; Boehm and Nabel 2002). Ang II mediates the majority of its cardiac, renal and adrenal function by the activation the AT_1 receptor. The role of the AT_2 receptor is less well defined; evidence suggests that it generally opposes effects mediated by the AT_1 receptor through activating vasodilatory and anti-proliferative effects (Dinh, Frauman et al. 2001; Duke, Evans et al. 2005). Stimulation of the AT_2 receptor results in the activation of number of vasodilatory peptides, including bradykinin, NO and guanosine cyclic 3', 5' monophosphate (cGMP). The AT_2 receptor is expressed at lower levels in cardiovascular tissues than the AT_1 receptor, offering an explanation for the preference of Ang II for the AT_1 receptor. Gene transfer techniques have been exploited to investigate the role of the AT_2 receptor in the RAS. AT_2 receptor expressing lentivirus vectors (Metcalfe,

Huentelman et al. 2004) and Ad-directed AT_2 receptor overexpression (D'Amore, Black et al. 2005) have been utilised, with both approaches showing conflicting results. Whilst the lentivirus overexpression study demonstrated a preventative role for the AT_2 receptor in cardiac hypertrophy (Metcalfe, Huentelman et al. 2004), Ad-mediated AT_2 receptor overexpression resulted in cardiomyocyte hypertrophy (D'Amore, Black et al. 2005).

The deleterious effects on the cardiovascular system associated with Ang II including vasoconstriction and the promotion of cellular growth are mediated by the AT_1 receptor (Unger, Culman et al. 1998; Perazella and Setaro 2003). The effects of this include the modulation of blood pressure through vasoconstriction and sodium retention. These effects have been shown to be prevented by rAAV-mediated delivery of antisense AT_1 receptor gene, in which the development of hypertension (Phillips 1997), renal injury and cardiac remodelling was shown to be inhibited (Li, Yan et al. 2007). Ang II acts on the vascular smooth muscle cells in the walls of arterioles causing them to constrict and forcing the blood to be pumped harder through this narrowed space thus increasing blood pressure. Furthermore, Ang II can also indirectly regulate blood pressure by stimulating the adrenal cortex to produce and release the hormone aldosterone from the zona glomerulosa. Aldosterone enhances sodium conservation by the kidneys and gastrointestinal tract, which in turn induces import of chloride ions and most importantly water. It also encourages the loss of potassium and magnesium and can also stimulate thirst. As more water is retained in the blood, there is a higher circulating volume, resulting in pressure being maintained. So the RAS has a two-fold effect at raising and maintaining a high blood pressure.

Ang II is more than just a vasoconstrictor and has been shown to have a role in the activation of angiogenic and fibrotic cytokines, as well as in the promotion of macrophage recruitment and infiltration. It also causes ventricular hypertrophy and remodelling of the cardiac wall. Whilst hypertrophy is initially adaptive, progression of hypertrophy is eventually maladaptive and leads to heart failure. Thus it is vital that levels of Ang II in the circulation are well regulated.

Inhibition of components of the RAS has proven undoubtedly beneficially therapeutic in the management of a number of diseases, including hypertension and heart failure,

highlighting that overactivity of the RAS is associated with disease. ACE inhibitors (ACEi) and Ang II receptor blockers (ARBs) are effective drugs for the treatment of cardiovascular diseases and their associated pathophysiologies, probably as a result of blocking the vasoconstrictor, hypertrophic and pro-inflammatory actions of angiotensin II. Interestingly, after AT_1 receptor blockade and ACE inhibition, it was found that levels of ACE2 and Ang 1-7 were increased, demonstrating that these peptides may contribute to the beneficial effects or RAS blockade (Chappell, Pirro et al. 1998; Iyer, Ferrario et al. 1998; Igase, Strawn et al. 2005).

1.10.3 Angiotensin 1-7 (Ang 1-7)

Ang 1-7 can be produced though several pathways. It can be synthesised directly from Ang I by endopeptidase NEP (neutral endopeptidase) (Yamamoto, Chappell et al. 1992) and prolylendopeptidase (PEP) (Welches, Santos et al. 1991). Alternatively, it can be indirectly produced from Ang I through cleavage by ACE2 into an Ang 1-9 intermediate (Donoghue, Hsieh et al. 2000). This intermediate can then be further degraded into Ang 1-7 by the actions of ACE. Ang 1-7 can also be produced both directly and indirectly from Ang II. ACE2 hydrolyses Ang II with high efficiency to produce Ang 1-7, displaying a 400-fold higher catalytic efficiency with Ang II as a substrate than with Ang I (Vickers, Hales et al. 2002). Finally, Ang 1-7 can be formed from Ang II by PEP and prolylcarboxypeptidase (PCP).

The G-protein coupled receptor mas has been identified as the receptor for Ang 1-7 (Santos, Simoes e Silva et al. 2003). Ang 1-7, acting though the mas receptor, is seen to produce effects that are opposite to those described for Ang II (Ferrario, Chappell et al. 1997). Levels of Ang 1-7 are increased in failing human heart ventricles, indicating that it may play a cardioprotective role in heart failure (Zisman, Keller et al. 2003). This is supported by the finding that Ang 1-7 reduced the incidence of arrhythmias after induced myocardial injury in isolated rat hearts (Ferreira, Santos et al. 2001). Ang 1-7 has additionally been shown to induce diuresis, to enhance the vasodilatory actions of bradykinin (Paula, Lima et al. 1995; Fernandes, Fortes et al. 2001) and to exert antiproliferative actions (Strawn, Ferrario et al. 1999). All these actions point to a role for Ang 1-7 in the counterregulatory arm within the RAS.

1.10.4 Angiotensin converting enzyme 2 (ACE2)

ACE2 is a member of the M2 zinc metalloproteinase family and a recently identified homologue of ACE with their catalytic domains sharing a 42% amino acid identity (Soubrier, Alhenc-Gelas et al. 1988; Ehlers and Riordan 1991; Tipnis, Hooper et al. 2000). Both ACE and ACE2 are highly expressed in vascular endothelial cells, however ACE2 expression is thought to be tissue specific and restricted to the heart, kidney and testis (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000). However, recent work has revealed ACE2 is abundantly present in humans in the epithelia of the lung and small intestine, thought to be associated with possible routes of entry for the severe acute respiratory syndrome-associated coronavirus (SARS-CoV), as ACE2 has been identified as the functional receptor for SARS-CoV (Hamming, Timens et al. 2004). Unlike ACE, ACE2 functions as a carboxypeptidase rather than a dipeptidyl carboxypeptidase (Donoghue, Hsieh et al. 2000) and is thought to counterbalance the vasopressor effects of ACE (Vickers, Hales et al. 2002). This transmembrane protein has been found to be insensitive to ACE inhibitors such as captopril and lisinopril *in vitro* (Tipnis, Hooper et al. 2000), showing that ACE2 has a distinct substrate and inhibitor specificity from ACE.

ACE2 has been proposed as a critical component of the RAS, thought to elicit cardioprotective effects by balancing the plethora of negative effects that Ang II exerts on the cardiovascular system (Ferrario, Chappell et al. 1997; Ferrario 1998). ACE2's role in the RAS is thought to lie in the inhibition of Ang II production, through two differing pathways. ACE2 primarily hydrolyses Ang II and less efficiently Ang I, (Tipnis, Hooper et al. 2000) resulting in Ang 1-9 and Ang 1-7 production. Ang 1-9, which itself is of unknown function, is further hydrolysed to Ang 1-7 by the actions of ACE (Donoghue, Hsieh et al. 2000). Ang 1-7 has been shown to have vasodilatory effects and it has been suggested that it acts through the G-coupled protein receptor *mas*. Thus, ACE2 may play a pivotal role in the RAS by reducing concentrations of the pro-fibrotic, pro-proliferative vasoconstrictor Ang II and raising levels of the anti-fibrotic, anti-proliferative vasodilatory peptide Ang 1-7 (Ferrario, Chappell et al. 1997; Ferrario 1998) As such, manipulation of ACE2 expression and function has potential utility in the treatment of cardiovascular disease. However, the role of ACE2 in the RAS remains ambiguous, with studies into its

interactions generating conflicting results. In two ACE2 knockout mice studies, one showed that these mice had severe cardiac contractility defects (Crackower, Sarao et al. 2002), whilst the other study found no effects on cardiac dimensions (Gurley, Allred et al. 2006). One possible reason for these discrpencies could lie in the genetic backround of these mice. In studies to investigate ACE2 overexpression, ACE2 transgenic mice were found to display a high incidence of sudden death (Donoghue, Wakimoto et al. 2003), whilst lentivirus-mediated ACE2 overexpression provided cardioprotective effects (Diez-Freire, Vazquez et al. 2006). And so the role of ACE2 is complex and remains ambiguous.

ACE2 also metabolises, with high specificity, a range of biologically active peptides other than peptide mediators of the RAS. The hydrolytic activity of ACE2 was tested against a panel of 126 biological peptides. Of these, 11 were found to be hydrolysed by ACE2 and with high affinity for 3 of the peptides (Vickers, Hales et al. 2002). Ang II, apelin-13 and dynorphin A 1-13 were hydrolysed with highest affinity. ACE2 is known to also hydrolyse Ang I, des-Arg^{9}-bradykinin, neurotensin 1-13, kinetensin (Donoghue, Hsieh et al. 2000), β-Casomorphin, Neocasomorphin and Apelin-36 amongst others (Vickers, Hales et al. 2002). Apelin-13 and -36 have both been shown to exert cardioprotective effects *in vivo*. In rodent models of myocardial ischemia-reperfusion (I/R) injury, apelin-13 and apelin-36 reduced infarct size by 43% and 33% respectively demonstrating cardioprotective activities (Simpkin, Yellon et al. 2007). Not much is known about the role of neocasomorphin or β-Casomorphin, yet derivatives of the latter have been implicated in vasorelaxation (Fujita, Suganuma et al. 1996). The hydrolysis of these peptides by ACE2 *in vivo* remains to be established, but may prove to be relevant and play a vital role in both the regulation of the RAS and in the pathophysiology of disease.

1.11 Aims of Thesis

The principle aim of this thesis was to assess the effect of ACE2 overexpression *in vivo* on heart function and blood pressure in relevant disease model, the stroke prone spontaneously hypertensive rat (SHRSP). In order to achieve cardiac overexpression of ACE2 we first had to identify a suitable gene delivery vector. This was achieved through the following techniques:

- The evaluation both *in vitro* and *in vivo* of candidate heart targeting peptides identified through phage display in which the heart vasculature was probed for heart-specific endothelial markers.

- The peptides were used to modify Ad5, Ad19p and AAV2 vectors to assess if they increased the selectivity of these vectors to endothelial cells of the vasculature.

- Comparison and optimisation of rAAV6 and rAAV9 vector-mediated gene delivery to the myocardium *in vivo* in SHRSP.

- Incorporation of cardiac-specific promoter MLC-2v into rAAV6 vectors to assess transcriptional regulation.

- Evaluation of ACE2 overexpression using rAAV6.

Materials and Methods

2.1. Chemicals

All chemicals unless otherwise stated were obtained from Sigma Chemical Company (Poole, UK) and were of the highest grade obtainable. All oligonucleotides were obtained from MWG-Biotech (Edersberg, Germany).

2.2. Cell Culture

All tissue culture work was performed using a biological safety class II vertical laminar flow cabinet in sterile conditions. Cell lines were maintained in the appropriate cell culture media (Table 2.1) and incubated at 37°C in a 5% CO_2 atmosphere.

2.2.1. Maintenance of established cell lines

Cells were grown as a monolayer and media was replenished every 3 days. Cells were routinely passaged at approximately 80% confluence to prevent overgrowth and loss of surface contact. To passage, cells were washed twice in phosphate buffered saline (PBS) (Biowhittaker, Berkshire, UK) and incubated in a minimal volume of trypsin-EDTA at 37ºC, 5% CO_2 (Gibco, Invitrogen, Paisley, UK) for approximately 5 minutes, until the majority of cells had detached. The action of trypsin-EDTA was blocked by the addition of an equal volume of media containing serum. Cells were harvested by centrifugation at 480 × g and resuspended in fresh media for passaging or plating. For plating cells were counted in a haemocytometer to enable them to be seeded at the required density.

2.2.2. Cryo-preservation and resuscitation of cell lines

Cells were harvested as described in section 2.2.1 and re-suspended at a density of approximately $1\text{-}2\times10^6$ cells/ml in complete cell culture media supplemented with

10% (v/v) dimethyl sulphoxide (DMSO). Cell suspensions were aliquoted into sterile 2 ml

Cell type	Description	Cell culture medium used
HCAEC	Primary human coronary artery endothelial cells	Endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 1% (v/v) penicillin and 100 µg/ml streptomycin (both Gibco, Paisley, UK).
293T	Transformed human embryonic kidney cell line	Minimum Essential Media (MEM) (BioWhittaker, UK) supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Gibco, Paisley, UK)
Cos7	African green monkey SV40 transformed kidney fibroblasts	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
HeLa	Human cervical carcinoma cell line	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
L6	Rat skeletal muscle myoblasts	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
HT1080	Human fibrosarcoma cells	Dulbecco's minimum essential media (DMEM) with glutamax-1, 4500mg/L glucose, 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 4 mM L-glutamine, 1.5 mM xanthine, 0.016mM mycophenolic acid and 50x HT supplement (all Gibco, Paisley, UK).
HepG2	Human hepatocellular carcinoma cell line	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
RGE	Rat glomerular endothelial cell line	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
H9C2	Rat heart myoblasts	MEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
633	Derived from A549 cells. Inducibly express Ad5 E1A and constitutively express the Ad5 fiber protein	DMEM with glutamax-1, 4500mg/L glucose, 10% (v/v) FCS, 1% (v/v) penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, 200 µg/ml hygromycin B, 300 µg/ml zeocin and 200 µg/ml neomycin sulphate (all Gibco, Paisley, UK).

Table 2.1 Media used to culture cells used in this study.

cryo-preservation vials and cooled at a constant -1°C/minute to -80°C using isopropanol. Vials were then stored indefinitely in liquid nitrogen. Cryo-preserved cells were resuscitated by thawing at 37°C and then transferring them drop by drop to a T-25 flask containing 5ml of pre-warmed cell culture media and incubated overnight at 37°C. The following day they were placed in fresh media.

2.3. Animal Models

All animals were housed under controlled environmental conditions. Temperature was maintained at ambient temperature with 12 h light/dark cycles. Rats were fed standard rat chow (rat and mouse No.1 maintenance diet, Special Diet Services) and water provided *ad libitum*.

Work with experimental animals was in accordance with the Animals Scientific Procedures Act 1986 under the project license held by Professor A.F. Dominiczak, License Number 60/2874. Inbred colonies of stroke-prone spontaneously hypertensive (SHRSP) and Wistar Kyoto (WKY) strains were maintained "in-house" by brother, sister mating and routine in lab microsatellite screening was used to confirm homozygosity of all loci within a random group from each strain. WKY rats for the phage studies were obtained from Harlan, Oxfordshire, UK.

2.4. Phage methods

All phage experiments were carried out using the T7Select415-1b phage display system (Novagen, EMD Biosciences, Darmstadt, Germany), which use the T7 capsid protein to display peptides on the surface of the phage, and can display in high copy numbers (415 per phage). All candidate peptide-phage and a non-recombinant control phage (with no peptide insertion) were obtained as a kind gift from Professor E. Ruoslahti, Burnham Institute, USA. Candidate peptides (Table 2.2) had been isolated by 3 rounds of selection on *ex vivo* murine heart cells, followed by 3 rounds of selection *in vivo* (Zhang, Hoffman et al. 2005). All phage amplification and titering was performed using *E.coli* strain BL21 (Novagen, EMD Biosciences, Darmstadt, Germany).

2.4.1. Amplification of phage

Phage were amplified using the liquid lysate amplification protocol as described in the Novagen T7 select system manual. Briefly, a single BL21 colony was picked from a freshly streaked plate and amplified overnight in 25 ml M9LB (1.25 ml 20× M9 salts, 0.5 ml 20% glucose, 25 µl 1M $MgSO_4$, 25 ml LB), in an orbital shaker at 37°C and 180 rpm. Five ml of overnight culture was added to 500 ml LB in a 2 L flask, and incubated for approximately 3 hours at 37°C in an orbital shaker at 180 rpm. The absorbance at 600 nm was read at intervals and the culture was grown until OD_{600} 0.5-1.0. Separately, 100 µl phage was added to 25 ml of culture and grown until lysis had occurred. This was then added to the 500 ml culture and was incubated with shaking at 37°C for between 1-3 hours, until cell lysis occurred. This was determined visually when the solution appeared clear but contained long thin strands and was also confirmed by a decrease in the OD_{600}. Ten percent (v/v) 5 M NaCl was added to the culture, which was then centrifuged at 8000 × g for 10 minutes to remove cell debris. The supernatant was transferred to a sterile bottle for Polyethylene glycol (PEG-8000) precipitation.

2.4.2. Purification of phage

1/6 volume of 50% (v/v) PEG-8000 was added to the supernatant and thoroughly mixed before incubating at 4°C overnight. The supernatant was then centrifuged at 3185 × g for 30 minutes at 4°C. The pellet was resuspended in 2 ml TBS and transferred to microcentrifuge tubes. To the phage, 1/6 volume of 20% PEG-8000/2.5M NaCl was added and then left overnight at 4°C, then centrifuged for 30 minutes at 12600 × g and 4°C. The pellet was resuspended in 1 ml TBS and left on ice for 1 hour before centrifugation for 10 minutes at 12600 x g and 4°C. The pellet was then resuspended in 1 ml 0.02% sodium azide and left at room temperature for 20 minutes before a further centrifugation of 10 minutes at 12600 × g and 4°C. Supernatants were then pooled and stored. For short-term storage, phage were kept at 4°C. For longer term storage 10% (v/v) sterile 80% (v/v) glycerol was added to the phage, which was stored at -80°C.

2.4.3. Quantification of phage by plaque assay (titering)

A single BL21 colony was picked from a freshly streaked plate and amplified overnight in 25 ml LB, supplemented with 5 ml M9 salts (20 g/L NH_4Cl, 60 g/L KH_2PO_4, 120 g/L $NaH_2PO_4.7H_2O$), 2 ml 20% (v/v) glucose, 0.1 ml 1 M $MgSO_4$, in an orbital shaker at 37°C and 180 rpm. LB plates were pre-warmed in a 37°C incubator. Serial dilutions of phage were made in LB. Agar top (10 g/L bactotryptone, 5 g/L yeast extract, 1 g/L $MgCl._6H_20$, 7 g/L agarose) was melted and 3 ml aliquots were made and placed in a 50°C waterbath. 250 µl of BL21 culture was added to 100 µl of each dilution of phage. This was then added to an aliquot of agar top and poured onto an LB plate. Once set, plates were inverted and placed in a 37°C incubator for 3 hours or at room temperature overnight. The following day, the number of plaques present at each dilution was counted and used to calculate an average titre for the phage stock using the formula:

Phage titre (pfu)/ml) = Number of plaques × dilution factor × 10

2.4.4. Sequencing of phage peptides

Individual plaques were picked from the agar using a glass pipette. Each plaque was placed in 250 µl BL21 liquid culture grown overnight (as for phage amplification). This was incubated in an orbital shaker at 37°C with shaking at 180 rpm for approximately 3 hours, until bacterial lysis occurred. Five µl of the lysate was used as a template for PCR of the peptide insertion region of the 10B gene using the primers T7 Super Up (5'-AGCGGACCAGATTATCGCTA-3') and T7 down (5'-AACCCCTCAAGACCCGTTTA-3'). Each PCR contained 200 µM each dNTP (Promega, Southampton, UK.), 1.25 U Taq DNA polymerase (Promega, Southampton, UK.) and 0.125 µM each primer in 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton X-100. The reactions were subjected to 40 cycles of denaturing at 94°C for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 1 minute. PCR products were analysed by agarose gel electrophoresis. PCR products were cleaned using AmpPure (Agencourt Bioscience Corporation, MA, USA) as per manufacturer's instructions. Purified PCR products were resuspended in 40 µl water and 10 µl used in the sequencing reaction.

Sequencing reactions contained 3.2 pmoles primer, 0.5 µl v3.1 Ready Reaction mix (Applied Biosystems, MA, USA), 4 µl v3.1 sequencing buffer (Applied Biosystems, MA, USA) in a 20 µl reaction. The cycle conditions were denaturing at 96°C for 50 seconds, annealing at 50°C for 20 seconds and extension at 60°C for 3 minutes, for 25 cycles. Sequencing products were cleaned using CleanSEQ (Agencourt Bioscience Corporation, MA, USA) as per manufacturer's instructions. Results were analysed on the ABI 3730 automated sequencer using SeqScape v2.0.

2.4.5. In vitro biopanning

A 6-well plate was placed at 4ºC for 10 minutes prior to two PBS washes. 1 ml of biopanning media (DMEM supplemented with 1% (w/v) BSA) was added to each well, along with 1×10^9 pfu of relevant phage. The plate was then incubated for 1 hour at 4ºC and then washed 5 times in PBS with 1% (w/v) BSA, for 5 minutes per wash. 1 ml 0.2 M glycine (pH 2.2) was added to the wells and then left on ice for 10 minutes before the addition of 200 µl 1M Tris. This solution was removed, discarded and replaced with 1 ml Tris/EDTA. Cell were then scraped and left to lyse for 1 hour on ice. After centrifugation at 10,000 × g for 2 minutes, the supernatant was kept for titration.

2.4.6. In vivo phage work

In vivo phage work was performed in male 12 week old Harlan WKY rats. Rats were anesthetised by halothane (4%). All animals received 5×10^{10} pfu phage via femoral vein injection which was left to circulate for 15 minutes. Blood samples were taken by cardiac puncture before rats were perfused through the heart with heparinised saline, and organs were removed and stored on ice before snap freezing.

2.4.6.1. Extraction of phage from tissues

Phage were extracted from approximately 250 mg of tissue. Tissues were placed in a fast RNA green biopulveriser lysis matrix tube (Qbiogene, CA, USA) containing 500 µl ice cold DMEM-PI (DMEM supplemented with 1% (w/v) BSA, 1 mM PMSF, 1 µg/ml leupeptin, 2 µg/ml aprotinin). The tissues were homogenised in a Fastprep system (Qbiogene) by performing six 45 second runs at a speed of 5.5. Samples were

placed on ice every two runs to prevent overheating. The homogenate was removed and an additional 500 µl DMEM-PI was added to the tube. An additional 2 runs were performed, and then the buffer was pooled with the first aliquot. A further 2 runs were performed with another 500 µl buffer. To each homogenate 100 µl 1% non-ident P40 (NP40) was added and incubated on ice for 5 minutes. This was followed by the addition of 30 µl 1% sodium azide, which acts as a preservative to help prevent the growth of bacteria. Samples were stored at 4°C for up to 5 days before titering was performed.

2.5. Lipofectamine Transfection

Lipofectamine™ 2000 (Invitrogen) is a proprietary cationic lipid formulation that allows the transfection of nucleic acids into eukaryotic cells for the expression of protein.

Cells were seeded at an optimised density of 3×10^5 cells/well (6-well plate) and left for 18 hours. This was to achieve an optimum confluence of 85-90%. A DNA (µg) to Lipofectamine™ 2000 (µl) ratio of 1:1 was normally used, but was optimised for each cell line. 250 µl of pre-warmed Opti-MEM® I Reduced Serum Medium (Invitrogen, UK) was added to an eppendorf tube and 3 µl of Lipofectamine™ 2000 added to this. To a further 250 µl of Opti-MEM® I was added 3 µg of DNA in an eppendorf tube. The contents of the DNA tube were then combined with the lipofectamine tube and mixed well by tapping. After 20 minute incubation at room temperature, the complexes were added one drop at a time to the cells, whose medium had meanwhile been replaced with 1 ml Opti-MEM® I Reduced Serum Medium. Cells were incubated for 6 hours at 37°C and then 2 ml serum-containing medium added to the well. Overnight incubation was followed by fresh medium being added to the wells and then left for a further 24 hours.

2.6. Caesium chloride preparation of DNA

After overnight growth of culture and centrifugation, the resultant bacterial pellet was lysed with 25 ml ice-cold lysis solution (0.025 M tris pH 8, 0.01 M EDTA, 0.05 M glucose) for 30 minutes at 4ºC. The lysate was cleared with 50 ml room temperature

alkaline SDS (0.2M NaOH, 2.5 ml 20% w/v SDS) for 5 minutes at 4ºC. 30 ml potassium acetate solution (3M potassium acetate, 3.45 ml glacial acetic acid) was added to precipitate chromosomal DNA and left at 4ºC for 15 minutes. Centrifugation was performed at 9794 × g, for 10 minutes at 4ºC and the supernatant decanted through a double layer of gauze into a fresh sterile bug pot containing 60 ml isopropanol. Care was taken to avoid contamination with the precipitate. The bug pot was placed at -20 ºC for 1 hour before centrifugation at 9794 × g for 10 minutes at 4ºC to pellet plasmid DNA. The supernatant was discarded and the pellet resuspended in 4 ml of 1 × TE (pH 8). The plasmid DNA solution was added to a Falcon tube containing 5g CsCl and 300 µl ethidium bromide (10 mg/ml stock) and placed on a roller mixer for 15 minutes. The tube was centrifuged at 1985 × g for 10 minutes at room temperature. The supernatant was removed and the refractive index determined using a refractometer and adjusted to 1.396-1.398 using either saturated CsCl or topping up solution (5g CsCl, 4 ml 1× TE, 300 µl EtBr). The solution was placed in 13.5 ml ultracentrifuge tubes and tube lids heat sealed. Tubes were centrifuged at 308426 × g at 16 ºC, for 18 hours using an optima™ L-80 XP ultracentrifuge (Beckman Coulter, UK). Supercoiled plasmid DNA was carefully removed by piercing the tube. To remove the EtBr, an equal volume of isopropanol was added and mixed gently. After settling, the upper organic layer was discarded and the original volume made up using 1 × TE. A further 5 isopropanol washes were carried out. The plasmid solution was dialysed in a collodion bag (Sartorius AG, Germany) against 5 L of 1 × TE at 4 ºC overnight.

2.7. Production of adenoviruses

2.7.1. Production of recombinant Ad5

High titre stocks of recombinant Ad5 were produced by large-scale amplification of a plaque pure stock of Ad5 in 293 cells. Low passage 293 cells were grown to 80% confluence then infected with a multiplicity of infection (MOI) of approximately 1 plaque forming unit (pfu)/cell. The media was changed every 3 days until the cytopathic effect of the Ad caused the cells to detach from the flask. Cells were then fed by adding 10 ml media to each flask until the majority of cells had detached. Cells were harvested by centrifugation at 850 ×g for 10 minutes at room temperature. The

pellet was resuspended in 10 ml of PBS and an equal volume of ArkloneP (trichlorotrifluoroethane). The tube was inverted for 10 seconds then gently shaken for 5 seconds so the solutions were mixed without vigorous shaking (as this results in detachment of the Ad fiber from the capsid). The mixing was repeated. The suspension was centrifuged at 850 × g for 15 minutes at room temperature. The upper aqueous layer containing the virus was removed. An additional 10 ml of PBS was added to the remaining solvent layers and the process was repeated. The aqueous layers were pooled and stored at –80°C until purified on a CsCl gradient.

2.7.2. Production of fiber gene deleted Ad with the lac Z transgene (Ad5.lacZ.ΔF)

633 cells were maintained in selective media (Table 2.1) until they were expanded into 20 × T-150 flasks, where they were cultured in MEM supplemented with 10% (v/v) foetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Twenty-four hours before cells were infected 0.3 μM dexamethasone was added to the media. Cells were infected with fiber gene deleted Ad5 (MOI 2000 virus particles (vp) /cell) when they were approximately 80% confluent. When the cytopathic effect had caused the majority of cells to detach, the cells were harvested by centrifugation at 850 × g for 10 minutes at room temperature. The pellet was resuspended in 10 ml of PBS. Virus was released from cells by three rapid freeze/thaw cycles and cellular debris was removed by centrifugation at 850 × g for 15 minutes at room temperature. The supernatant was stored at -80°C until it was purified on a CsCl gradient.

2.7.3. Cloning of peptides into Ad5 and Ad5/Ad19p fiber genes

Oligonucleotides (Table 2.2) were obtained from MWG-Biotech (Edersberg, Germany). Overlapping oligonucleotides encoding the peptides flanked by the *Bsp*E1 restriction site were used for cloning peptides into the Ad5KO1 vector. These overlaps were not required for Ad19p fiber as blunt ligation was used. Oligos were annealed by mixing 1 μM of each oligo in 100 μl reaction and heating to 98°C for 10 minutes, then cooling to 50ºC over 1 hour.

Virus	Plasmid	Peptide	Sequence of oligo encoding the vascular targeting peptide
Ad Control (AdCTL)	pDV111 (Nicklin *et al*,. 2001c)	None	None
AdKO1	pDV137 (Nicklin *et al*,. 2001c)	CRPPR	5'–CCGGATGCCGTCCGCCTCGGG–3'
			3'–TACGGCAGGCGGAGCCCGGCC–5'
		CGRKSKTVC	5'–CCGGATGCGGTCGGAAGTCGAAGACGGTTTGCG–3'
			3'–TACGCCAGCCTTCAGCTTCTGCCAAACGCGGCC–5'
		CARPAR	5'–CCGGATGCGCGCGTCCTGCTCGTG–3'
			3'–TACGCGCGCAGGACGAGCACGGCC–5'
		CPKRPR	5'–CCGGATGCCCTAAGCGTCCGCGGG–3'
			3'–TACGGGATTCGCAGGCGCCCGGCC–5'
		CRNSWKPNC	5'–CCGGATGCCGAAACTCGTGGAAGCCTAATTGCG–3'
			3'–TACGGCTTTGAGCACCTTCGGATTAACGCGGCC–5'
		CRSTRANPC	5'–CCGGATGCCGTAGTACTCGTGCTAATCCTTGCG–3'
			3'–TACGGCATCATGAGCACGATTCGGAACGCGGCC–5'
		CPKTRRVPC	5'–CCGGATGCCGTAGTACTCGTGCTAATCCTTGCG–3'
			3'–TACGGGATTCTGCGCAGCTCAAGGAACGCGGCC–5'
		CSGMARTKC	5'–CCGGATGCTCTGGTATGGCTCGTACTAAGTGCG–3'
			3'–TACGAGACCATACCGAGCATGATTCACGCGGCC–5'
Ad5/19p	pDV145 mod (Denby *et al*., 2007)	CRSTRANPC	5'–TGCCGTAGTACTCGTGCTAATCCTTGC–3'
			3'–ACGGCATCATGAGCACGATTAGGAACG–5'
		CSGMARTKC	5'–TGCTCTGGTATGGCTCGTACTAAGTGC–3'
			3'–ACGAGACCATACCGAGCATGATTCACG –5'
		CPKTRRVPC	5'–TGCCCTAAGACGCGTCGAGTTCCTTGC–3'
			3'–ACGGGATTCTGCGCAGCTCAAGGAAGC–5'
		CRPPR	5'–GTGCCGTCCGCCTCGG–3'
			3'–CACGGCAGGCGGCGCC–5'
		CRPPR-mod	5'–GGATCGTCGTGCCGTCCGCCTCGGGGATCGTCG–3'
			3'–CCTAGCAGCACGGCAGGCGGAGCCCCTAGCAGC
		3CRPPR	5'TGCCGTCCGCCTCGGGGAGGAGGATCGTGCCGTCCGCCT CGGGGAGGAGGATCGTGCCGTCCGCCTCGG–3'
			3'ACGGCAGGCGGAGCCCCTCCTCCTAGCACGGCAGGCGG AGCCCCTCCTCCTAGCACGGCAGGCGGAGCC'–5'

Table 2.2 Plasmids used to produce Ad vectors and the sequences of oligos encoding the vascular targeting peptides.

Fifty µg of each plasmid (pDV147, pDV111 or pDV137) was digested overnight at 37°C with 5 U/µl enzyme *Eco47*III (Promega, Southampton, UK) or 10 U/µl *Bsp*E1 (New England BioLabs, Hitchin, UK) in a 100µl reaction using enzyme buffer D or buffer 3 respectively.

Dephosphorylation of 2.5 µg of digested plasmid was performed using 5 U shrimp alkaline phosphatase (SAP) (Promega, Southampton, UK) by incubation at 37°C for 15 minutes. SAP was inactivated by incubation at 65°C for 15 minutes. Dephosphorylated plasmid and oligo duplexes were ligated using Quick T4 ligase (New England BioLabs, Hitchin, UK). Twenty ng vector, 1 µM annealed oligo, 1 µl ligase and 10 µl of the supplied buffer were mixed in a total volume of 20 µl and incubated at room temperature for 5 minutes. Ligated plasmids were then transformed into JM109 competent *E. coli* (Promega, Southampton, UK) using a standard heat shock protocol. Briefly, 10 µl of the ligation reaction was incubated with 50 µl competent cells on ice for 30 minutes. The reaction was placed in a 42°C water bath for 30 seconds and then placed back on ice for 2 minutes. To the tube was added 950 µl of SOC media (20 g/L bactotryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose), which was then placed in an orbital shaker for 1 hour at 37°C with 180 rpm shaking. One hundred µl of culture was plated onto LB agar plates (10 g/L bactotryptone, 5 g/L bactoyeast extract, 5 g/L NaCl, 15 g/L agar, pH 7.5) supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C.

Several colonies were picked from each plate and amplified overnight in 5ml LB. Plasmid DNA was isolated using the Qiagen plasmid mini preparation kit (QIAGEN Ltd., Crawley, UK) as per manufacturer's instructions. Restriction digestion of individual clones was performed to determine which plasmids contained a single copy of the inserted oligonucleotide duplex. Clones were sequenced to confirm they contained a single copy of the insertion in the correct orientation. Sequencing reactions contained 250 ng plasmid DNA, 3.2 pmoles primer 5 (primer for Ad5/19p plasmids 5'-TCTTTGATTGTGGTCGCAGG-3', primer for the AdKO1 plasmids 5'-CACTTGAGTTGTGTCTCCTCCACC-3'), 1 µl v3.1 Ready Reaction mix (Applied Biosystems, MA, USA), 4 µl v3.1 sequencing buffer (Applied Biosystems, MA, USA) in a 20 µl reaction. The cycle conditions were denaturing at 96°C for 45

seconds, annealing at 50°C for 25 seconds and extension at 60°C for 4 minutes, for 25 cycles. Sequencing products were cleaned using CleanSEQ (Agencourt Bioscience Corporation, MA, USA) as per manufacturer's instructions. Results were analysed on the ABI 3730 automated sequencer and using SeqScape v2.0.

Large scale plasmid DNA preparations of correctly sequenced plasmids were then carried out using the Qiagen Plasmid Maxi Preparation Kit (Qiagen Ltd, Crawley, UK) following the manufacturer's instructions. Briefly, a 200 ml culture was grown overnight in a 2 litre flask in an orbital shaker at 180 rpm and 37°C. Bacteria were harvested by centrifugation at 8000 × g for 10 minutes at 4°C. The cells were lysed by alkaline lysis and centrifuged at 20000 × g for 30 minutes at 4°C to remove cell debris. The supernatant was removed and centrifugation repeated for 15 minutes. The supernatant was applied to a QIAGEN tip to bind the DNA. A medium salt wash (1.0 M NaCl, 50 mM MOPS, pH 7, 15% v/v isopropanol) was used to remove RNA, proteins and low molecular weight impurities DNA was eluted in a high salt buffer (1.25 M NaCl, 50 mM Tris.HCl, pH 8.5, 15% v/v isopropanol). Isopropanol precipitation was used to purify and concentrate the DNA. The plasmid was resuspended in TE buffer (pH 8.0, 10 mM Tris.Cl, pH 8.5) and stored at -20°C. Glycerol stocks of positive colonies were produced by mixing 150 µl sterile glycerol with 850 µl of culture and stored at -80°C.

2.7.3.1. Production of fiber-modified and pseudotyped Ads

Peptide modified viruses were produced using the previously developed transfection/infection protocol where the modified fiber gene is expressed from a plasmid (Von Seggern, Kehler et al. 1998; Jakubczak, Rollence et al. 2001; Nicklin, Von Seggern et al. 2001). All the plasmids express the Ad fiber gene from the CMV IE promoter.

Ad vectors with genetically modified fibers were produced in 293T cells that express the adenovirus E1A gene, which is essential for virus replication. Ten × 10 cm^2 tissue culture Petri dishes (Nunc, Wiesbaden, Germany) of 70% confluent 293T cells (maintained in MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine) were transfected with plasmid expressing

the modified fiber gene using a calcium chloride differential pH method. Briefly cells were washed twice in PBS, and then placed in 4.5 ml DMEM supplemented with 10% (v/v) FCS, 25 mM HEPES pH 7.9. A solution containing 960 µl media (DMEM supplemented with 25 mM HEPES pH 7.1), 48 µl 1M $CaCl_2$, and 21 µg plasmid DNA per plate was made up. This was added slowly while the plate was gently rocked. As a positive control, one plate was transfected with plasmid pMV10 which expresses the *lacZ* gene. Following overnight incubation at 37°C the cells were washed in PBS then placed in 10 ml of the standard cell culture media. The transfection efficiency was assessed using the pMV10 transfected plate. If 70-80% of cells were positive then cells were infected with 2000 vp/cell Ad5.lacZ.ΔF (an E1, E3 and fiber gene deleted first generation adenovirus). When the cytopathic effect had caused the majority of cells to detach, the cells were harvested by centrifugation at 850 × g for 10 minutes at room temperature. The pellet was resuspended in 10 ml of PBS. Cells were lysed by three freeze/thaw cycles. The suspension was centrifuged at 850 × g for 15 minutes at room temperature. The supernatant was stored at -80°C until purified on a CsCl gradient.

2.7.4. Adenovirus purification using CsCl density gradient centrifugation

To purify and concentrate crude Ad stocks, centrifugation on CsCl density gradients was used. Fourteen ml cellulose-nitrate ultra-clear centrifuge tubes (Beckman Coulter Ltd, Buckinghamshire, UK) were sterilised with 70% ethanol and then washed with sterile water. A CsCl gradient was produced by sequentially layering 2 ml of CsCl with a density of 1.45 g/cm^3, 3 ml of CsCl with a density of 1.32 g/cm^3 and 2 ml 40% glycerol. The crude Ad supernatant was overlayed and the tube filled with PBS. The tube was then loaded into a Sorvall Discovery 90 rotor container, placed in the rotor (RPS4OT-859) and centrifuged at 90,000 x g for 1.5 hours at room temperature with maximum acceleration and free deceleration. Following centrifugation a band containing complete virus can be seen. This was removed by piercing the tube below the virus band with a 22 GA needle and drawing off the band in the minimum volume without disrupting the other bands.

Extracted virus was transferred to a Slide-A-Lyzer Dialysis Cassette (MW cut of 10,000) (Perbio Science UK Ltd., Northumberland, UK) for dialysis. The virus was dialysed against 2 L of 0.01 M Tris pH 8 / 0.001 M EDTA for approximately 2 hours then buffer was replaced and the dialysis repeated overnight. The buffer was changed and supplemented with 10% (v/v) glycerol and dialysis was continued for a further 2 hours. The virus was carefully removed from the cassette, aliquoted and stored at -80^{o}C.

2.7.5. Determining Ad virus particle titres

Particle titre of fiber modified Ad vectors is calculated based on the protein content of the virus stock using the Micro BCA (bicinchoninic acid) assay kit (Pierce, Rockford, IL, USA). Briefly, 8 bovine serum albumin (BSA) standards ranging from 200 μg/ml to 0.5 μg/ml were prepared and 150 μl of each was pipetted in duplicate into a 96 well plate. 1, 3 and 5 μl of virus made up to 150 μl in PBS were also used in duplicate. One hundred and fifty μl of BCA working reagent was added to each well then incubated at 37°C for 2 h. The absorbance at 570 nm was measured using a Wallac Victor^{2} plate reader (Wallac, Turku, Finland). Background absorbance was subtracted from the samples and standards and the amount of protein present in each virus was then calculated from the standard curve. The virus particle titre was then calculated using the established formula: 1 μg protein = 1 $\times 10^{9}$ viral particles (Von Seggern, Kehler et al. 1998).

2.7.6. Western blotting

Before using a new virus stock it was important to confirm that the virus capsids had the fiber protein incorporated. To achieve this SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blotting were performed using the monoclonal anti-fiber antibody 4D2 (Neomarkers Fremont, CA, USA).

To detect fiber monomers, reducing conditions and a 12% polyacrylamide gel (containing 40% (v/v) polyacrylamide (30%), 11.25 mM Tris pH 8.8, 0.1% (v/v) SDS, 300 μl ammonium persulphate (APS) and 30 μl TEMED) were used. To detect

fiber trimer, non-reducing conditions and a 7.5% gel (containing 25% (v/v) polyacrylamide (30%), 11.25 mM Tris pH 8.8, 0.1% (v/v) SDS 300 µl APS and 30 µl TEMED) were used. A 4% stacking gel containing 13.3% (v/v) polyacrylamide (30%), 3.75 mM Tris pH 6.8, 0.1% SDS 300 µl APS and 30 µl TEMED) was used with each gel.

Twenty µg ($8x10^{10}$ vp) were mixed with an equal volume of reducing loading dye (125 mM Tris pH 6.8, 4% (v/v) SDS, 10% (v/v) glycerol, 0.006% (v/v) bromophenol blue, 2% (v/v) β-mercaptoethanol) or non-reducing loading dye (125 mM Tris pH 6.8, 4% (v/v) SDS, 10% (v/v) glycerol, 0.006% (v/v) bromophenol blue). For reduced conditions virus was heated to 95°C for 5 minutes before the gel was loaded. Samples were electrophoresed at 200 V in running buffer (0.025 M Tris-HCl, 0.2 M glycine, 0.001 M SDS) for approximately 3 hours.

Proteins were transferred onto Hybond-P membrane (Amersham Bioscience UK Limited, Buckingham, UK) overnight at 30 V in transfer buffer (0.025 M Tris, 0.2 M glycine, 20% (v/v) methanol, 0.01% (v/v) SDS). The membranes were then blocked in TBS-T (150 mM NaCl, 50 mM Tris, 0.1% (v/v) Tween-20) + 10 % (w/v) fat-free milk powder (blocking buffer) for 2 hours with shaking. The membrane was incubated for 1 hour at 37°C with the anti-fiber antibody diluted to 1:500 in blocking buffer. The membrane was washed twice in blocking buffer at room temperature for 5 minute. The secondary antibody, rabbit anti-mouse horseradish peroxidase (HRP) (Neomarkers Fremont, CA, USA) was diluted 1:1000 in blocking buffer and incubated with the membrane at room temperature for 1 hour. The membrane was then washed four times in blocking buffer for 15 minutes at room temperature. An additional three washes of 5 minutes in TBS-T were performed. Proteins were visualised using the ECL detection system (Amersham Biosciences UK Limited, Buckinghamshire, UK) as per manufacturer's instructions. Films were exposed for varying lengths of time, ranging from 10 seconds to overnight.

2.8. In vitro infections

2.8.1. In vitro infection with adenovirus

Cells were seeded in 96-well plates at a seeding density of 1×10^4 cells/well and incubated overnight at 37°C to produce 70-80% confluence. Viruses were diluted to the desired concentration in PBS. Wells were infected with the required multiplicity of infection (MOI) of virus and incubated for 3 hours at 37°C. Cells were washed in PBS then placed in fresh media and incubated at 37°C for 48 hours before transgene expression was measured.

2.8.2. In vitro infections with AAV

Cells were seeded in 96-well plates and incubated overnight at 37°C to produce 70-80% confluence. Viruses were diluted to the desired concentration in PBS. Wells were infected in triplicate with the required MOI of virus and incubated for 24 hours at 37°C. Cells were washed in PBS then placed in fresh media and incubated for a further 72 hours at 37 °C.

2.9. In vivo virus biodistribution

Male 11 week old WKY were administered a single bolus intravenous injection of an adenovirus construct at 3×10^{11} vp/rat under general anaesthesia (2% isoflurane, vol/vol). Animals were sacrificed 5 days post infusion.

2.9.1. DNA extractions

DNA was isolated from tissue samples using the QIAamp DNA Mini Kit (Qiagen, CA, USA) as per manufacturer's instructions. Briefly, approximately 25 mg tissue (10 mg for spleen) was placed in 200 µl SDS-containing lysis buffer with proteinase K and incubated overnight at 56°C. A further 200 µl of buffer was added to the samples that were then heated to 70°C for 10 minutes. For blood samples, 200 µl of blood was mixed with 200 µl of buffer and incubated at 56°C for 10 minutes. The same protocol was then followed for both sample types. Ethanol (200 µl) was added to the samples,

mixed and then loaded onto a QIAamp Spin Column. Samples were centrifuged for 1 minute at 6000 x g to adsorb the DNA onto the silica-gel membrane of the spin column. The spin column was washed with buffers AW1 and AW2 and then DNA was eluted in 100 µl deionised water by centrifugation at 6000 x g for 1 minute. The concentration of DNA in each sample was measured using the ND1000 Spectrophotometer (Nanodrop, DE, USA).

2.9.2. Quantitative Real Time PCR

Real-time PCR (RT-PCR) was used to quantify the number of virus genome particles in tissue extracts. This procedure of quantitative measurement is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle and can be measured directly. Acquisition of data occurs when PCR amplification is still in the exponential phase. The SYBR Green detection system (Applied Biosystems) was used to carry out the RT-PCR on the Taqman^{TM} machine. SyBr Green PCR core reagents kit (Applied Bioscience, UK) with 200 nM *lac*Z specific primers, forward (5' ATC TGA CCA CCA GCG AAA TGG 3') and reverse (5' CAT CAG CAG GTG TAT CTG CCG 3') were used to amplify *lac*Z DNA. The following conditions were used: denaturation- 95^{o}C for 10 mins; amplification- 95^{o}C for 15 sec; annealing- 60^{o}C for 1 minute (50 cycles); dissociation - 95^{o}C for 15 sec; 60^{o}C for 15 sec and 95^{o}C for 15 sec.

A *lacZ* quantification standard curve was produced from serial dilutions of each virus preparation. Total DNA (100 ng) was used in each reaction and all samples and standards were analysed in duplicate using TaqMan data analysis software.

2.10. Histology

Tissues were excised and immediately fixed by either 10% formalin or x-gal fix (0.1 M phosphate buffer supplemented with 5mM EGTA and 2mM $MgCl_2$) overnight and then transferred to PBS. Tissues were then paraffin embedded and single tissue sections with 6 µm thickness mounted onto a sialanised glass slide. Slides were baked at 65°C for 3 hours, then at 40°C overnight.

2.10.1. Coating slides in aminoalkylsilanes

Blank glass slides were placed in a solution of 2% 3-aminopropyltriethoxysaline (APES) in dry acetone for 30 seconds. They were then rinsed once in acetone for 10 seconds, and then rinsed twice in deionised water for 5 minutes before being dried at 42°C overnight.

2.10.2. Immunohistochemistry

Paraffin was removed from the sections by 2 x 7 minute washes in Histoclear (Fisher Scientific, Leicestershire, UK). Sections were rehydrated by passing through an alcohol gradient of 100%, 95%, 70% ethanol for 7 minutes each. Slides were then washed in deionised water for 5 minutes. Endogenous peroxidase activity was quenched by incubating slides for 30 minutes in 0.3% (v/v) methanol-hydrogen peroxide at room temperature. The slides were then washed twice in water for 5 minutes. Antigen unmasking if required was carried out (section 2.4.2.1). IHC was performed using Vectastain ABC rabbit IgG kit or universal IgG kit (Vector Laboratories, Peterborough, UK). Briefly, sections were placed in blocking solution (goat or horse serum) and incubated for 1 hour at room temperature in a humidified chamber to prevent sections from drying out. The primary antibody and the negative control antibody were diluted in blocking solution (For dilutions see table 2.3). Antibodies were incubated on the sections overnight at room temperature in a humidified tray. Slides were washed 3 times in PBS, for 5 minutes each. The secondary antibody biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse/rabbit IgG (Vector Laboratories, Peterborough, UK) was diluted to 0.01mg/ml in blocking solution and incubated on the slides for 30 minutes at room temperature. Slides were washed in PBS 3 times, for 5 minutes each. The avidin and biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories, Peterborough, UK) was then incubated on the slides for 30 minutes at room temperature. This was followed by 3 further 5 minute washes in PBS. Slides were then incubated for 5 minutes in DAB chromogen solution (3,3’ diaminobenzidine, hydrogen peroxide, and nickel solution diluted in water) (Vector Laboratories, Peterborough, UK). Slides were washed in water for 5 minutes then nuclei were counter-stained by incubation in haematoxylin for 30 seconds. Slides were washed for 5 minutes in running water.

Sections were dehydrated by incubation in 70% ethanol, 95% ethanol, 100% ethanol then Histoclear for 7 minutes each. Sections were mounted using Histomount (National Diagnostics, Georgia, USA). Nuclei of β–gal positive cells appeared dark blue/purple.

Antibody	**Animal raised in**	**Source**	**Clone Number**	**Concentration used at**
Mouse IgG	Mouse	Dako, Denmark	N/A	Equivalent to primary antibody
Rabbit IgG	Rabbit	Dako, Denmark	N/A	Equivalent to primary antibody
Goat-anti rabbit HRP	Goat	Dako, Denmark	N/A	0.1 µg/ml
Mouse-anti rabbit HRP	Mouse	Dako, Denmark	MR12/53	0.1 µg/ml
ACE2	Rabbit	Santa Cruz, CA, USA	H-175	20 µg/ml
β-galactosidase	Rabbit	MP Biomedicals		0.36 µg/ml
CRIP-II	Chicken	Genway Biotech Inc	aa1-208	1 µg/ml
MPC-II	Rabbit	Made by collaborator		1 µg/ml
BC-10	Rabbit	Made by collaborator		1 µg/ml
T7	Rabbit	Chemicon	T7(masmtggqqmg)	0.02 mg/ml
RECA-1	Mouse	Abd Serotec	HIS52	1 µg/ml
Ab-4	Rabbit	Neomarkers Fremont	4D2	0.4 µg/ml

Table 2.3 Antibodies used in experimental procedures

2.10.2.1. Antigen retrieval

Formalin fixation forms protein cross-links that mask the antigenic sites in tissue specimens. Antigen retrieval methods are designed to break these protein cross-links, and therefore unmask antigens. Two methods were used; sodium citrate buffer and trypsin. Briefly, sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) was heated in microwave until temperature reached 95-100°C. Slides were immersed in the buffer and incubated for 15 minutes. Slides were then washed twice

in deionised water and the immunohistochemical protocol followed from the blocking step.

For trypsin retrieval methods, sections were covered with trypsin working solution (0.1% trypsin, 0.1% $CaCl_2$, pH 7.8) and incubated for 10 minutes at 37°C. Sections were then washed in PBS and then blocking carried out.

2.10.3. Haematoxylin and Eosin Staining

After the removal of paraffin and rehydration of slides as described in section 2.10.2, sections were stained in haematoxylin for 2 minutes. Slides were then washed in running tap water for 5 minutes then placed in eosin for 2 minutes, before a further 5 minute wash in running water. Slides were dehydrated then mounted in Histomount. Nuclei appeared blue/purple whereas cytoplasm was stained pink.

2.10.4. Picrosirius Red Staining

After the removal of paraffin and rehydration of slides (as described in section 2.10.2), sections were incubated for 90 minutes at room temperature under dark conditions in sirius red F3B (0.1% (w/v) sirius red F3B in saturated picric acid). Slides were then washed twice for 3 minutes in 0.01 N HCl followed by two 3 minute washes in deionised water. Slides were dehydrated then mounted in Histomount before being viewed under linear polarised light. Collagen was stained various shades of red.

2.10.5. Masson's Trichrome Staining

Slides were deparaffinised through alcohol gradient to deionised water and then incubated in Bouin's solution at room temperature overnight. Slides were washed under tap water until the yellow colour had been removed. Sections were stained for 5 minutes in Weigert's Iron Haematoxylin solution (Weigert's Iron Haematoxylin solution was prepared by mixing equal parts of Solution A (1% Haematoxylin in ethanol) and Solution B (ferric chloride 1.2% (w/v) and Hydrochloric acid, 1% (v/v)). Slides were then washed in running tap water for 5 minutes before being stained in

Biebrich Scarlet-Acid Fucshin (Biebrich scarlet, 0.9% (w/v), acid fuchsin 0.1% (w/v), in acetic acid, 1.0% (v/v)) for 5 minutes and then rinsed in deionised water. Slides were placed in Working Phosphotungstic/Phosphomolybdic Acid Solution (1 volume of Phosphotungstic Acid 10% (v/v), 1 volume Phosphomolybdic Acid 10% (v/v) with 2 volumes of deionised water) for 5 minutes and then in Aniline Blue Solution (Aniline blue, 2.4% (w/v) and acetic acid, 2% (v/v)) for 5 minutes. Sections were washed in Acetic Acid, 1% (v/v), for 2 minutes before being dehydrated through alcohol, cleared in histoclear then mounted. Cell nuclei appeared black, cytoplasm and muscle fibers appeared red and collagen stained blue.

2.11. Immunocytochemistry

Cells were fixed on coverslips in 4% paraformaldehyde at room temperature for 15 minutes. After three 5 minute washes in PBS, cells were permeabilised in 0.1% Triton for 15 minutes then washed a further 3 times in PBS. Cells were then incubated with the primary antibody (1 µg/ml diluted in PBS and 20% (v/v) serum of animal in which antibody was raised) for 30 minutes. Cells were then washed three times in PBS before being incubated with the secondary FITC labelled antibody (1 µg/ml diluted in PBS and 20% (v/v) serum) for 30 minutes. After three 5 minute PBS washes, the back of the coverslip was washed in water and the coverslip mounted onto a glass slide using Vectashield (Vector Laboratories, Peterborough, UK) (containing propidium iodide) and set with nail polish.

2.12. Visualisation of β-galactosidase (β-gal) expression in infected cells

Following infection, cells were washed in PBS then fixed in 50 µl 2% paraformaldehyde by incubation on ice for 20 minutes. Cells were then washed in PBS and 100 µl X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) stain (77 mM Na_2HPO_4, 23 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 3 mM $K_4Fe(CN)_6$, 0.05% (v/v) 20 mg/ml X-gal dissolved in dimethyl formamide) was added to each well and incubated overnight at 37°C. Cells were washed in PBS and placed in fresh PBS.

2.12.1. Detection of β-gal expression in tissues

For tissue and whole limb staining, tissues were fixed in 2% paraformaldehyde before incubation in X-gal stain.

2.12.2. Quantification of β-gal expression in cell and tissue lysates

Unless otherwise stated, to quantify β-gal expression the Tropix Galacto-Light Plus system (Applied Biosystems, MA, USA) was used. This assay could not be used to quantify β-gal expression from rat tissues as rat blood inhibits this assay for unknown reasons. For rat tissues, the CPRG assay was used (section 2.12.2.1).

Briefly, infected cells were washed in PBS then lysed in 80 µl of Lysis Solution (0.2% (v/v) Triton X-100 in PBS). Twenty µl of each sample was transferred to a white 96-well plate. Standard curves of recombinant β-galactosidase protein ranging from 0-20 ng and 0-20 pg were produced in duplicate. Galacton Plus chemiluminescent substrate was diluted 1:100 in reaction buffer (100 mM sodium phosphate pH 8, 1 mM $MgCl_2$). Seventy µl was then added to each well and incubated for 1 hour at room temperature. One hundred µl of light emission accelerator was added to each well. Luminescence was measured using a Wallac Victor^2 plate reader (Wallac, Turku, Finland). β-galactosidase activity was then normalised to protein content of samples to give relative light units per mg protein (RLU/mg protein).

2.12.2.1. Quantification of β-gal expression by CPRG assay

The chlorophenol red β-d-galactopyranoside (CPRG) assay is a quantitative assay to detect differences in β-gal expression. This assay is based on the ability of β-galactosidase to catalyse the hydrolysis of β-galactosides, including lactose and the galactoside analog CPRG. β-Galactosidase converts the yellow-orange CPRG substrate into galactose and the chromophore chlorophenol red, yielding a dark red solution. The amount of substrate converted can easily be detected.

First the tissues were ground using a mortar and pestle on an ethanol and dry ice bath. Lysis buffer was added to the samples to a concentration of 2 ml/g homogenate and samples were then freeze/thawed three times. They were then further diluted 1:1 in

Doc buffer (10 mM tris, 0.5g/100ml deoxycholate, pH 8.4) and centrifuged for 40 minutes at 16000 × g at 4ºC. The supernatant was kept as the working samples and 25 µl of sample was added to the well of a clear 96-well plate. 100 µl CPRG substrate solution was then added to each well, before incubating the plate at room temperature until the red colour developed (10 minutes - 4 hours). Luminescence was measured using a Wallac Victor^2 plate reader (Wallac, Turku, Finland). β-galactosidase activity was then normalised to β-gal MU / mg protein.

2.13. Determination of protein concentration in cell and tissue lysates

The amount of protein in cell lysates was determined using the BCA assay kit (Pierce, Rockford, USA) as per manufacturer's instructions. A standard curve was generated using dilutions of BSA ranging from 2000 µg/ml to 25 µg/ml. 200 µl of BCA working reagent was added to 25 µl of cell lysate or standard, in duplicate in a 96 well plate. The plate was incubated at 37°C for 30 minutes. The absorbance was measured at 570 nm on the Wallac Victor^2 plate reader (Wallac, Turku, Finland).

2.14. AAV methods

2.14.1. Production of rAAV2 vectors

All rAAV2 vectors were produced, purified and titered by Dr. H. Buening (University of Cologne, Germany) using a previously described method (Nicklin, Buening et al. 2001; Perabo, Buning et al. 2003).

2.14.2. Production of rAAV6 in the laboratory for in vitro studies

rAAV6 is made from the plasmids pDGM6 and the expression cassette pAAV *lacZ*. pDGM6 is the packaging/helper plasmid including the serotype 6 reading frame. It provides genes E2A, E4 and VA, which when transfected into E1A-expressing 293T cells, allows the replication of AAV *in trans*. The cells were transfected with a total of 21 µg DNA per transfection plate, in a ratio of 3:1 of each plasmid (15.75 µg helper

plasmid pDGM6 and 5.25 μg AAV *lacZ* plasmid), according to the method previously described for the production of fiber-modified and pseudotyped Ads (section 2.5.3.2). Lysates were then freeze-thawed 3 times before being centrifuged at 10,000 × g for 10 minutes to remove tissue debris. The supernatant was overlayed onto a sucrose gradient consisting of 2.4 ml of a solution of 40% sucrose plus 0.01% BSA in TBS. Crude viral particles were pelleted by centrifugation at 100,000 × g for 16 hours at 4°C. The pellets were then combined in 5 ml DNase Buffer. 1000 units of DNase I (Promega) were added and the samples incubated for 1 hour at 37°C. Two hundred and fifty μl 0.5M EDTA was then added before centrifugation at 10,000 × g for 2 minutes. Supernatant was removed and kept at 4°C overnight.

A HiTrap™ heparin HP column (Amersham Biosciences) was then equilibrated with DNase buffer, by means of a peristaltic pump. The virus was then bound to the column then washed with Ringer's solution. A further wash of Ringer's solution/0.5% N-lauryol-sarcosine was applied to the column before the virus was eluted in 200 mM NaCl Ringer's solution and again in 400 mM NaCl Ringer's solution.

Elutions were titered by carrying out a micro-BCA assay. Serial dilutions of rAAV2 (of a known titre) were used to construct a standard curve. Micro-BCA values from serial dilutions of rAAV6 were compared against the standard curve.

2.14.3. Production of rAAV6 vectors for in vivo studies

rAAV6 vectors were produced by Dr J Allen and Dr P Gregorevic through collaboration with the Chamberlain laboratory (University of Washington, Seattle, USA). A titre of 1.2×10^{13} vp was obtained, which was determined by Southern blot. Briefly an oligo probe derived from a common sequence, in this case the polyadenylation tail of the CMV promoter, is hybridized to southern blots containing dilutions of the vector preparation and plasmid standards to determine the genome titre of the preps. Vector genome content was also confirmed in house by quantitative real time PCR using primers to the *lacZ* gene.

2.14.4. Cloning of murine ACE2 into rAAV6 vector and production of virus

To clone the ACE2 AAV shuttle plasmid, pTYF-EF1a_IRES-eGFP containing the ACE2 cDNA (a kind gift from Dr M Raizada, Department of Physiology and Functional Genomics, University of Florida, USA) was digested using *Nhe*I and *Sal*I restriction sites, and cloned into the *Xba*I and *Sal*I sites in pAAV-MCS (Stratagene, CA, USA). pAAV-MCS-ACE2 was then packaged into AAV6 vectors (Gregorevic, Blankinship et al. 2004) (rAAV6:CMV*lacZ*, rAAV6:hPLAP and rAAV6:ACE2) by Dr J Allen and Dr P Gregorevic through collaboration with the Chamberlain laboratory (University of Washington, Seattle, USA) as previously described (Gregorevic, Blankinship et al. 2004).

2.14.5. Production of rAAV9

Pseudotype-9 rAAV (rAAV9:CMV*lacZ*) was purchased from University of Pennsylvania Vector Laboratories. The vectors were purified by two cycles of caesium chloride gradient centrifugation and titres were determined by a quantitative dot-blot assay.

2.14.6. ACE2 activity assay

To confirm that pAAV-ACE2 overexpressed functional ACE2, it was tested *in vitro*. ACE2 activity in Cos7 and HeLa cells following pAAV-ACE2 lipofectamine transfection was determined using an assay based on the use of Fluorogenic Peptide Substrate VI (FPS VI) (R&D Systems, Minneapolis, USA). ACE2 cleaves an amide bond between the fluorescent group and the quencher group (Pro and Lys), resulting in an increase in fluorescence in the presence of ACE2 activity at excitation and emission spectra of 320 and 405 nm, respectively. Briefly, protein was isolated from transfected cells using lysis buffer (75 mM Tris pH 7.5, 1 M NaCl, and 0.5 µM $ZnCl_2$) and the protein content determined by BCA. Samples were normalised to an arbitrary quantity and made up to 50 µl. To the samples the following was added; 100 µM FPS VI, 10 µM ACE inhibitor captopril and reaction buffer (1 M NaCl, 75 mM Tris and 0.5 mM ZnCl, pH 7.5) in a final volume of 100 µl.

To determine specific ACE2 activity, the experiment was also carried out in the presence of 100 µM ACE2 inhibitor DX600 (Phoenix Pharmaceuticals, Inc, California, USA). Fluorescence was monitored every 50 seconds for 2500 seconds using a spectrophotometer (Spectramax, Molecular Devices).

2.14.7. RNA extractions

RNA was extracted from the cells using an RNeasy midi kit (Qiagen). Briefly, samples were lysed and then homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC) containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to the lysate to provide ideal binding conditions. The lysate is then loaded onto the RNeasy silica-gel membrane. RNA binds, and all contaminants are efficiently washed away. Pure, concentrated RNA is eluted in water. The procedure provides enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs) are selectively excluded.

2.14.7.1. DNase treatment of RNA

DNase digestion is required for RNA applications that are sensitive to very small amounts of DNA (e.g. RT-PCR analysis). This was carried out on RNA samples using TURBO DNA-free™ (Ambion, Texas, USA). To a 40 µl RNA sample was added 0.1 volumes 10x TURBO DNase Buffer and 1 µl TURBO DNase (2 U/µl). After 20 minutes incubation at 37°C, 0.1 volume DNase inactivation reagent was added and mixed for 2 minutes at room temperature. The sample is then centrifuged at 10,000 × g for 90 seconds and supernatant kept as DNA free sample.

2.14.7.2. cDNA synthesis

One µg of RNA was used to synthesise cDNA using a QPCR cDNA synthesis kit (Stratagene, CA, USA) as per manufacturer's instructions. Briefly, RNA samples were mixed with 10 µl of first strand master mix (2×), 3 µl of oligo (dT) primer and 1 µl of AffinityScript RT/ RNase Block enzyme mixture in a final volume of 20 µl. The reaction was incubated at 25 ºC for 5 minutes to allow primer annealing, then at 42ºC for 15 minutes to allow cDNA synthesis. The reaction was finally incubated at 95ºC for 5 minutes to terminate the cDNA synthesis reaction.

2.14.8. In vivo infusion of rAAV6

Male 6 week old SHRSP were administered a single intravenous injection of increasing doses of rAAV6:CMV*lacZ* (2×10^{11}, 1.5×10^{12} and 3×10^{12} vp/rat) in the presence or absence of recombinant human VEGF-165 (20 µg/100 g body weight) or rAAV9:CMV*lacZ* or rAAV6:MLC2v at identical doses under general anaesthesia (2% isoflurane, vol/vol). Animals were sacrificed 2 weeks or at 12 weeks post infusion. Male 8 week old animals were infused with 3×10^{12} vp rAAV6:hPLAP or rAAV6:ACE2. Control animals were infused with 200 µl PBS and enalapril was supplied in the drinking water at 0.1 mg/ml.

2.15 Systolic blood pressure measurements

Systolic blood pressure monitoring was carried out weekly by non-invasive computerised tail cuff, which is based on the plethysmographic method (Davidson, Schork et al. 1995). A pneumatic pressure sensor was attached to the tail distal to a pneumatic pressure cuff, both under the control of a Programmed Electro-Sphygmomanometer. Systolic blood pressure values from each animal were determined by averaging a minimum of six separate indirect pressure measurements.

2.16 Echocardiography

Transthoracic echocardiography was carried out by Dr K Gilday and Mrs E Beattie at the University of Glasgow. Briefly, it was performed using an Acuson Sequoia c512 ultrasound system with a 15-MHz linear array transducer. Non-invasive acquisitions of 2-D guided M-mode images at a depth of 2 mm were recorded at the tip of papillary muscles. Rats were anesthetized with 1.5% isoflurane in O_2 and placed in the left lateral decubitus position on a heated pad. Three-lead electrocardiogram (ECG) was obtained using sub-dermal electrodes adapted with fine needles and secured on the front limbs and the right hind limb. The thickness of the posterior and anterior walls of the LV chamber and the LV chamber diameter during systole and diastole were measured in a short axis view using the leading edge-to-leading edge convention. All parameters were measured over at least three consecutive cardiac cycles. Pulse-wave and colour Doppler were used to measure the velocity of blood

through the mitral valve and to qualitatively examine the valve for evidence of mitral regurgitation from the apical four-chamber. The sample volume was placed at the tip of the mitral leaflets and adjusted to the position at which velocity was maximal; the sample volume was set at 2.5 mm. All Doppler spectra were recorded for 5-10 cardiac cycles at a sweep speed of 150 mm/s.

2.16.1 Formulae used in echocardiography assessments

Ejection fraction was defined as follows: EF = [(LVEDV - LVESV)/LVESD × 100], where LVEDV is left ventricular end diastolic volume and LVESV is left ventricular end systolic volume. Fractional Shortening was derived from: FS = [(LVEDD - LVESD) / LVEDD × 100], where LVEDD is left ventricular end diastolic diameter and LVESD is left ventricular end systolic diameter. Cardiac output was derived from: CO = [(ESV - EDV) × HR], where HR is heart rate. Change in interventricular septal wall thickness (ISWT) was measured using the formula [(AWTs-AWTd)/AWTs × 100], where AWT is anterior wall thickness, s is systole and d is diastole.

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2.17 Small vessel myography

Myography experiments were carried out by Mrs A. Spiers at the University of Glasgow. Briefly, basal NO bioavailability was determined in vascular rings from small mesenteric arteries which were cleaned of connective tissue and fat and divided into 3-mm rings. Isolated rings were placed in a small vessel four-channel myograph. Each ring was mounted on two parallel stainless steel wires of 40 μm diameter, one of which was connected to a micrometer, the other fixed to a force transducer in wire myograph organ baths maintained at 37ºC, containing 5ml Krebs buffer (composed of, in mM, 130 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 14.9 $NaHCO_3$, 5.5 glucose; continuously bubbled with 95% O_2/5% CO_2) for isometric tension recording. The resting tension of the rings was set using the normalisation procedure previously described (Mulvany and Halpern 1977). Briefly, each ring was subjected to stepwise radial stretching by adjustment of the micrometer. At each step the effective transmural pressure was calculated using the LaPlace equation. This process was continued until the ring was stretched to an internal circumference equivalent to a

transmural pressure of 100 mmHg. The micrometer was then adjusted to set the internal circumference of the ring to 90% of this length (a value termed "$0.9L_{100}$"), a setting that is optimal for force generation. The rings were then washed with fresh Kreb's solution, prior to the commencement of the experimental protocol. Basal NO bioavailability was calculated using the difference between responses to contractile agonists in the presence and absence of the NOS inhibitor, NG-nitroarginine methyl ester (L-NAME), and expressed as area under the curve across the full concentration–response range for the contractile agonist. The augmentation of the contractile response in the presence of L-NAME is an index of the degree of basal NO activity in the vessel.

2.18 Statistical analysis

All *in vitro* experiments were carried out in triplicate on three independent occasions. Results shown are representative and values are mean ± standard error of the mean (SEM). Student's unpaired t-test or ANOVA was used to analyse the results, which were considered significant when $p<0.05$ after Bonferonni corrections.

2.18.1 In vivo statistical analysis

Comparisons were made using one way or two way ANOVA. Statistical analysis was performed in Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). For all tests, $P<0.05$ is considered statistically significant after Bonferroni corrections or Tukey's post analysis. The results represent mean values and SEM of the data. For vascular function, EC50 was analysed using the student's t test.

Chapter 3

Targeting the Cardiac Vasculature

3.1 Introduction

We aimed to assess the effects of sustained overexpression of ACE2 in the vasculature. In general terms, the treatment of cardiovascular disease would greatly benefit from the generation of reagents that specifically localise to defined vascular beds including the heart. Targeted gene delivery would increase the efficacy of gene transfer vectors, whilst limiting vector induced side effects. By exploiting the molecular diversity of the endothelium, peptides that bind to specific receptors that are expressed on the surface of blood vessels can be identified that will allow cell-specific targeting, either of gene delivery systems (Biermann, Volpers et al. 2001; Nicklin, Von Seggern et al. 2001) or of bioactive peptides (Ellerby, Arap et al. 1999). These unique receptors may be expressed in an organ specific or disease specific manner, allowing the identification of highly specific ligands that home to these molecular targets. This complex molecular address system can be manipulated to develop systemically administrable therapies. Peptide discovery may be mediated through the use of either phage (Pasqualini and Ruoslahti 1996; Zhang, Hoffman et al. 2005) or AAV2 libraries (Michelfelder, Lee et al. 2007).

Bacteriophage T7 is a commercially available obligate lytic phage that infects enteric bacteria and more specifically *Escherichia coli*. It is appealing for use in display technologies as it is robust and fast growing phage. Kits are now available that allow the creation of novel T7 phage libraries from T7select® vectors, or pre-made libraries can also be supplied. T7 phage display allows the efficient cloning and display of targeting peptides as C-terminal fusions to the 10B major capsid protein. Peptides are displayed on the surface of the virion, fully accessible for interaction with other ligands. High, mid, or low copy number of peptide are displayed per phage, depending on the T7 vector system used and the size of insertion.

Random peptide phage libraries have been used to recover targeting peptides through *in vitro* or *in vivo* approaches. Pasqualini and Rouslahti were the first group to demonstrate the utility of phage display peptide libraries in organ targeting *in vivo* in mice (Pasqualini and Ruoslahti 1996). Phage recovered from the brain and kidney blood vessels were repeatedly recovered, amplified and intravenously infused into mice to ultimately enrich the population of phage targeting these organs. Brain and

kidney localising phage were identified that had increased selectivity for these tissues. This approach has been used to identify ligands that bind to tissue-specific vasculature. Targeting peptides that home to the vasculature of a specific organ have been identified for lung, pancreas, skin (Rajotte, Arap et al. 1998), skeletal and cardiac muscle (Samoylova and Smith 1999) and even diseased regions of atherosclerotic blood vessels in low density lipoprotein receptor knockout mice (Houston, Goodman et al. 2001). In 2005, phage that selectively home to the murine heart vasculature were identified through a combination of *ex vivo* and *in vivo* phage display techniques (Zhang, Hoffman et al. 2005). Three rounds of *ex vivo* biopanning on murine myocardial cells was succeeded by three rounds of *in vivo* biopanning in mice and resulted in the identification of heart targeting peptides. Target receptors for some of these targeting peptides were also identified and were found to be preferentially expressed in heart blood vessels and in the endocardium (Zhang, Hoffman et al. 2005). Phage expressing the heart targeting proteins were found to have a homing selectivity of 20- to >300-fold greater than insertless phage.

As viral vectors are limited by their natural tropisms, the modification of these vectors can greatly aid their subsequent transduction profile. Phage display-derived peptides have previously been used to retarget adenoviral vectors to ovarian cancer cells (Dmitriev, Krasnykh et al. 1998), renal cell carcinoma cells (Haviv, Blackwell et al. 2002) and primary vascular smooth muscle cells as well as primary human endothelial cells (Biermann, Volpers et al. 2001), through the insertion of the RGD peptide into the HI loop of the fiber of the vector. AAV2 vectors have also been modified by targeting peptides, with success being found *in vivo* shown by reduced vector accumulation in the liver and selective transgene expression of endothelial cells in the vena cava (White, Nicklin et al. 2004). Lung and brain targeting peptides inserted into the capsid of AAV2 vectors were shown to target to the vascular beds of the specific organs after systemic injection in rats (Work, Buening et al. 2006). Heart-homing peptides can potentially be used to retarget viral vectors for use in cardiovascular gene therapy. This method may allow the efficacy and selectivity of viral vectors to be improved.

Here I aimed to increase the efficiency of vector targeting to the vasculature through viral vector tropism modification via phage display-derived peptides.

3.2 Results

3.2.1 Targeting peptides

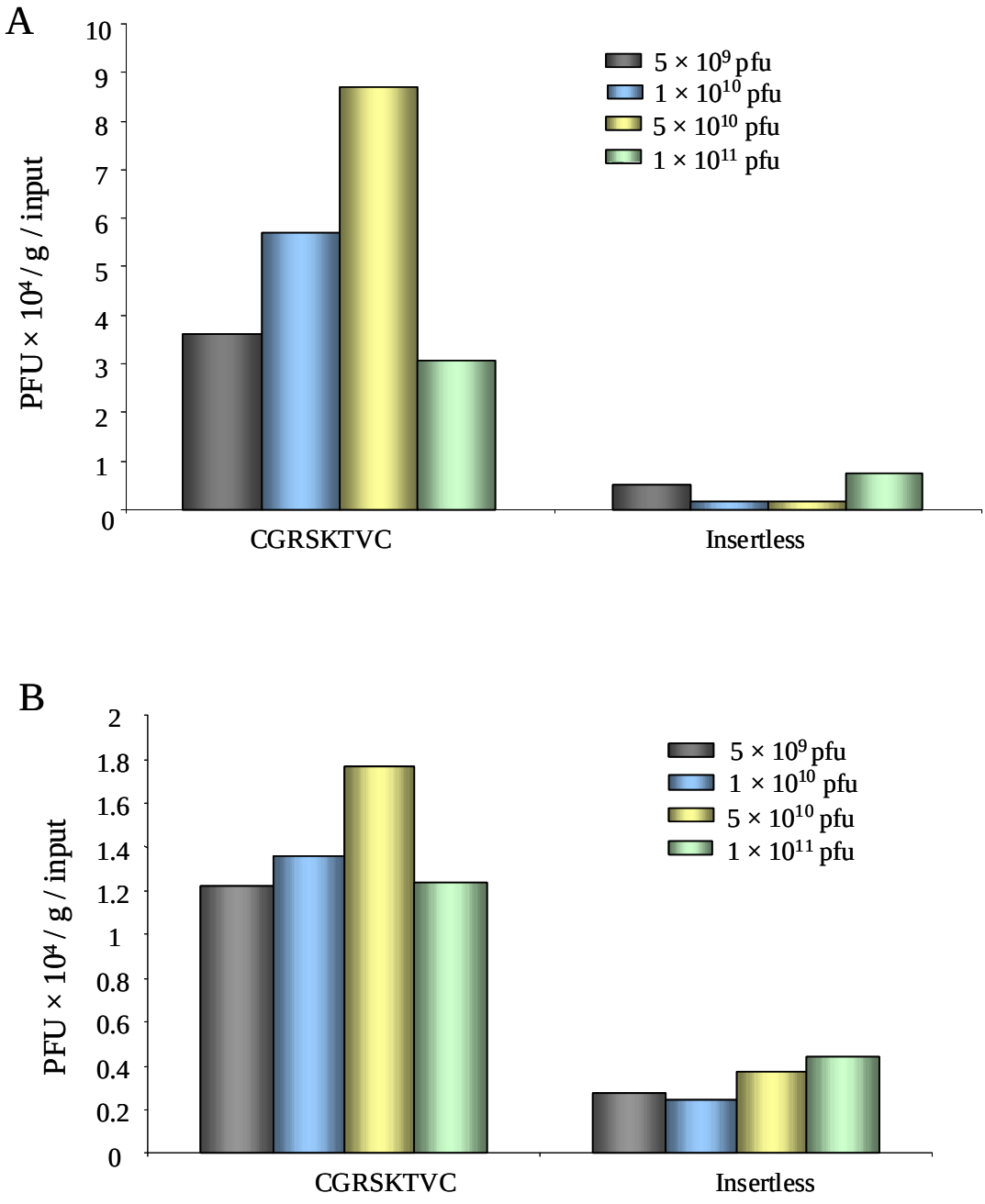
Candidate peptides, isolated by three rounds of selection on *ex vivo* heart cells, followed by three rounds of selection *in vivo*, were supplied through collaboration with E. Rouslahti (Zhang, Hoffman et al. 2005). T7 phage displaying the selected peptides (Table 3.1), were grown to high titres and purified. Before use, the phage were sequenced to check the sequence of the inserts (Table 3.1). A dose-response study was then carried out *in vivo* in which 4 different doses (5×10^9, 1×10^{10}, 5×10^{10} and 1×10^{11} pfu) were compared between the non-recombinant phage and a recombinant targeting phage chosen at random (CGRSKTVC) in WKY strain rats (Figure 3.1). As there is a large, rapid and non-specific uptake of phage by the liver following systemic injection, we aimed to saturate non-specific binding in the liver (Figure 3.1B) and in the heart. By comparing phage recovered per gram of heart tissues, normalised to input dose, an optimal dose of 5×10^{10} pfu was decided upon from which to base further experiments. This was the dose that allowed for maximum recovery of specific-binding phage from the heart (Figure 3.1A).

All six phage were then tested *in vivo*. Phage recovery showed that three of the peptides can be seen to selectively target the heart in comparison to the non-recombinant phage and the other major organs (Figure 3.2 and Table 3.2). These three potential candidates were identified as CRPPR, CSGMARTKC and CRSTRANPC. All three demonstrated an increase in ability to home to the heart, when compared to insertless phage (Figure 3.2 and Figure 3.3). CRSTRANPC showed the highest selectivity with a 2000-fold higher specificity than the insertless phage. CRPPR has 450 fold and CSGMARTKC 85 fold higher homing to the heart compared to the non-recombinant phage. CPKTRRVPC was also shown to target the heart in comparison to non-recombinant phage, with a 13 fold higher homing ability. However, this targeting was not limited to the heart and CPKTRRVPC also showed increased transduction in the lung. Because of the heart targeting potential, further work was carried out with this phage. Phage displaying the CGRSKTVC and CPKRPR peptides were discarded at this point as they were not seen to be selective at heart targeting, instead targeting the lung and spleen respectively (Figure 3.2).

CGRSKTVC	5’ TGCGGTCGGAAGTCGAAGACGGTTTGC 3’
CRPPR	5’ TGCCGTCCGCCTCGG 3’
CPKTRRVPC	5’ TGCCCTAAGACGCGTCGAGTTCCTTGC 3’
CRSTRANPC	5’ TGCCGTAGTACTCGTGCTAATCCTTGC 3’
CSGMARTKC	5’ TGCTCTGGTATGGCTCGTACTAAGTGC 3’
CPKRPR	5’ TGCCCTAAGCGTCCGCGG 3’

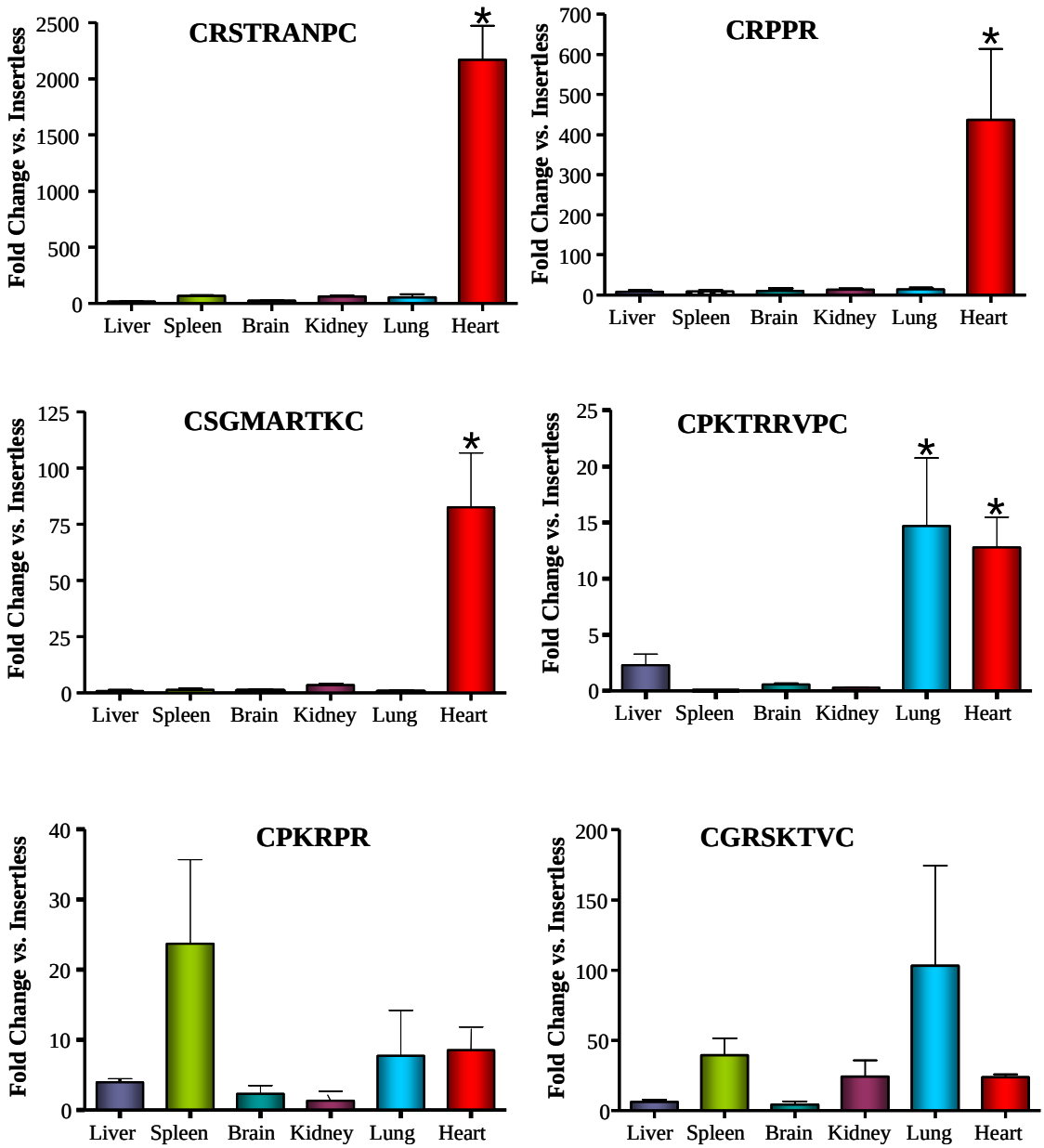
Table 3.1 Heart targeting peptides.

Selective heart targeting peptide sequences isolated through successive rounds of *ex vivo* and *in vivo* phage biopanning (Zhang, Hoffman et al. 2005).



<u>Figure 3.1.</u> Dose-response study.

Four different increasing doses (5×10^9, 1×10^{10}, 5×10^{10} or 1×10^{11} pfu) of CGRSKTVC or insertless phage were infused into 12 week old WKY rats. Phage were recovered from (A) heart and (B) liver and analysed as PFU/g tissue/ input (n=1/dose).



<u>Figure 3.2.</u> Cardiac targeting capacity of selected phage.

Recovery of phage from WKY infused with $5x10^{10}$ pfu of indicated phage. The cardiac endothelial homing peptides were displayed in T7 phage and intravenously injected into rats. Data are presented as a fold change in comparison to the nonrecombinant phage in the major organs (n=3/group). *$p<0.05$ for heart vs. liver, spleen, brain, kidney and lung by one way ANOVA and Bonferonni's post hoc analysis.

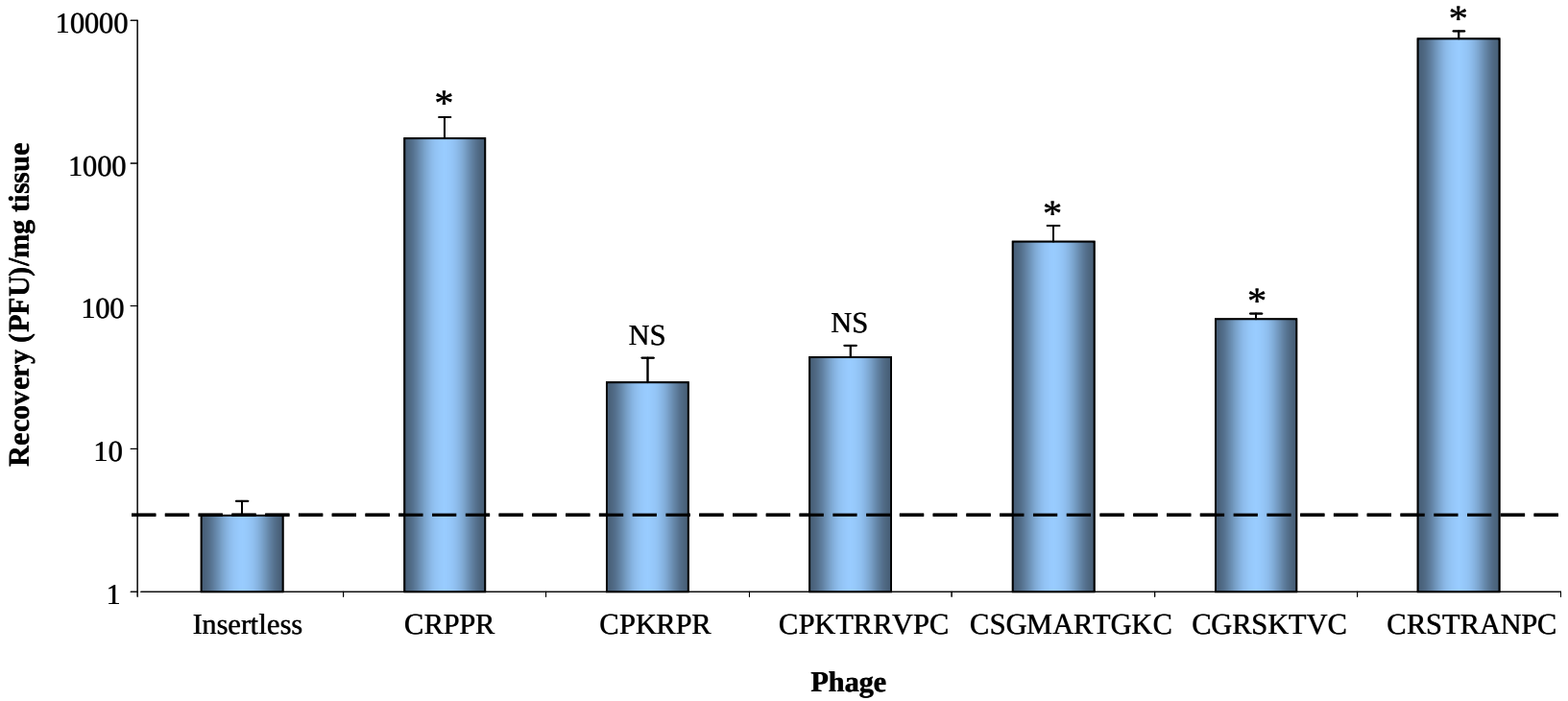


Figure 3.3. Phage recovery from heart.

Recovery of phage from the hearts of WKY infused with $5x10^{10}$ pfu of indicated phage (n=3/group). Data are presented as mean recovery (PFU)/ mg tissue ± SEM. *$p<0.05$ as compared to insertless by unpaired two-tailed t test.

Immunohistochemical analysis confirmed the targeting of the four selected peptides to the hearts of these rats (Figure 3.4). Whilst histology revealed that CSGMARTKC was found to primarily target the vascular endothelial cells, T7 phage displaying the peptides CPKTRRVPC, CRPPR and CRSTRANPC were also found throughout the myocardium, suggesting that the latter three peptides are not endothelial specific in the WKY heart (Figure 3.4).

3.2.2 Evaluation of targeting phage in vitro

The putative endothelial molecules (receptors) that the targeting peptides bind to were isolated by Zhang et al (Zhang, Hoffman et al. 2005). C-terminal 92 amino acids of heart LIM protein (cystein rich protein - CRIP-II) was identified as the receptor for CRPPR, bladder cancer-associated protein (human) homologue (BC-10) for CPKTRRVPC and CSGMARTKC, and an unnamed protein product similar to integral membrane protein CII-3 (MPCII-3) was suggested as the receptor for CRSTRANPC. To assess the expression of the receptors *in vivo* in the WKY rat heart, immunohistochemical staining was carried out on sections of heart with antibodies against the three receptors (Figure 3.5). Selective staining was observed from CRIP-II and BC-10 receptors but not from the MPCII-3 receptor. Thus the expression of CRIP-II and BC-10 in the WKY heart was confirmed, but the expression of MPCII-3 could not be determined. Western blot analysis was then carried out following transfection of plasmids expressing these putative receptors into COS cells to confirm their expression (Figure 3.6). Only CRIP-II expression was detected by this method. In a second attempt to confirm expression of the receptors from each plasmid, immunocytochemistry was carried out, which again confirmed expression of the CRIP-II receptor, but not of BC-10 or MPCII-3 (Figure 3.7). Thus the functionality of two of the plasmids expressing the putative receptors BC-10 and MPCII-3 could not be ascertained. Consequently, further work with the receptors was not carried out.

3.2.3 Peptide-modified Ad19p vectors

The selected heart targeting peptides (CRPPR, CRSTRANPC, CSGMARTKC and CPKTRRVPC) were inserted into the HI loop of the Ad19p fiber. The Ad19p vector was chosen for insertion of the targeting peptides as these vectors have previously

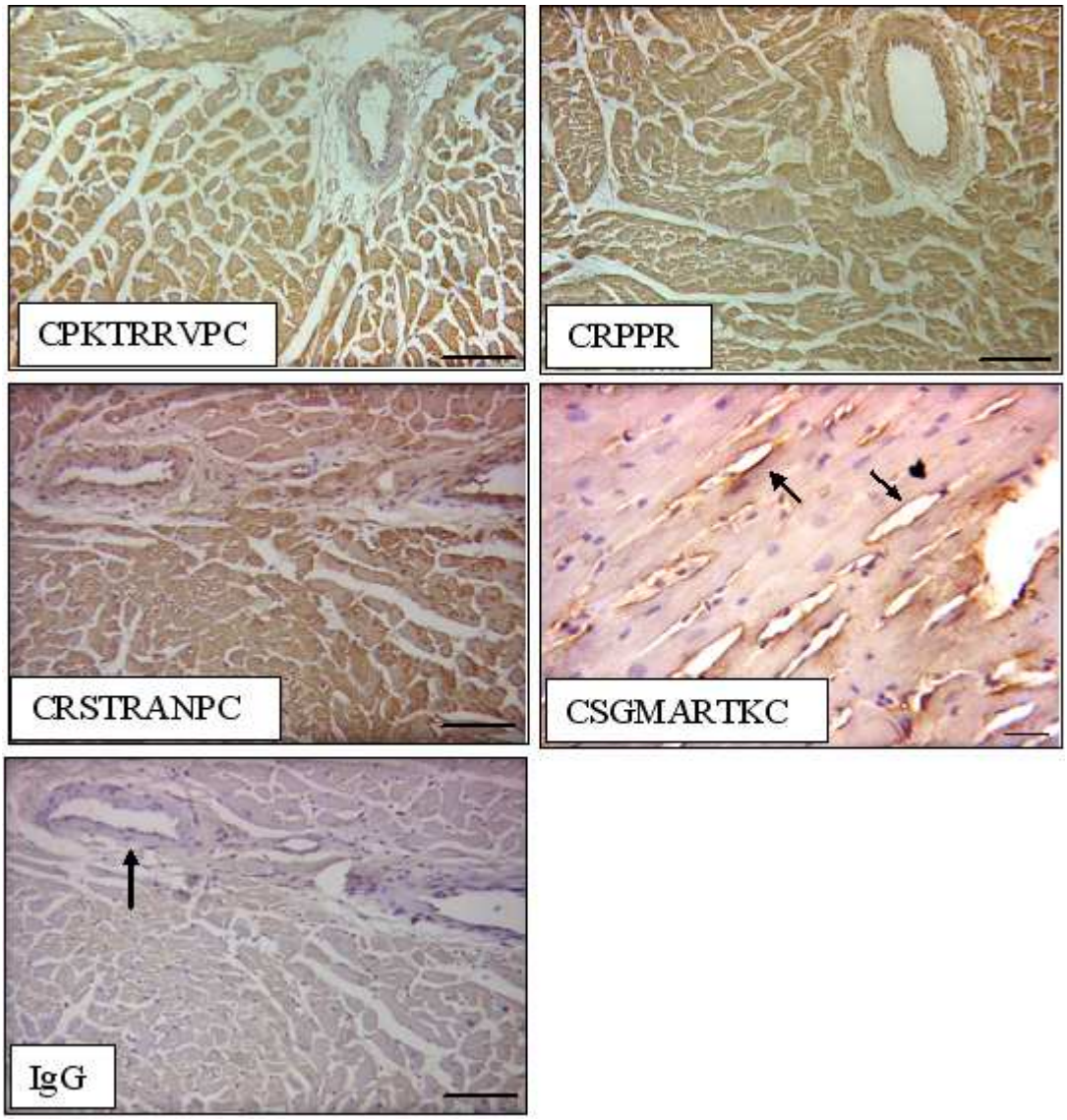


Figure 3.4. Histological analysis of T7 phage.

Histological analysis of vascular targeting with an anti-T7 antibody was carried out on sections of heart from animals in all 4 groups at termination, 15 minutes post-infusion (n=3/group). Scale bar = 50 μm, magnification × 20 or scale bar = 100 μm, magnification × 40. Black arrows = endothelial cells.

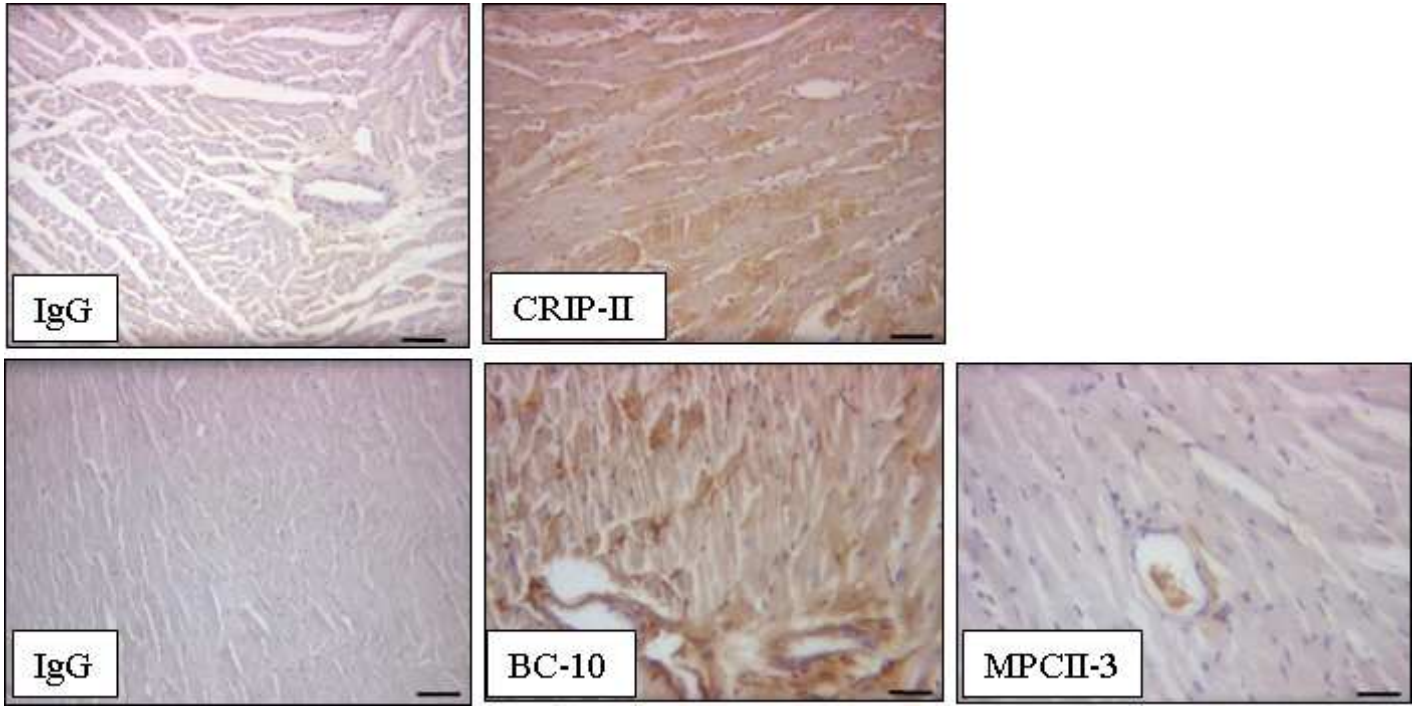


Figure 3.5. Expression of the receptors in WKY heart.

Immunohistochemistry with an anti-CRIP-II, anti-BC-10 or anti-MPCII-3 antibody was carried out on heart sections from WKY rat. Scale bar = 30 μm, magnification × 20.

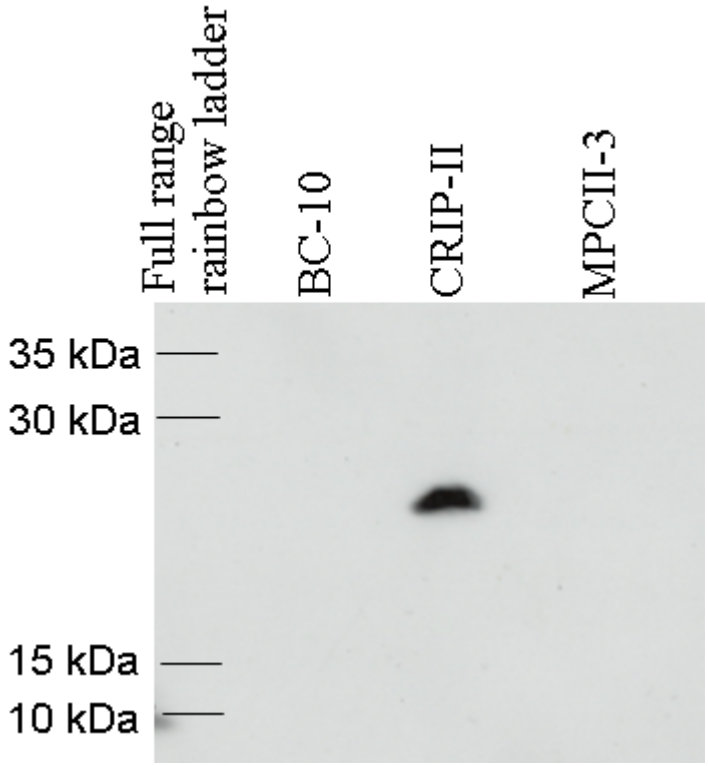
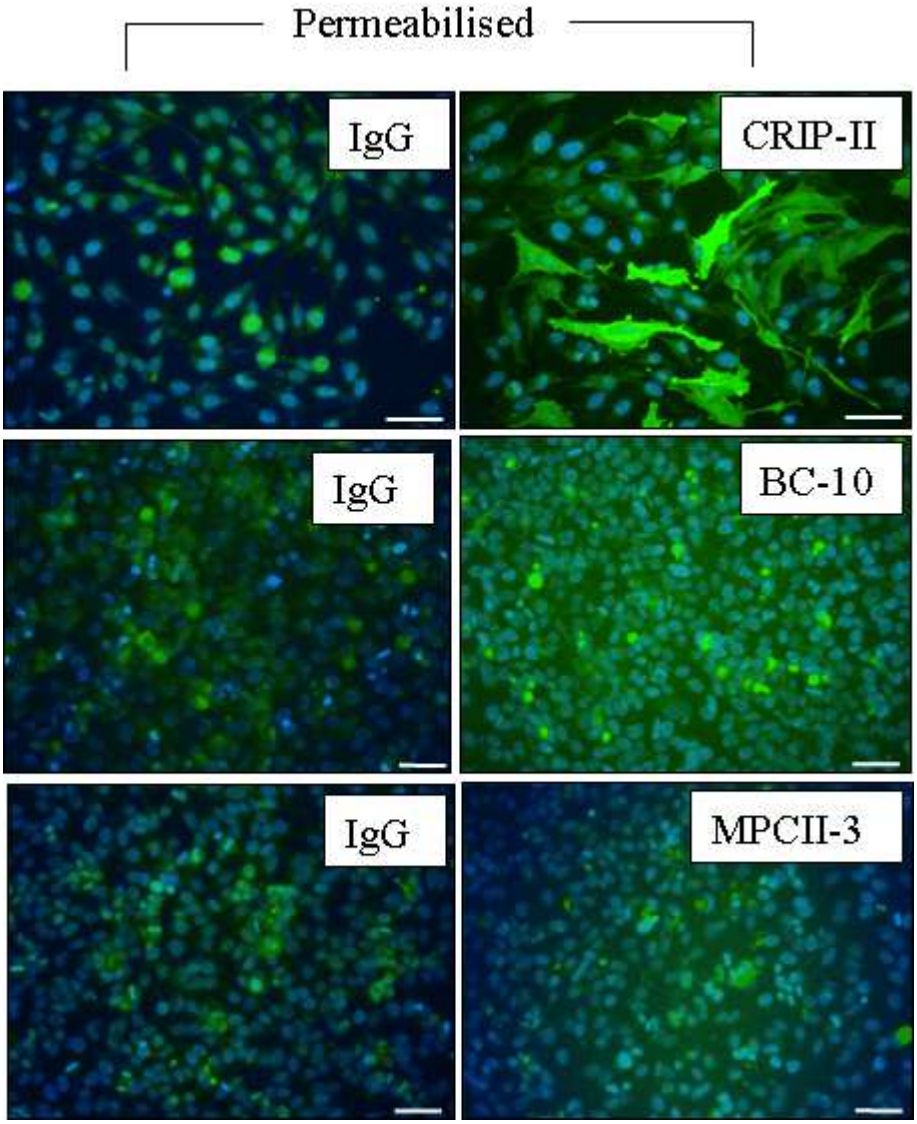


Figure 3.6. Expression of candidate phage receptors.

Cells were transfected with the plasmids expressing each putative receptor fused to a HIS tag. Following 48 hours lipofectamine-mediated transfection, cells were harvested and lysed. Expression of the three receptors BC-10, CRIP-II, MPCII-3 was tested by western immunoblotting with an anti-HIS tag antibody and detected on a 12% gel under reducing conditions.



<u>Figure 3.7.</u> Analysis of function of receptor expressing plasmids.

Plasmids expressing the receptors CRIP-II, BC-10 and MPCII-3 were transfected into HeLa cells and immunocytochemistry carried out 48 hours later in permeabilised conditions. Scale bar = 50 µm, magnification = × 4 or magnification = × 10.

been shown to lack tropism for mouse, rat, and human hepatocytes *in vitro* and have demonstrated greatly reduced transduction of liver after systemic injection into rats (Denby, Work et al. 2004). Two modified versions of the CRPPR peptide were also inserted into this loop in the Ad19p capsid. The peptides, GSSCRPPRGSS (CRPPR-mod) and CRPPRGGGSCRPPRGGGSCRPPR (3-CRPPR), were created by the insertion of spacers with the sequence GGG to generate peptides that might protrude further from the capsid of the vectors, thus allowing enhanced binding. To see if Ad19p tropism had been modified by the insertion of these targeting peptides, they were first tested *in vitro*. Infection of human coronary artery endothelial cells with the peptide-modified vectors revealed no increased transduction of control Ad19p (no peptide) (Figure 3.8). Levels of transduction were not increased above control uninfected wells and were not enhanced above those of unmodified Ad19p vectors, which showed the highest transduction of all the vectors tested (Figure 3.8).

In vivo testing of the seven vectors revealed unmodified Ad19p displayed the highest heart uptake, as shown by Q-PCR (Figure 3.9). Q-PCR confirmed the lack of native hepatic tropism of the Ad19p vectors. However, it also confirmed that no modified heart targeting tropism had been bestowed upon these vectors with the insertion of the selected peptides (Figure 3.9). Immunohistochemistry showed evidence of staining in the hearts of CSGMARTKC, CPKTRRVPC, CRSTRANPC and to a lesser extent CRPPR peptide-modified Ad19p vector infused rats. High levels of staining were observed in the hearts of unmodified Ad19p vector infused rats. Thus immunohisotchemistry confirmed that no specific staining was observed in the hearts of the peptide-modified vector infused animals that was not either present in the PBS infused hearts, or greater than that seen for the unmodified Ad19p vector (Figure 3.10).

3.2.4 Retargeting AdKO1

To test the ability of the phage-display derived targeting peptides to retarget alternative viral vectors, the selected candidates were inserted into the HI loop of the AdKO1 vector, which is CAR-binding ablated. These vectors were then tested *in vitro* for their ability to transduce human coronary artery endothelial cells. AdKO1 CRPPR,

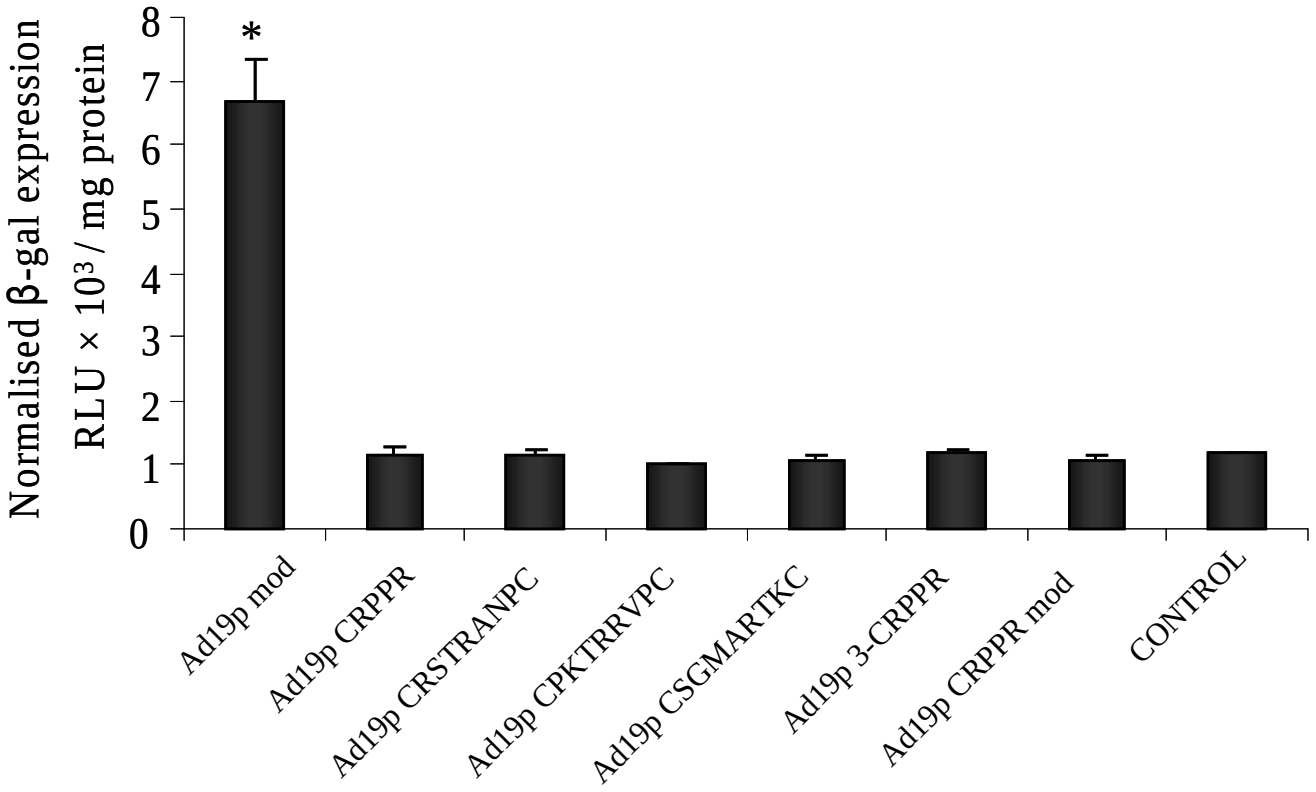


Figure 3.8. ***In vitro*** **Ad19p transduction.**

Ad19p peptide modified vectors were tested *in vitro* in HUCAEC (n=3/group). *$p<0.05$ as compared to control as determined by one way ANOVA and Bonferonni post test. β-gal expression quantified by Tropix Galacto-Light Plus assay.

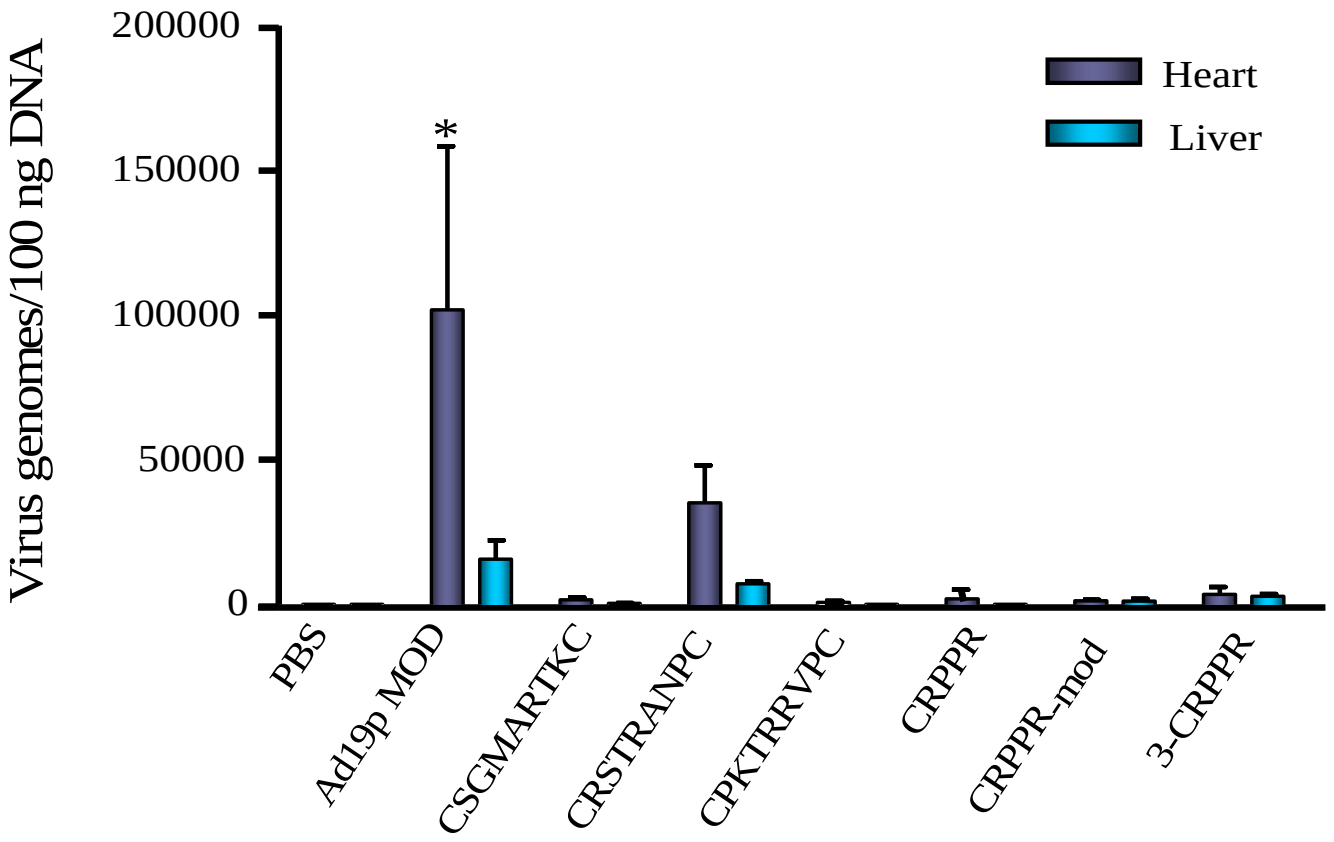


Figure 3.9. Ad19p peptide modified vectors *in vivo*.

3×10^{11} vp were infused into 12 week old male WKY rats (n=4/group). 5 days post-infusion organs were removed and DNA extracted. Q-PCR was carried out to quantify relative vector genome numbers. * $p<0.05$ as compared to Ad19p mod by 2 way ANOVA and Bonferonni's post hoc analysis. N=4/group.

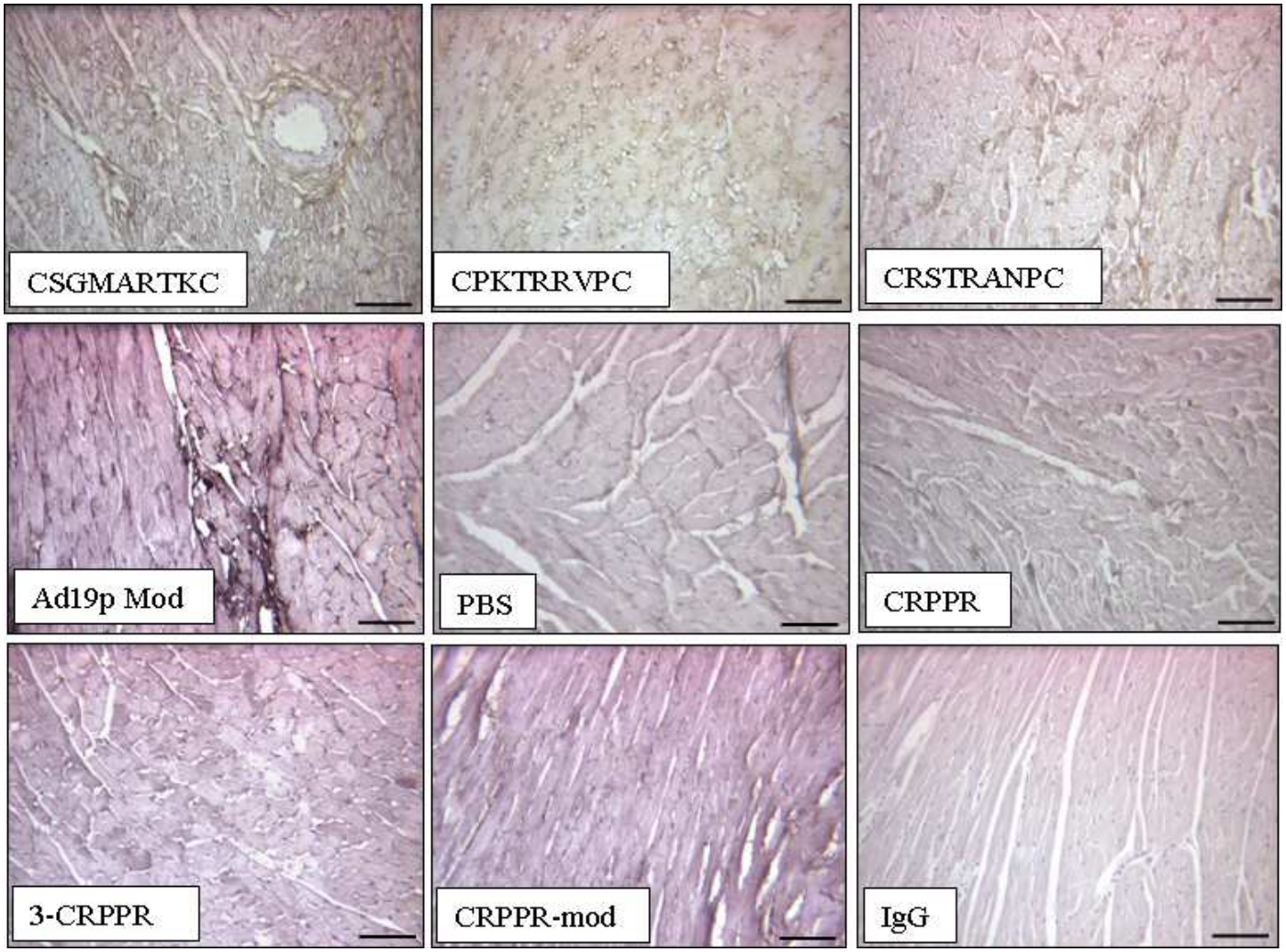


Figure 3.10 Histological analysis of Ad19p mediated *lacz* expression in the heart.

Immunohistochemical anaylsis with an anti-β galactosidase antibody was carried out on sections of heart from the Ad19p vector infused animals at termination (n=4/group). Scale bar = 50 μm, magnification × 20.

AdKO1 CRSTRANPC and AdKO1 CPKTRRVPC all demonstrated increased transduction of the cells in comparison to control uninfected cells (Figure 3.11). However, AdKO1 CSGMARTKC showed no increase in transduction of the endothelial cells. These results suggest that this configuration is useful but cannot be assessed *in vivo* as the AdKO1 vector is known to be sequestered by the liver, despite its CAR-binding ablation (Mizuguchi, Koizumi et al. 2002; Rittner, Schreiber et al. 2007). However, it has very recently been discovered that CAR is not important in binding *in vivo* and it is the interaction of the hexon with blood coagulation factors that are involved (Waddington, McVey et al. 2008). This discovery would allow for the development of methods to test these vectors *in vivo*.

3.2.5 Peptide-modified rAAV2 vectors

Once the peptides had been cloned into the capsid of the rAAV2 vector, the vectors were tested *in vitro* (Figures 3.12 and 3.13). Only three of the peptides could successfully be packaged into rAAV2 vectors; these were CRPPR, CPKTRRVPC and CSGMARTKC. Insertion of targeting peptides into the HSPG binding site in the rAAV2 capsid has been shown to reduce the hepatic tropism of the vectors (White, Nicklin et al. 2004; Work, Buening et al. 2006). To assess whether the natural hepatic tropism of rAAV2 vectors had been altered, HepG2 cells were infected with the peptide-modified vectors (Figure 3.12). As expected, rAAV2-wild type showed high levels of transduction of the hepatocytes. In contrast, the other three peptide-modified vectors showed no hepatic transduction (Figure 3.12). Next, human coronary artery endothelial cells were transduced with the peptide-modified vectors to determine their transduction efficiency of vascular endothelial cells (Figure 3.13). RAAV2 CRPPR showed low levels of transduction in HUCAECs when used at a dose of 10,000 genomic particles/cell. Disappointingly, the other three vectors, wild type rAAV2, rAAV2 CPKTRRVPC and rAAV2 CSGMARTKC demonstrated no transduction of the vascular endothelial cells, indicating that peptide-modification of rAAV2 with each individual peptide did not enhance tropism for HUCAEC (Figure 3.13). The low titre of these vectors did not allow for them to be tested *in vivo*.

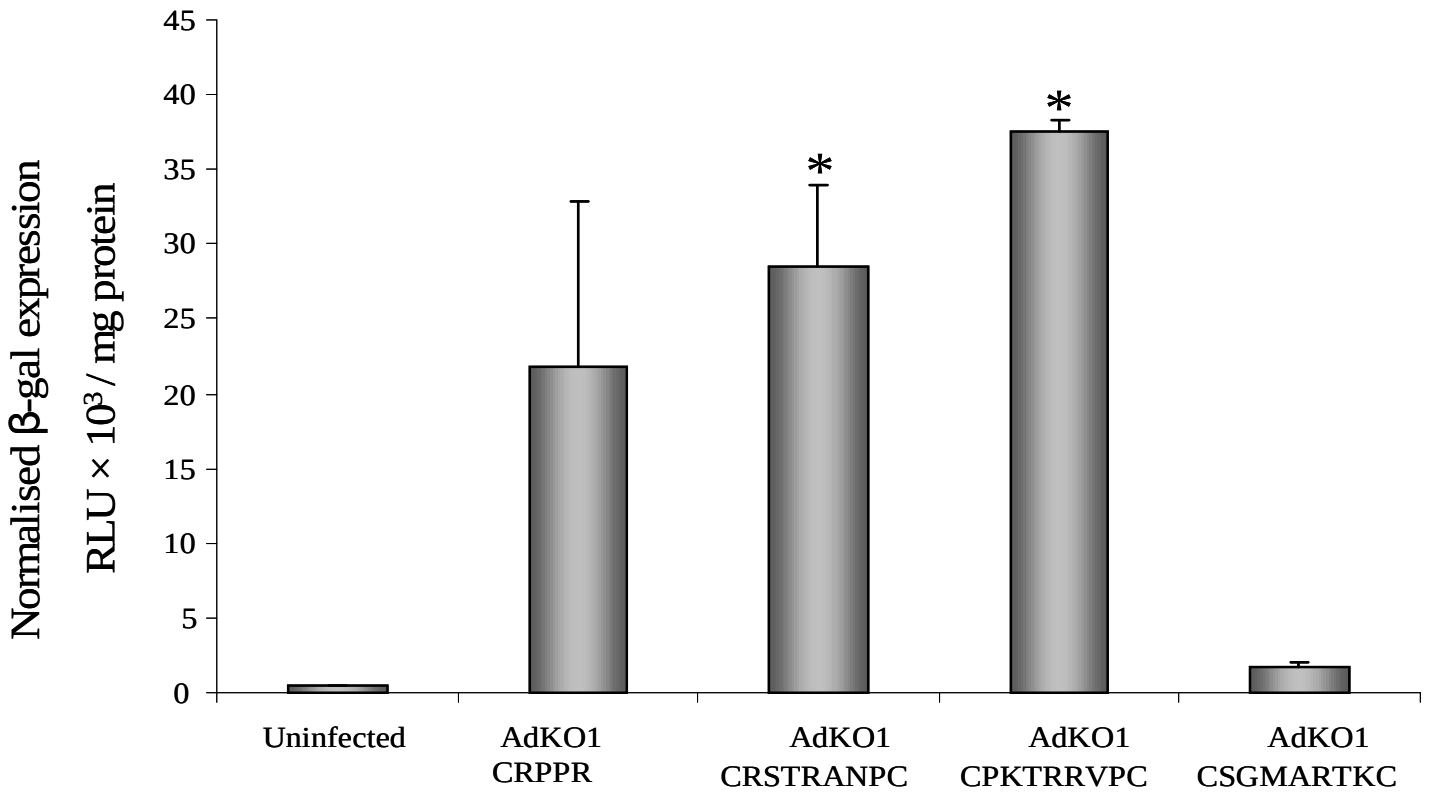
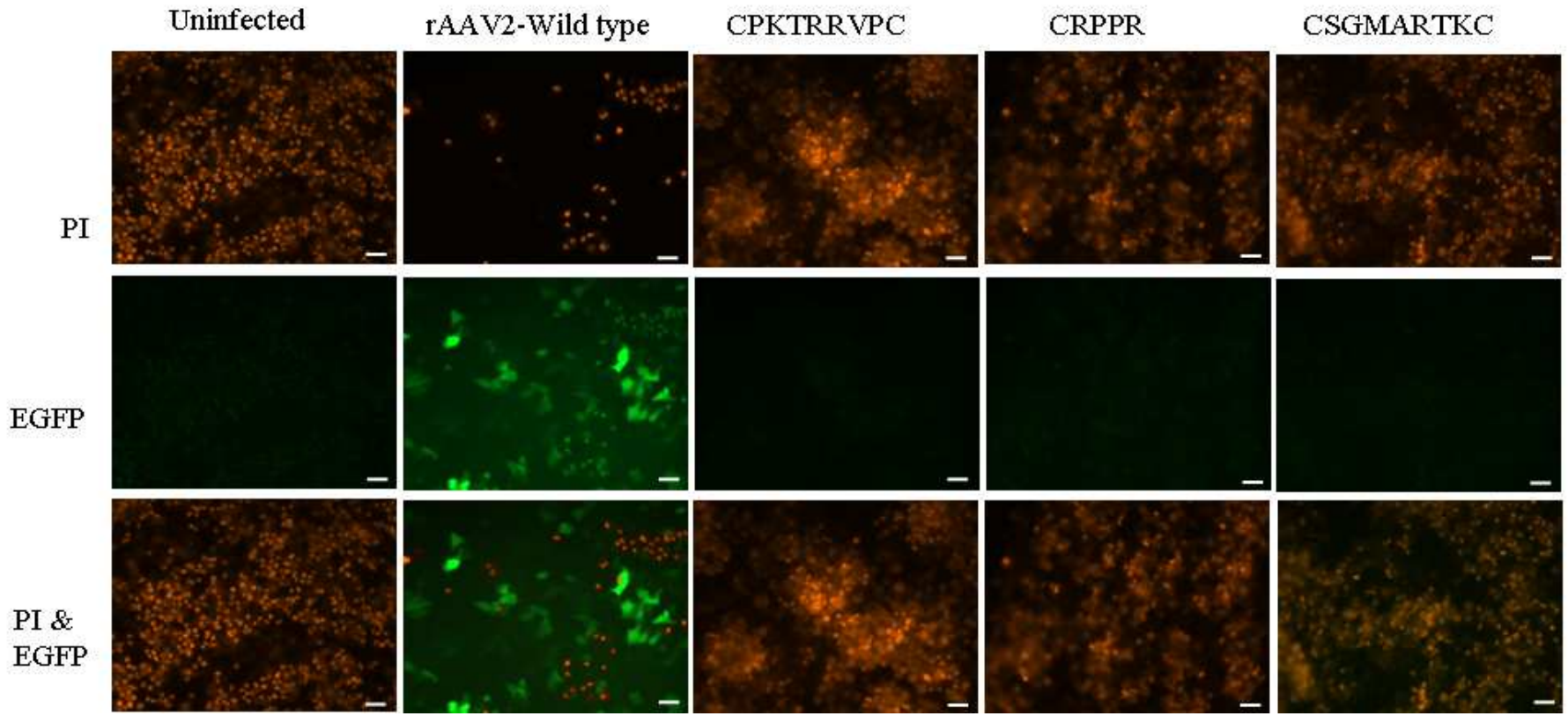


Figure 3.11. Retargeting of AdKO1 peptide modified vectors.

The four heart targeting peptides were cloned into the HI loop of AdKO1. Vectors were tested *in vitro* in human coronary artery endothelial cells (HUCAEC) (n=3/group). *p<0.05 AdKO1 CRSTRANPC and AdKO1 CPKTRRVPC vs. uninfected by one way ANOVA and Bonferonni posttest. β-gal expression quantified by Tropix Galacto-Light Plus assay.



<u>Figure 3.12.</u> rAAV2 infection of HepG2 cells.

HepG2 cells were infected with rAAV2 vectors at 10,000 particles/cell (n=6). Transduction ability of the vectors was observed 72 hours later by EGFP transgene expression and propidium iodide counterstaining. Scale bar = 50 µm, magnification × 10.

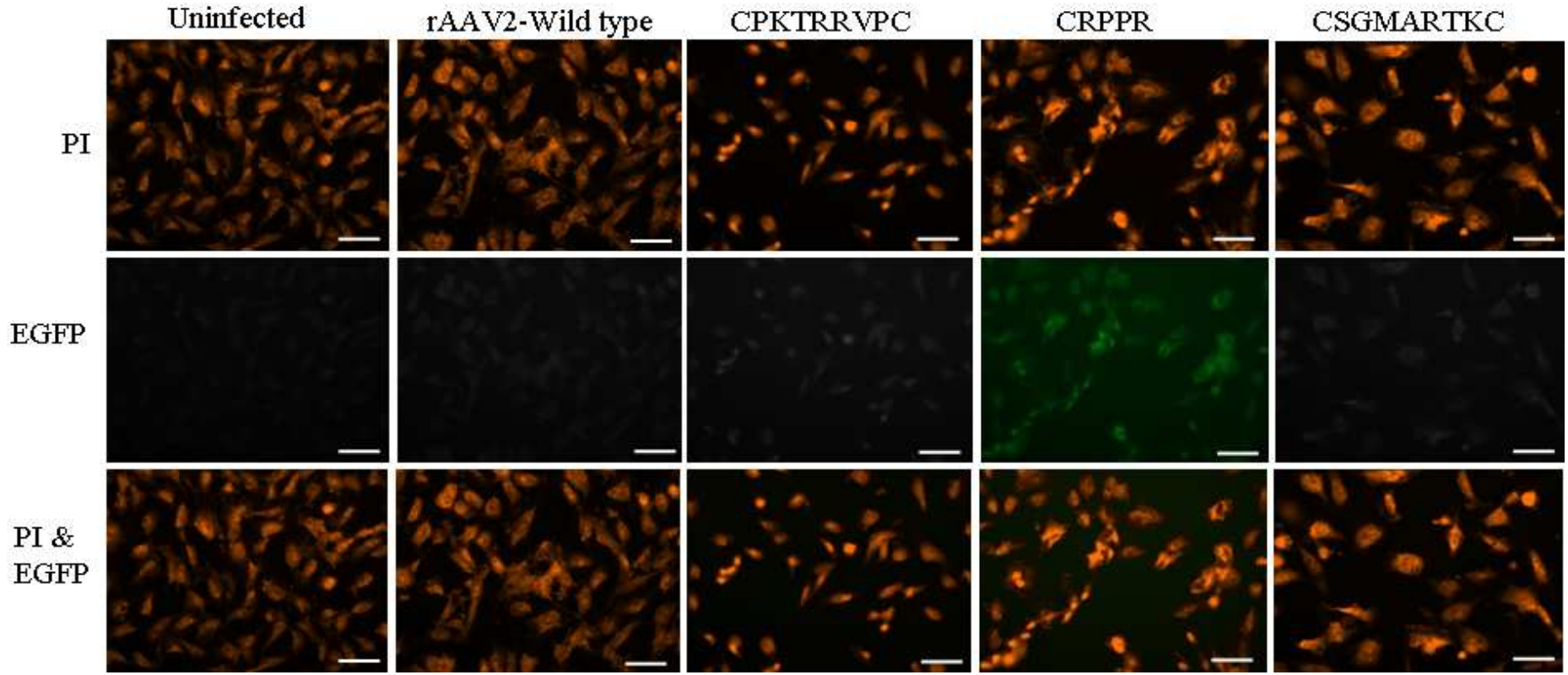


Figure 3.13 rAAV2 infection of human coronary artery endothelial cells.

HCAEC were infected with rAAV2 vectors at 10,000 particles/cell (n=6). Transduction ability of the vectors was observed 72 hours later by EGFP transgene expression and propidium iodide counterstaining. Scale bar = 50 μm, magnification = × 20.

3.3 Discussion

We demonstrate that heart targeting peptides isolated from a random phage-display library show increased selectivity for the heart in comparison to non-recombinant phage when intravenously infused into WKY rats. The heart specificities of the phage were up to 2000-fold higher than that of non-recombinant phage. Originally isolated through a combination of *ex vivo* biopanning of murine endothelial cells and *in vivo* biopanning in mice, four out of the six peptide's homing abilities were shown to be conserved cross-species.

The identification of the cognate receptors for these four targeting peptides not only aids the assembly of a ligand-receptor vascular map (Sergeeva, Kolonin et al. 2006), but also allows for the possibility of efficiently targeting therapeutic compounds to specific receptors on vascular endothelial cells (Balestrieri and Napoli 2007). It also allows the possibility of identifying other targeting peptides that display higher affinities to the identified vascular endothelial markers. However, in contrast to the studies by Zhang et al (Zhang, Hoffman et al. 2005), which confirmed high levels of expression of all three putative receptors in the hearts of mice, we could only localise two out of three receptors to the heart, with no detection of MPCII-3 expression being found in the heart of WKY rats by immunohistochemical analysis. The discrepancy in receptor expression may be due to a species difference between WKY rats and mice in expression of the vascular target MPCII-3. However, the corresponding peptide, CRSTRANPC, was shown to have the highest fold change in heart targeting capacities of all the phage tested, and so it is unlikely that its receptor is not expressed in the WKY rat heart. It remains to be shown whether this peptide binds to the same receptor in the rat as is does in the murine model.

The utility of bioactive peptides is not limited to the targeting of viral vectors. Targeting peptides can also be used to enhance tissue specific uptake of genes or drugs. Heart targeting peptides could be used to carry pharmacologically active compounds for improved targeted therapy. Peptides specifically targeting tumour neovasculature have been linked to liposomes carrying the chemotherapy drug

doxorubicin and have been shown to enhance the efficacy of the drug (Lee, Lin et al. 2007). Similar approaches could be applied to drugs for the treatment of cardiovascular diseases. Potentially, targeting peptides could be used for the delivery of cytoprotective drugs to the heart (McGuire, Samli et al. 2004) to prevent damage to the heart muscle that is associated with the treatments of non-cardiovascular disease, such as radiation and chemotherapy (Goethals, De Winter et al. 2002). The heart targeting peptides also have utility in the linking to other peptides, which could include antisense oligonucleotides and inhibitory peptides, such as novel oxidase homologues (NAD(P)H) inhibitors (Cifuentes and Pagano 2006). Targeting peptides have been studied in the context of the treatment of obesity. White adipose tissue vasculature targeting peptides linked to a proapoptotic peptide were shown to ablate white adipose tissue, leading to a potential targeted therapeutic for the treatment of obesity (Kolonin, Saha et al. 2004).

The identification of heart targeted peptides ultimately allows the potential for the generation of novel retargeted gene delivery vectors. However, we found that the phage-display derived cardiac targeting peptides proved disappointing in the context of viral vector retargeting. Ad19p vectors have a naturally reduced hepatic tropism in comparison to other Ad vectors (Denby, Work et al. 2004; Denby, Work et al. 2007). Therefore it was proposed that these vectors would provide the ideal context in which to study the isolated phage peptides. After engineering of the Ad19p fiber to accommodate the four heart targeting peptides, we found that there was no selective targeting, either to endothelial cells *in vitro* or to the heart after intravenous *in vivo* infusion. Intravenous injection of Ads has previously been reported to be hampered by a number of complex interactions with the blood (Shayakhmetov, Gaggar et al. 2005; Lyons, Onion et al. 2006; Parker, Waddington et al. 2006; Baker, McVey et al. 2007). However, as we found no convincing evidence of enhanced transduction of vascular endothelial cells *in vitro*, it is unlikely that this is the only reason for poor vascular transduction *in vivo*. The incorporation of the targeting peptides into the HI loop on the Ad19p fiber may have led to hampered production of virus by hindering virus packaging and assembly or could also have been detrimental to fiber trimerisation. However, targeting with 7-mer peptides has been shown to be efficient for renal gene delivery (Denby, Work et al. 2007). T7 phage display promotes the selection of stable ligands through the display of active conformations of the peptides

through the use of constrained configurations (Falciani, Lozzi et al. 2005). Despite this fact, binding properties of these selective peptides may have been changed through the incorporation into the viral envelope. It is also important to consider that these vascular targeting peptides were selected for their cell binding abilities and not for their abilities to aid vector cell entry or subsequent steps needed for genome nuclear translocation or transcription. They were also selected from a prokaryotic environment.

Despite the promising targeting *in vitro* results from the peptide-modified AdKO1 vectors, they have not be tested *in vivo* since recent studies have demonstrated that the biodistribution and transduction profiles of CAR binding-ablated AdKO1 is not significantly altered when tested *in vivo* (Alemany and Curiel 2001; Mizuguchi, Koizumi et al. 2002; Rittner, Schreiber et al. 2007). Recently, it has been shown that blood coagulation factors, predominantly factor X, enhance hepatocyte transduction by binding directly to the adenovirus capsid, bridging the virus to HSPG receptors, expressed abundantly in the liver and providing a CAR-independent means of cell transduction (Parker, Waddington et al. 2006; Waddington, Parker et al. 2007). The use of warfarin to globally down-regulate vitamin K dependent coagulation zymogens ablated liver uptake of CAR binding-ablated Ad5 (Parker, Waddington et al. 2006) and CAR binding Ad5 (Waddington, Parker et al. 2007). The utilisation of this technique to knock-out coagulation factors and significantly reduce liver uptake of the peptide-modified AdKO1 vectors would allow for their retargeting abilities to be tested *in vivo*.

The genetic insertion of targeting ligands into the capsid of rAAV2 vectors is a commonly applied approach to genetically retarget the vectors. However, often the insertion of a peptide can result in a reduction in yield of viral particles, through defects in genome encapsidation which inevitably leads to poor transduction efficiency (Douar, Poulard et al. 2003). The low titre of the rAAV2 vectors that were obtained would suggest similar results in our study.

In summary, these applied approaches did not lead to the identification of an efficient method for the delivery of potentially therapeutic genes to the vasculature. The cardiac vasculature targeting peptides isolated by T7 phage display did not retarget

the viral vectors tested. Because of their homing abilities when displayed as peptides, they may be useful as targeting peptides in combination with, for example drugs, to provide enhanced therapeutically beneficial properties.

Chapter 4

Characterisation of rAAV6 and rAAV9 vector-mediated transduction of the myocardium in vivo in the SHRSP

4.1 Introduction

A major challenge to overcome in cardiac gene therapy is the limited availability of vectors that provide efficient delivery via a minimally invasive route of administration. Recently, adeno-associated viruses (AAV) have emerged as promising vectors for cardiac gene transfer (Du, Kido et al. 2004; Wang, Zhu et al. 2005; Palomeque, Chemaly et al. 2007). To date eleven serotypes of AAV have been identified. However, the most characterised of these vectors, AAV2, has demonstrated poor tropism for many cells, including endothelial cells, and tends to transduce non-vascular tissue more readily than it does vascular tissue (Nicklin, Buening et al. 2001; Dishart, Denby et al. 2003). This disappointment in the performance of rAAV2 vectors in basic science and clinical trials has directed the focus of intense research efforts to the identification and development of novel serotypes. Exploitation of alternative AAV isolates with differing tissue tropisms could overcome this limitation faced by conventional AAV2 vectors.

Among the AAV serotypes tested so far, certain serotypes including AAV6, -8 and -9 have displayed powerful tropism for skeletal and cardiac muscle (Kawamoto, Shi et al. 2005; Wang, Zhu et al. 2005; Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006), achieving high cardiac transduction rates following local and systemic injection (Wang, Zhu et al. 2005; Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006). AAV6 has evolved through recombination between AAV1 and AAV2 and differs in only six amino acids in the capsid region from AAV1 (Gao, Vandenberghe et al. 2004). Intravenous delivery of recombinant AAV vectors pseudotyped with serotype 6 capsid proteins (rAAV6) leads to transduction of the skeletal musculature at levels >500-fold higher than rAAV2 vectors in mice (Blankinship, Gregorevic et al. 2004). Moreover, extensive transgene expression was observed throughout the entire skeletal musculature when mice were intravascularly administered rAAV6 in combination with vascular endothelial growth factor (VEGF) (Gregorevic, Blankinship et al. 2004). High level expression was dependent on co-administration with VEGF for vector administered at lower dose levels of 2×10^{11} vp/mice. Importantly, there was high levels of transduction of the cardiomyocyte population in mice receiving rAAV6 vectors in combination with acute vascular permeabilisation with VEGF (Gregorevic,

Blankinship et al. 2004). In a subsequent study with direct comparison to rAAV2 vectors, rAAV6 vectors were shown to transduce a 10-fold larger volume of the myocardium and achieve a 5-fold greater transgene activity after local myocardial delivery in mice (Kawamoto, Shi et al. 2005). The same study also demonstrated the capacity of rAAV6 vectors to mediate early onset transgene expression in comparison to rAAV2 vectors when the β-actin based hybrid promoter was used to accelerate the time to gene expression (Kawamoto, Shi et al. 2005). Recently, rAAV6 vectors have also been used to achieve high levels of transgene expression in rat hearts up to 24 weeks after local gene delivery (Palomeque, Chemaly et al. 2007).

One of the most recently identified AAV serotypes is AAV9, and has been shown to have similar properties to AAV8 being predominantly found in the liver and bone marrow and differing by 14.3% in capsid amino acid sequence (Gao, Vandenberghe et al. 2004). In a recent study, pseudotyped rAAV9 vector-mediated liver transduction was shown to correct haemophilia A in mice and dogs (Sarkar, Mucci et al. 2006), demonstrating the pre-clinical potential of alternative naturally occurring AAV vectors. Vectors based on AAV9 have also been shown to transduce murine myocardium at 5 to 10-fold higher levels than AAV8-pseudotyped vectors, resulting in over 80% cardiomyocyte transduction following tail vein injection with as low as 1×10^{11} particles per mouse (Inagaki, Fuess et al. 2006). In a direct comparison with rAAV1 vectors, rAAV9 vectors produce higher levels of transgene expression in mice and nonhuman primates (Pacak, Mah et al. 2006). The vector biodistribution profile of rAAV9 was not affected by age of the animals, displaying a similar preference for cardiac tissue over skeletal muscle in both neonates and adult mice. Transgene expression levels from rAAV9 vector increases overtime for at least 56 days post-injection in mice (Pacak, Mah et al. 2006). Stable transgene expression with rAAV9 vectors has been detected for at least 9 months (Limberis and Wilson 2006), illustrating the utility of this vector for the treatment of pathologies, such as cardiovascular disease, where long-term transgene expression is required. Furthermore, is has been shown that rAAV9 can be re-administered, despite the presence of neutralising antibodies without limiting effects on transgene expression levels (Limberis and Wilson 2006).

No studies to date have compared the capacity of rAAV6 and -9 to achieve cardiac transduction following intravenous injection into rats. We used stroke-prone spontaneously hypertensive rats (SHRSP), an established model of cardiovascular disease with genetically bred predisposition to hypertension and stroke sensitivity (Okamoto, Yamori et al. 1974). The colony of SHRSP was obtained by the selective breeding of a substrain of SHR which had been found to display a high incidence of spontaneous cerebrovascular disease. Moreover, SHRSP develop concentric left ventricular hypertrophy (LVH) in response to blood pressure elevation (Ohtaka 1980) that is evident at 12 weeks of age (Davidson, Schork et al. 1995) and also display endothelial dysfunction (McIntyre, Hamilton et al. 1997; Kerr, Brosnan et al. 1999). It is considered a relevant pathogenetic model for research into human disease as the SHRSP and humans share many pathophysiological similarities, such as local factors for stroke, (Yamori, Horie et al. 1976), and consistent with humans, male SHRSP maintain a higher blood pressure than females. Here, we document the cardiac gene delivery profiles of AAV6 and -9 in SHRSP.

Although rAAV6 efficiently transduces the myocardium in mice, it also targets all striated muscle. An immunological reaction can sometimes be observed after foreign gene transfer to skeletal muscles as a result of expression of the transgene in non-muscle antigen-presenting cells, (Hauser, Robinson et al. 2000; Hartigan-O'Connor, Kirk et al. 2001). The incorporation of a transcription-regulating element that limits transgene expression to the myocardium would reduce transgene expression in both skeletal muscle and non-muscular cells. Inclusion of a cardiac specific promoter may further improve the selectivity of this vector. The MLC-2v promoter is one of the most well characterised cardiac specific promoters (Small and Krieg 2004). It has been shown to drive AAV2 vector-mediated transgene expression at levels comparable to that of the CMV promoter (Phillips, Tang et al. 2002). This promoter has yet to be characterised in rAAV6 vectors.

Here we aim to characterise an efficient cardiac gene delivery vector and to introduce an element of transcriptional control to improve the efficiency of the chosen vector.

4.2 Results

4.2.1 Production of rAAV6 vectors

The transfection of pAAV*lacZ* and pDGM6 into 293T cells (encoding the E1 region of the Ad5 genome) produced rAAV6 vectors. pAAVlacZ harbours a β-galactosidase expression cassette flanked by ITR's, whilst the pDGM6 plasmid is the packaging and helper plasmid that also contains the serotype 6 capsid reading frame (i.e. contains AAV2 rep genes, AAV6 cap genes and Ad5 E2, E4 and VA genes). These two plasmids were used to make a small scale prep of rAAV6 with which to test *in vitro*. A microBCA assay detected the presence of rAAV6 and allowed the titering of rAAV6 in comparison to the protein levels of known titre rAAV2 vectors. This assay confirmed the production of rAAV6 vectors, albeit it at low levels, with a mean titre of 2.4×10^{10} gp/ml.

4.2.2 In vitro testing of rAAV6

Most studies have utilised rAAV6 vectors *in vivo* as a result of the inefficient transduction of cells *in vitro*. Therefore, the ability of rAAV6 vectors to transduce a variety of cell lines was tested by infecting cells with different doses of rAAV6. L6, H9C2, HeLa and HT1080 cell lines were infected with 5000, 10000 or 20000 gp/cell. Ad5 wild type was added to aid single stranded DNA conversion to double stranded DNA to enhance transgene expression and was added at a multiplicity of infection (MOI) appropriate for the cell line (L6 = MOI 5, HeLa = MOI 0.1, H9C2 = MOI 5, HT1080 = MOI 1). rAAV6 vectors showed no transduction of any cell line, in comparison to control uninfected cells (Figure 4.1A). Adenovirus co-infection did not greatly increase transduction of any of the cell lines, contrary to what would be expected (Figure 4.1A). Infection of HepG2 cells to compare rAAV6 vectors made in the laboratory and rAAV6 vectors made by collaborators with rAAV2 vectors showed that both batches of rAAV6 vectors were inefficient transducers, especially in comparison to rAAV2 (Figure 4.1B). Thus no optimal cell line could be found that would allow efficient transduction. This confirmed that rAAV6 vectors are poor at *in vitro* transduction, even cells of skeletal and cardiac origin.

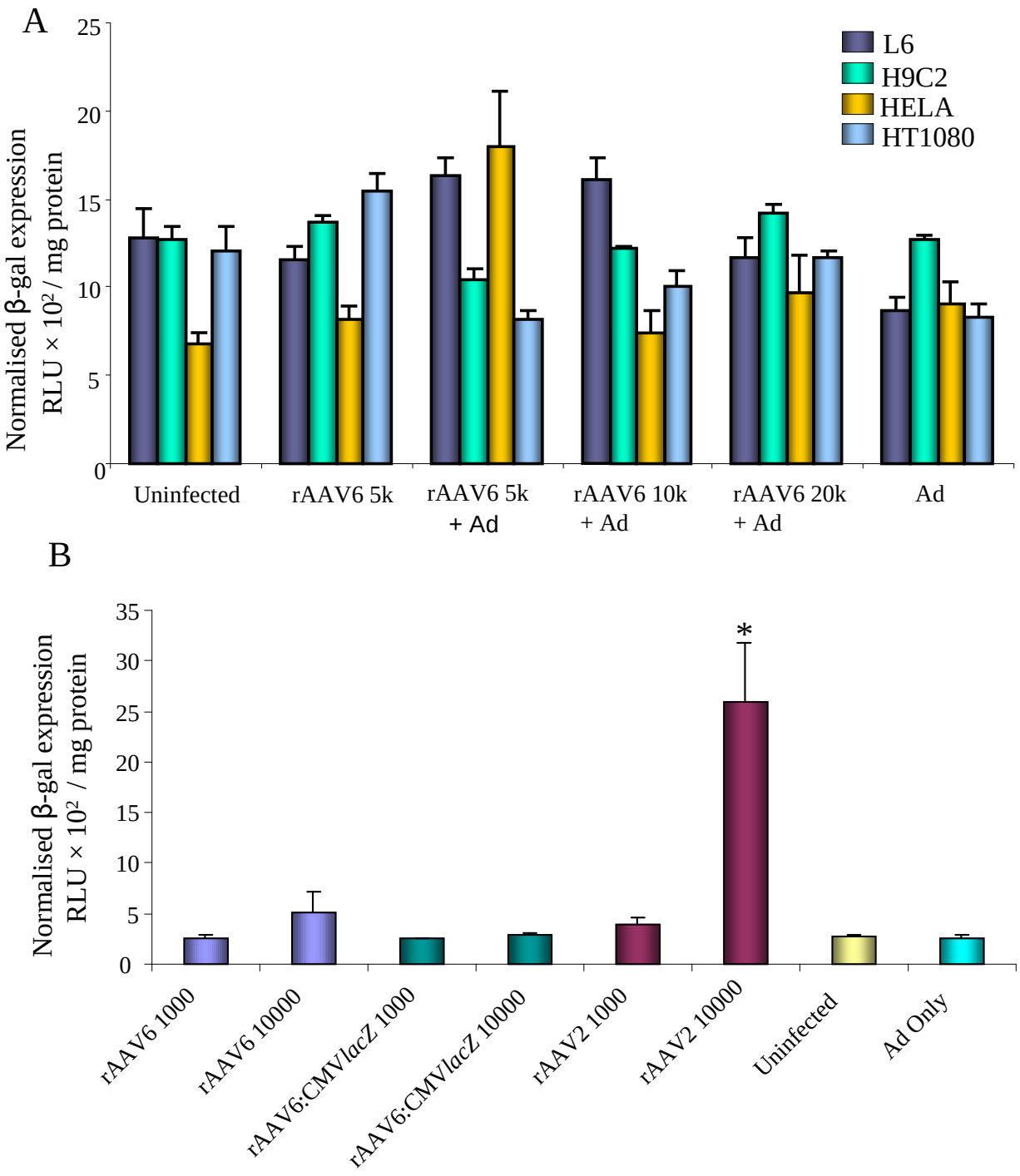


Figure 4.1 ***In vitro*** **transfections with rAAV6.**

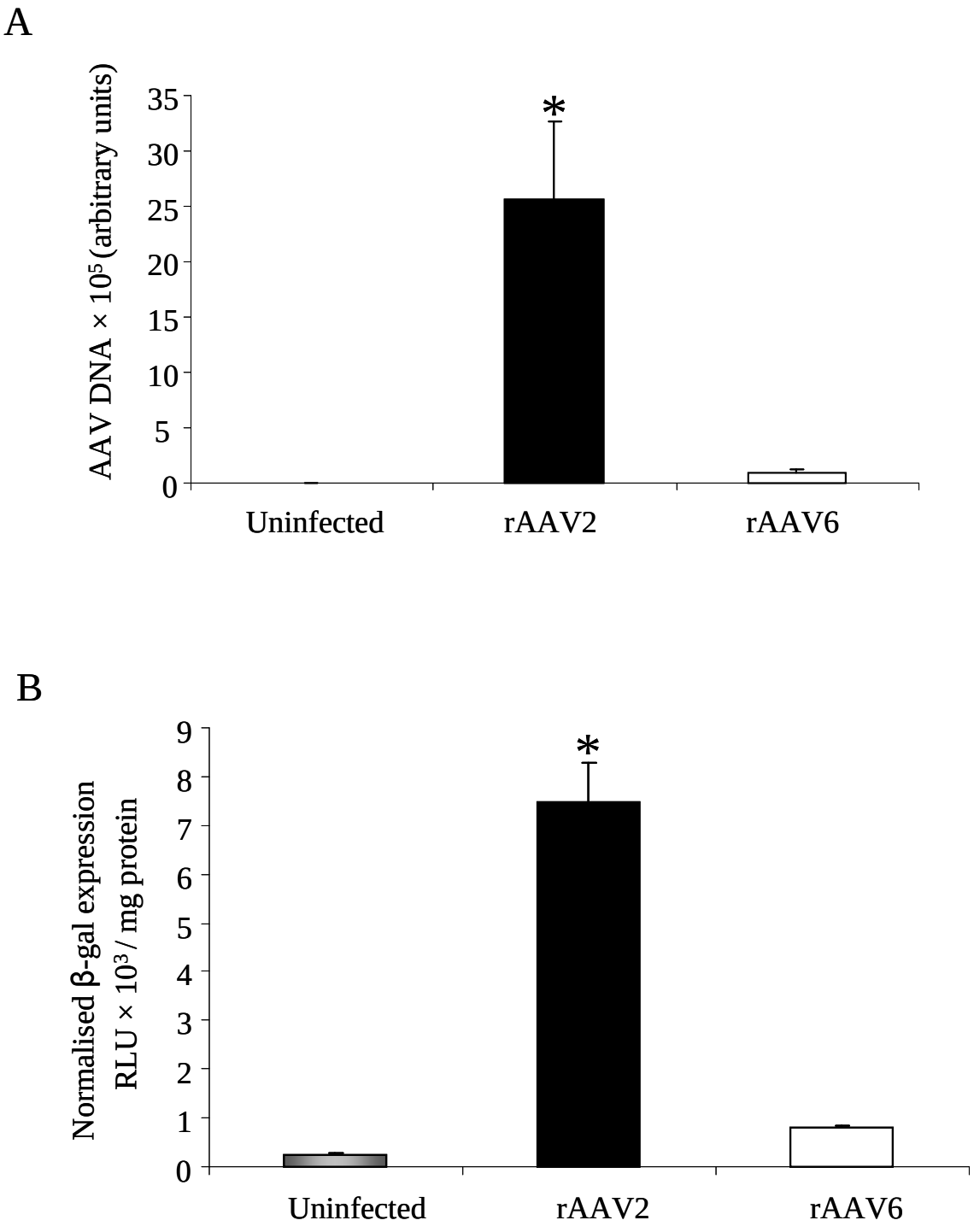
(A) Transduction of L6, H9C2, HeLa and HT1080 cell lines by rAAV6 vectors. Cells were infected with rAAV6 at MOI 5000, 10000 and 20000 gp/cell. Ad5 wild type virus was added at MOI appropriate for the cell line (n=6/group). (B) Transduction of HepG2 cells by rAAV6 made in the laboratory and rAAV6 from collaborators in comparison with rAAV2 (n=6/group). * $p<0.01$ as compared to uninfected by one way ANOVA and Bonferroni posttest. β-gal expression quantified by Tropix Galacto-Light Plus assay.

4.2.3 Binding and transduction of rAAV6

Because of the poor *in vitro* transduction profile of rAAV6 vectors, we investigated the binding (Figure 4.2A) and transduction (Figure 4.2B) properties of rAAV6 in comparison to rAAV2 vectors in HepG2 cells. rAAV6 vectors demonstrated very poor binding and transduction of HepG2 cells, in comparison to rAAV2 vectors. Because rAAV6 vectors had previously been used effectively *in vivo* (Blankinship, Gregorevic et al. 2004; Gregorevic, Blankinship et al. 2004), we proceeded to *in vivo* studies.

4.2.4 rAAV6 and rAAV9 biodistribution and transduction profiles in SHRSP following intravascular delivery

We first determined the capacity of rAAV6 and -9 vectors to transduce the SHRSP heart following a single bolus injection into the femoral vein. To determine the efficiency of *in vivo* gene transfer in rats mediated by rAAV6 and -9 vectors, male 6-week old SHRSP were administered a single intravenous injection of increasing doses of rAAV6:CMV*lacZ* (2×10^{11}, 1.5×10^{12} and 3×10^{12} vp/rat) in the presence or absence of recombinant human VEGF-165 (20 µg/100g body weight) or rAAV9:CMV*lacZ* at identical doses. VEGF co-administration with rAAV9 was not included in the study as preliminary results had revealed rAAV6 was capable of myocardial transduction in the absence of VEGF and so was considered unecessary and undesirable for a gene delivery vector.Both rAAV6 and rAAV9 mediated high level gene transfer in hearts, which was dose-dependent (Figure 4.3). Transgene expression in the SHRSP for rAAV6 was not modified by VEGF co-administration (Figure 4.3). Staining for transgene expression showed higher expression levels in the heart following rAAV6 transduction compared to rAAV9, although both vectors were relatively efficient (Figure 4.4). As expected (Gregorevic, Blankinship et al. 2004; Pacak, Mah et al. 2006) in non-cardiac muscle beds (skeletal), both rAAV6 and -9 vectors achieved high levels of gene delivery (Figure 4.5 and Figure 4.6). High levels of transgene expression were seen in the diaphragm of the rAAV9 transduced animals, which was absent in the rAAV6 transduced group. Similar vector biodistribution by X-gal staining was observed for both vectors in liver, lung and kidney (Figure 4.5 and Figure 4.6).



<u>Figure 4.2.</u> Binding and transduction profiles of rAAV2 and rAAV6.

HepG2 cells were infected with 10,000 gp/cell rAAV2 or rAAV6 vectors and compared for (A) binding and (B) transduction efficiencies (n=3/group). *$p<0.001$ rAAV2 vs. rAAV6 and uninfected by one way ANOVA and Bonferonni posttest. β-gal expression quantified by Tropix Galacto-Light Plus assay.

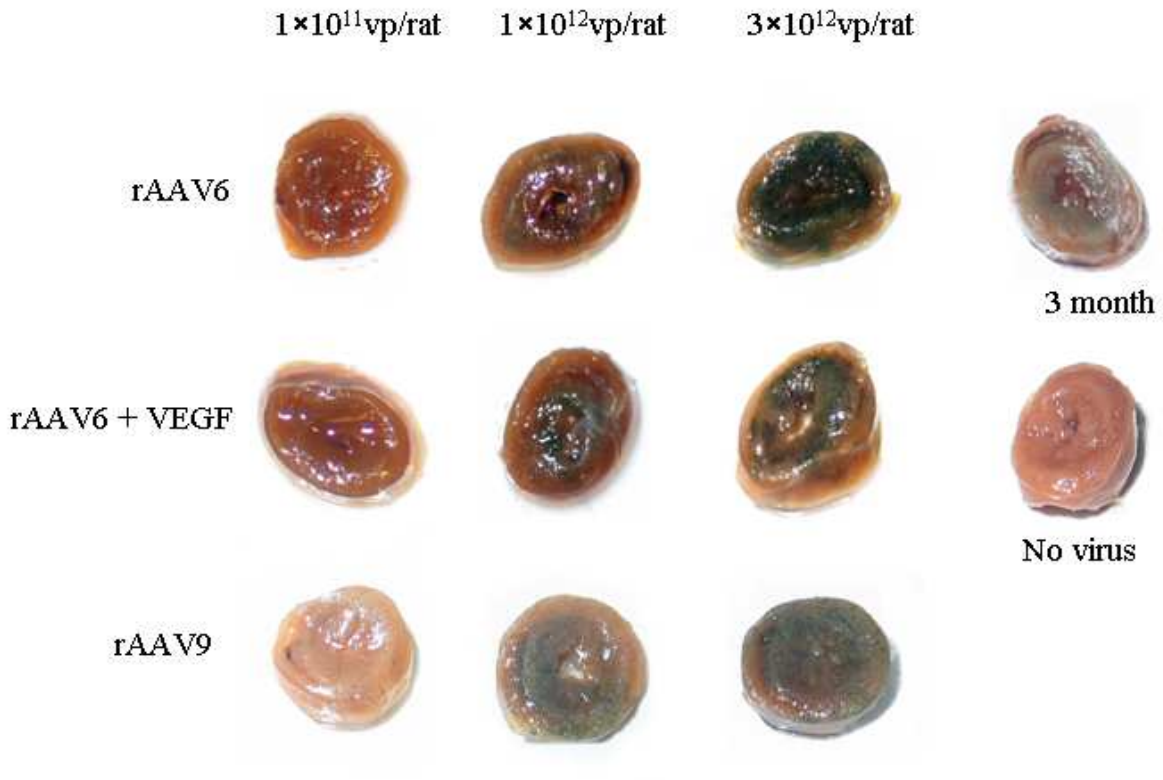


Figure 4.3. Transduction of cardiac tissue by rAAV6 and rAAV9.

SHRSP were infused with 3 different doses of rAAV6:lacZ or rAAV9:lacZ (1×1011 vp, 1×1012 vp or 3×1012 vp). Transverse slices of heart tissue were fixed and stained for β-galactosidase expression *en face* 14 days post-delivery of either rAAV6, rAAV6 + VEGF or rAAV9. n=1/group.

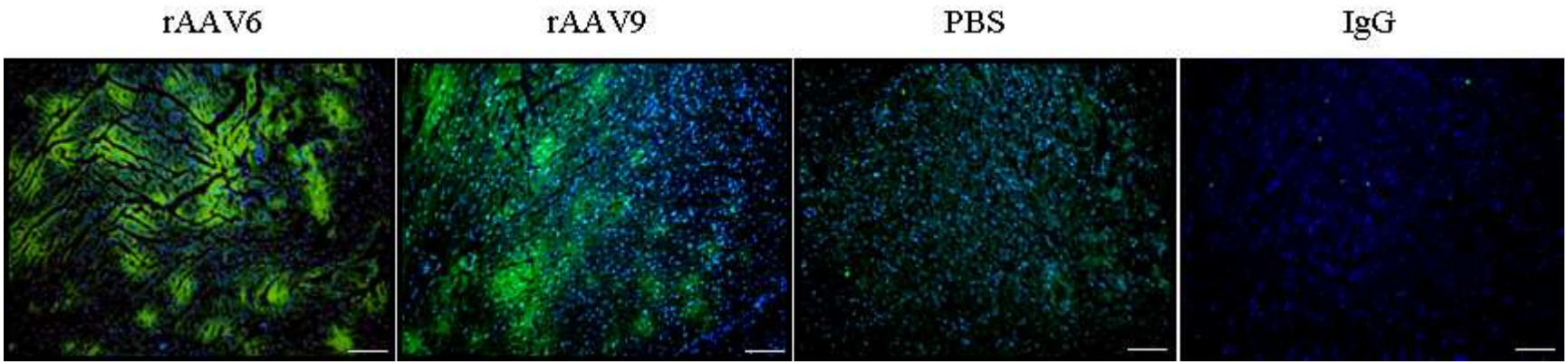
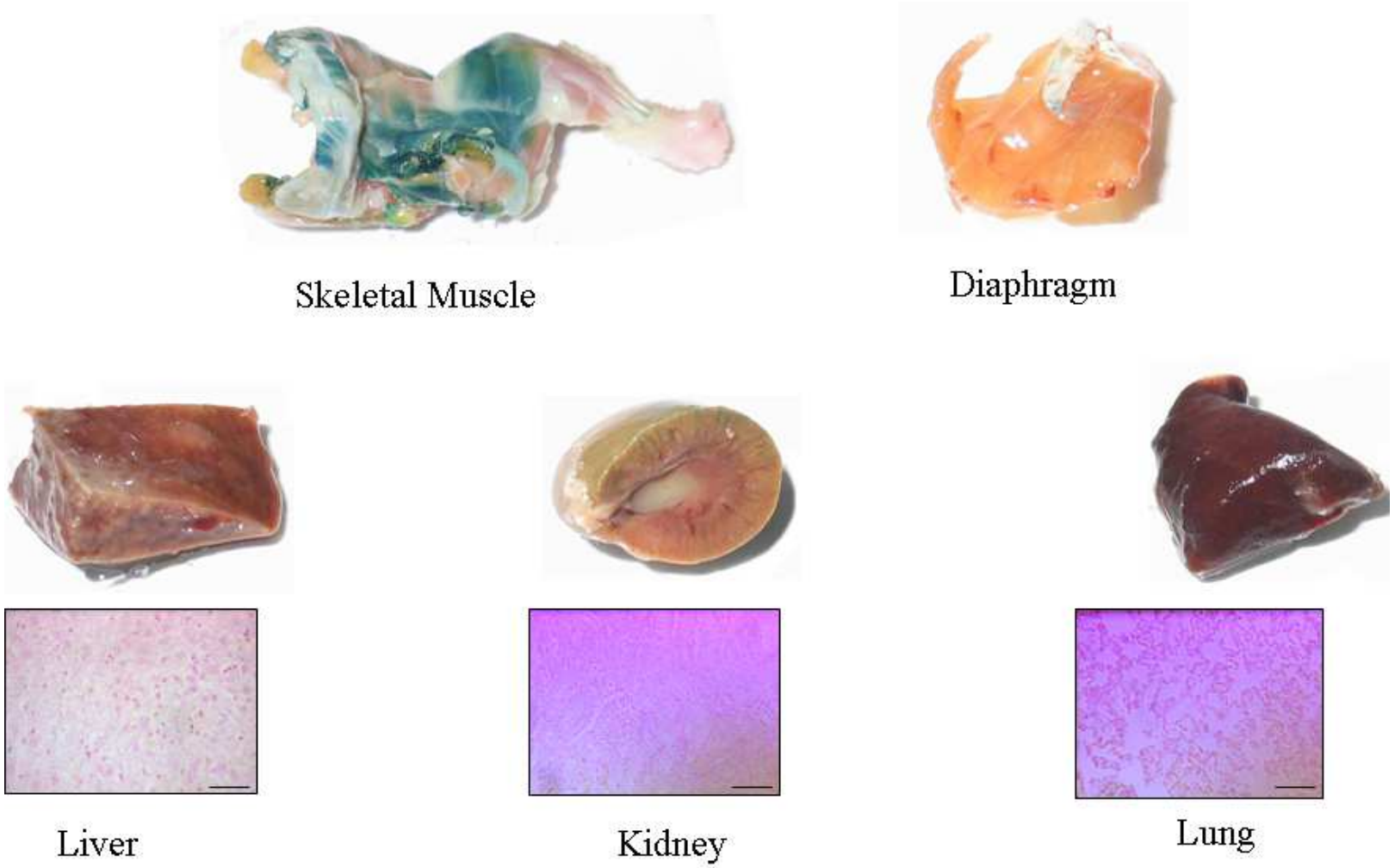


Figure 4.4. Detection of rAAV6 and rAAV9 vector-mediated transgene expression.

Immunofluorescence detection of β-galactosidase expression with nuclear counterstain DAPI, in sections of heart from rAAV6 and rAAV9-transduced animals and a PBS infused control animal and non-immune IgG control (n=1/group). Scale bar = 50 µm.



<u>Figure 4.5.</u> rAAV6:CMV*lacZ* transduction profile in non-cardiac tissue.

rAAV6:CMV*lac*Z vectors ($3x10^{12}$ vp/rat) were infused into 6 week old SHRSP rats (n=1). Tissues were stained for β-galactosidase after 14 days. Scale bar = 50 μm, magnification × 20**.**

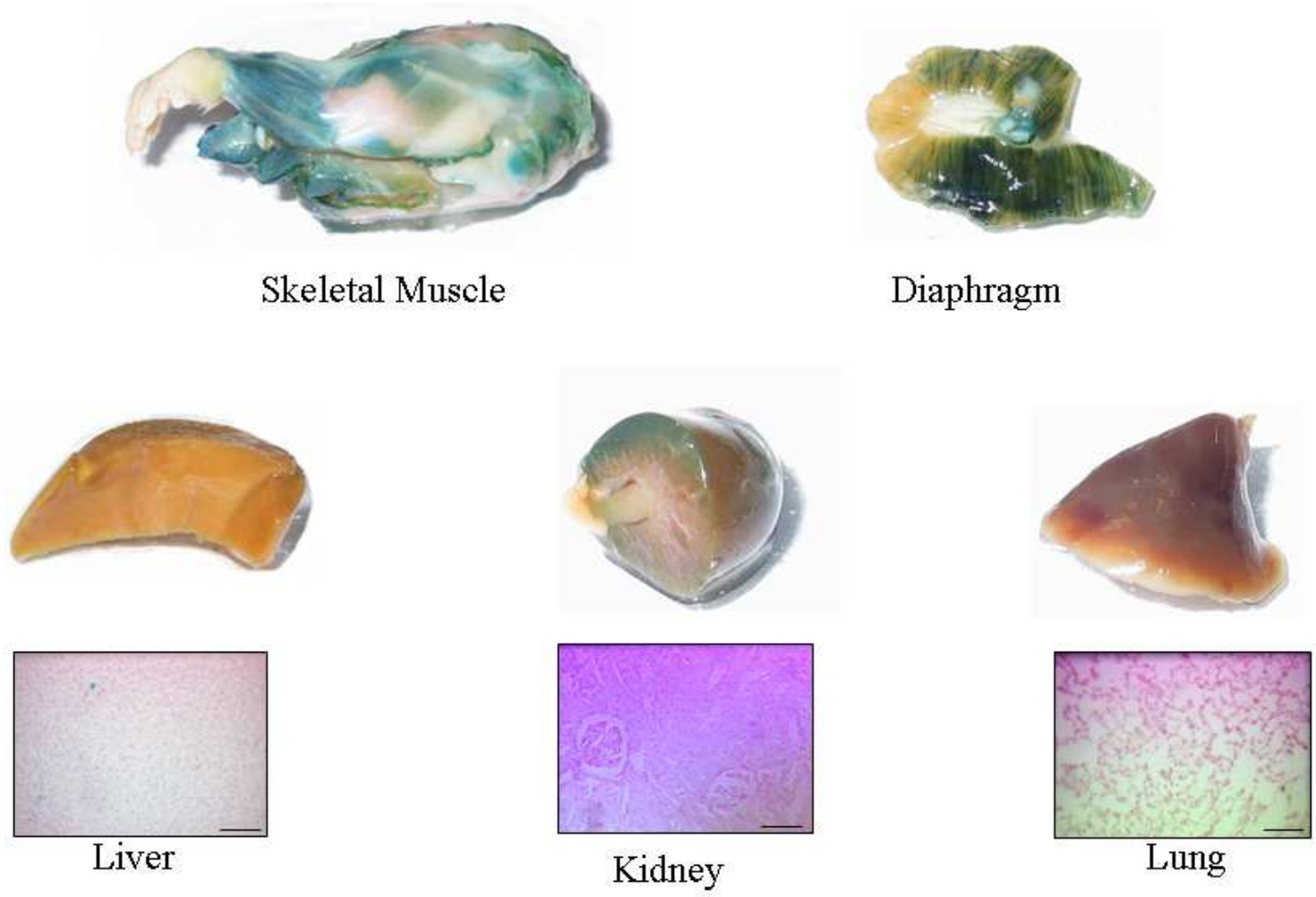


Figure 4.6. rAAV9:CMV*lacZ* transduction profile in non-cardiac tissue.

rAAV9:CMV*lac*Z vectors ($3x10^{12}$ vp/rat) were infused into 6 week old SHRSP rats (n=1). Tissues were stained for β-galactosidase after 14 days. Scale bar = 50 μm, magnification × 20.

Biodistribution studies to quantify vector genomes by TaqMan™ Q-PCR revealed marked differences in vector genome accumulation. In particular, accumulation of rAAV9-packaged genomes in the heart (Figure 4.7) was approximately 10-fold lower (at the highest dose) despite comparable genome levels in skeletal muscle (Figure 4.8A) The CPRG assay confirmed the comparable levels of transgene expression in the skeletal muscles (Figure 4.8B). Furthermore, biodistribution of rAAV9-packaged genomes to kidney was far higher than rAAV6, although immunohistochemical analysis revealed no obvious transgene expression (Figure 4.9).

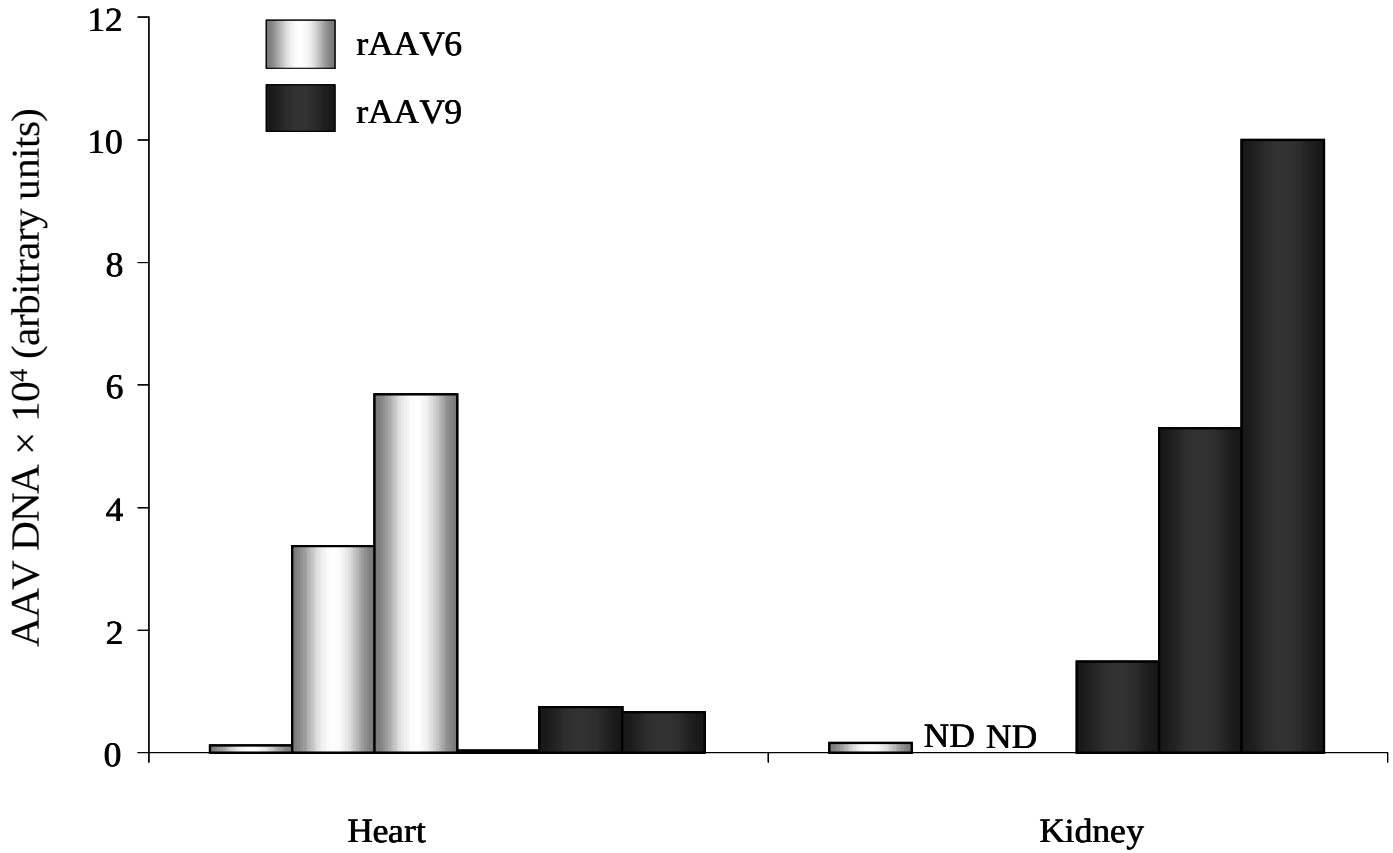
Taken together, we concluded that rAAV6 exhibited a more favourable profile for cardiac gene delivery than rAAV9, and represents a useful tool for studying the molecular mechanisms of cardiac disease.

4.2.5 Cloning of cardiac-specific promoter into AAV6 vector

To clone the MLC-2v AAV shuttle plasmid (Figure 4.10), 5 µg pMV10 containing a *lacZ* and corresponding poly-A tail was digested with 2.5 µl of *Xba*I and 2.5 µl *Hin*DIII. The released *lacZ* and poly-A tail (4.5 kb) were then gel purified and cloned into pCMV6-XL4 (Stratagene, CA, USA). The short version of the MLC-2v promoter (280 bp) was amplified by PCR using primers with flanking *Xba*I sites (MLC2v forward 5'CCCTCTAGATTAGACAATGGCAGGACCCA3' and MLC2v reverse 5'CCCTCTAGAAATTCAAGGAGCCTGCTGGC3'). PCR was performed using standard conditions, with the exception of a 2 minute extension time and an optimised annealing temperature of 65ºC. The gel purified PCR product was then cloned into the X*ba*I sites in pCMV6-XL4. This construct (4.7 kb) was then cut out of pCMV6- XL4 using *Not*I restriction sites and cloned into the corresponding sites in pAAV-MCS (Stratagene, CA, USA). The completed construct could then be tested.

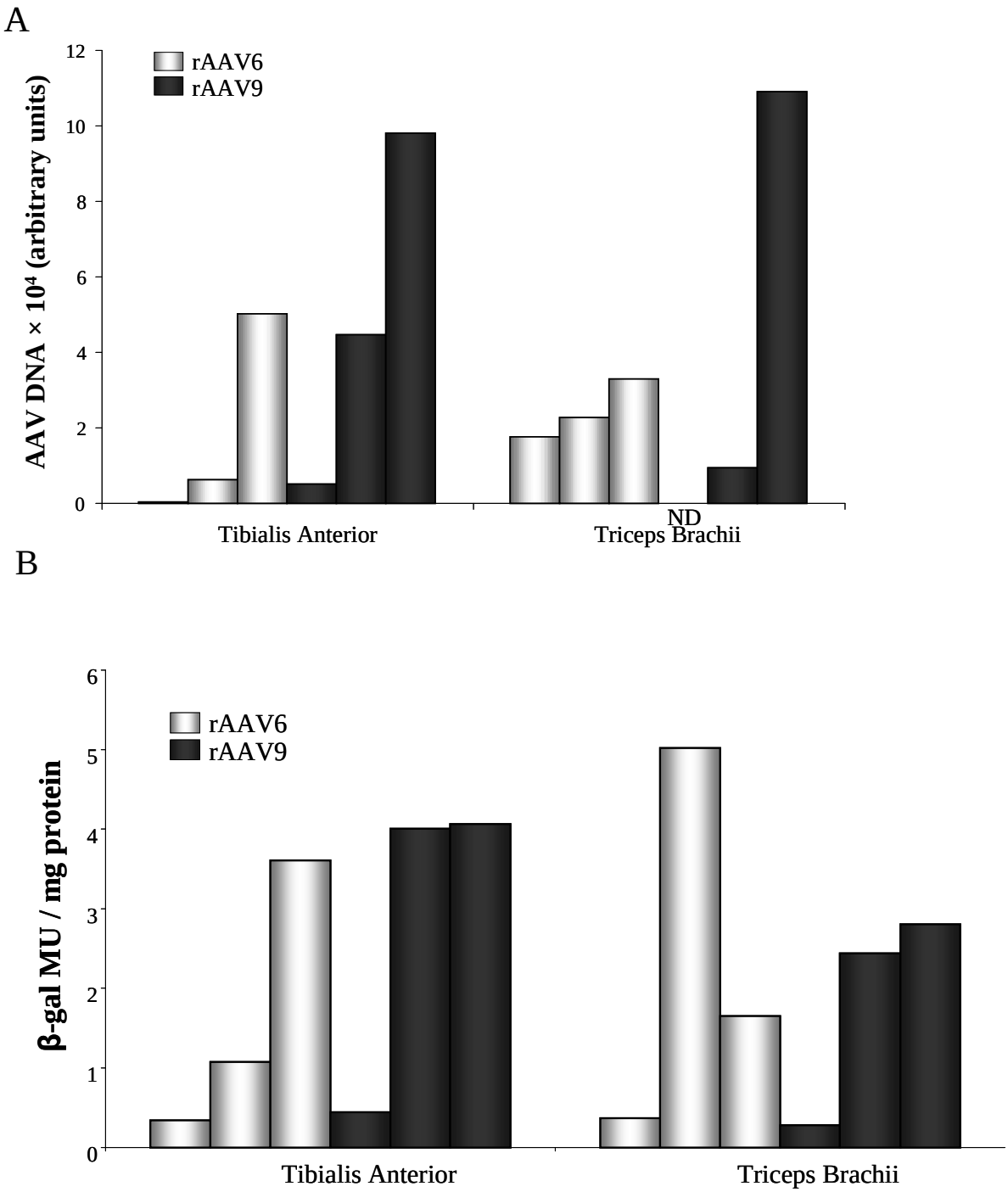
4.2.6 Characterisation of pAAV-MCS-MLC2v

The MLC2v promoter (Phillips, Tang et al. 2002) was cloned into the shuttle plasmid pAAV-MCS to allow packaging into rAAV6 vectors. Expression of the *lacZ* gene in various cell lines was carried out to test the function and specificity of the construct.



<u>Figure 4.7.</u> Transduction of tissues by rAAV6 and rAAV9.

SHRSP were infused with 3 different doses of rAAV6:CMV*lacZ* or rAAV9:CMV*lacZ* (1×10^{11} vp, 1×10^{12} vp or 3×10^{12} vp). Total DNA was extracted from heart and kidney and Q-PCR performed using *lac*Z primers. Data are plotted as mean quantity of viral particles in each tissue analysed. The three bars for each tissue with each virus represent the three different doses (left = lowest dose, right = highest dose). ND = not detectable. N=1/group.



<u>Figure 4.8.</u> Transduction of tissues by rAAV6 and rAAV9.

SHRSP were infused with 3 different doses of rAAV6:CMV*lacZ* or rAAV9:CMV*lacZ* (1×10^{11} vp, 1×10^{12} vp or 3×10^{12} vp). Total DNA was extracted from tibialis anterior and triceps brachii and (A) Q-PCR and (B) CPRG assay was carried out to quantify β-gal expression. The three bars for each tissue with each virus represent the three different doses (left = lowest dose, right = highest dose). ND = not detectable. N=1/group.

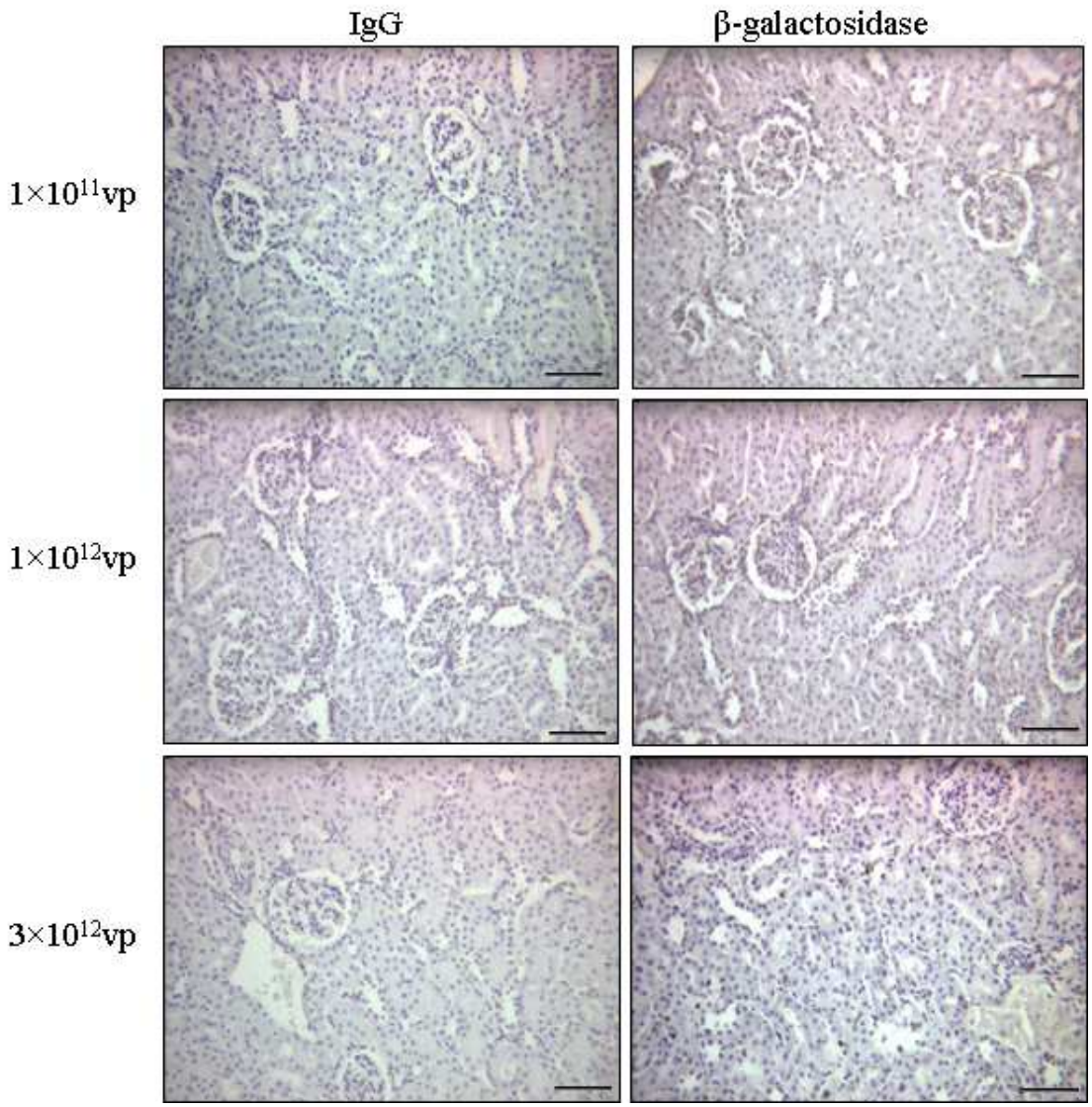


Figure 4.9. rAAV9:CMV*lacz* expression in the kidney.

Histological analysis with an anti-β galactosidase antibody was carried out on sections of kidney from the rAAV9:CMV*lacZ* infused animals at termination. Scale bar = 30 μm, magnification × 20, n=1/group.

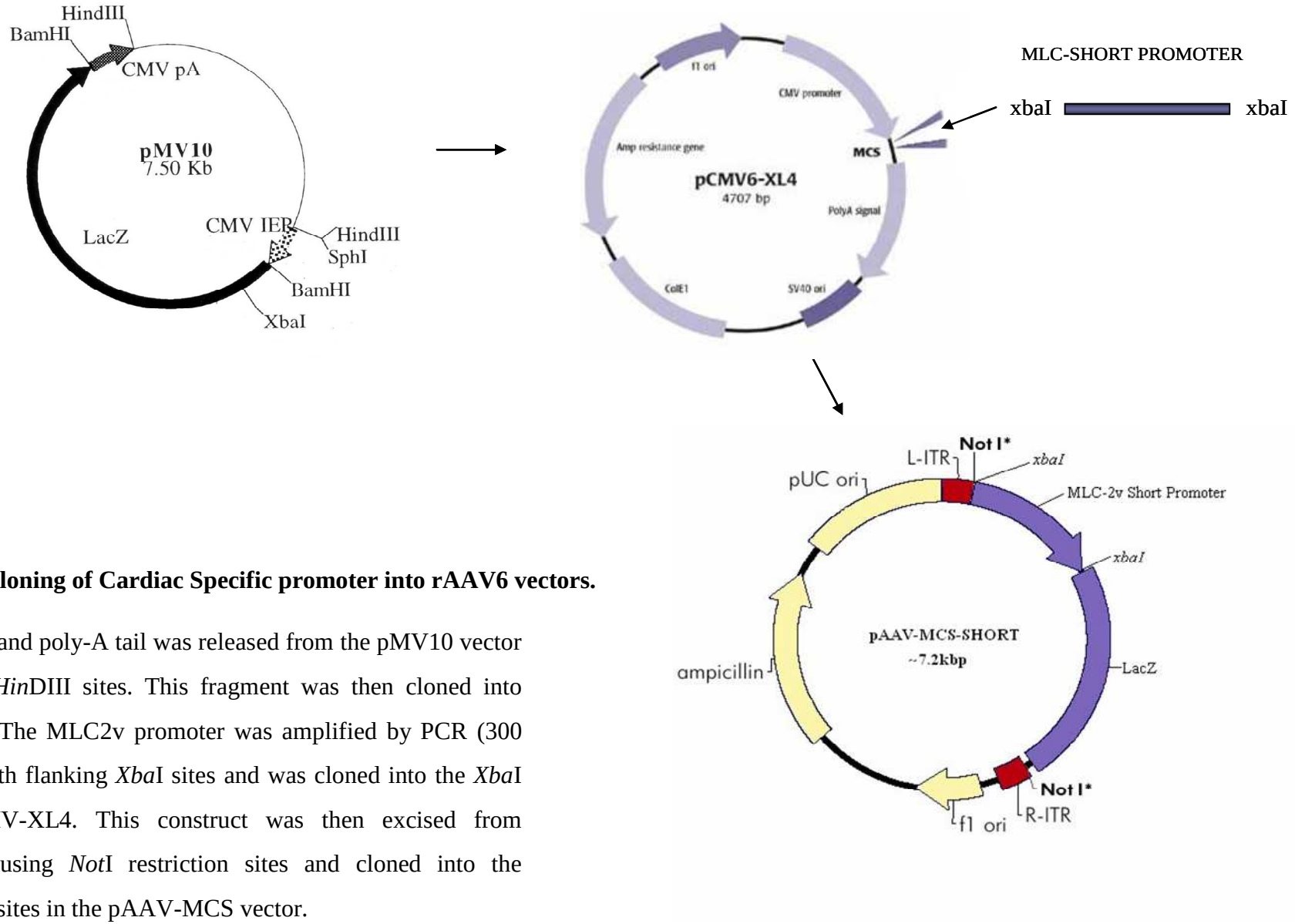


Figure 4.10. Cloning of Cardiac Specific promoter into rAAV6 vectors.

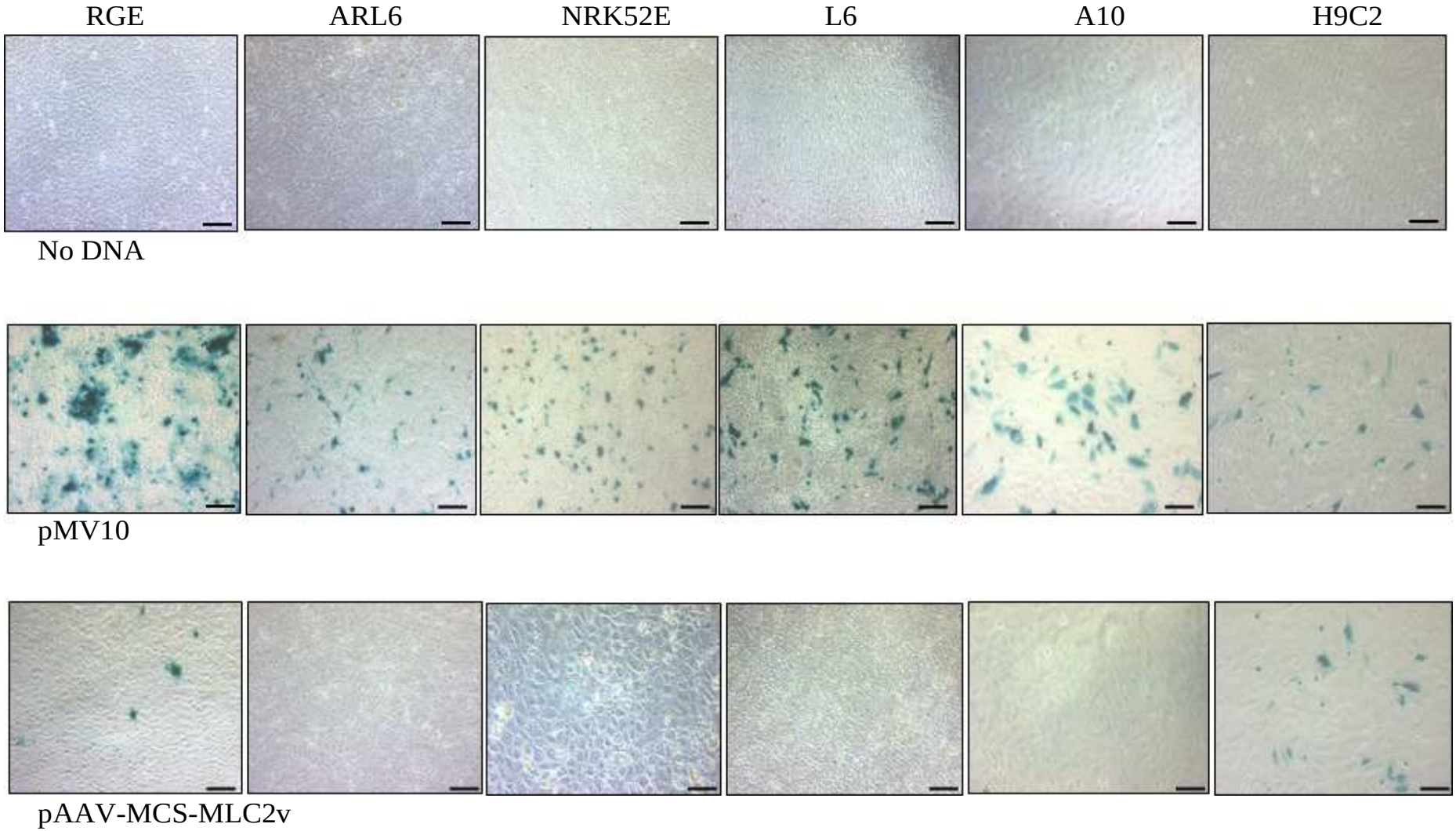
The *lacZ* gene and poly-A tail was released from the pMV10 vector on *Xba*I and *Hin*DIII sites. This fragment was then cloned into pCMV6-XL4. The MLC2v promoter was amplified by PCR (300 bp product) with flanking *Xba*I sites and was cloned into the *Xba*I sites of PCMV-XL4. This construct was then excised from PCMV6-XL4 using *Not*I restriction sites and cloned into the corresponding sites in the pAAV-MCS vector.

No β-galactosidase activity from pAAV-MCS-MLC2v was detected in ARL6, NRK52E, L6 or A10 cell lines (Figure 4.11). β-galactosidase activity was observed in H9C2 cells. A low level of β-galactosidase activity was observed in RGE cells. Quantification of β-galactosidase expression confirmed expression of control plasmid pMV10 in all cell lines tested, but not of pAAV-MCS-MLC2v (Figure 4.12). Expression from this plasmid was only detectable in H9C2 cells (Figure 4.12). Due to time restraints, RGE, NRK52E and A10 cell lines were not tested.

We next determined if the MLC2v promoter was active in the liver *in vivo*. Hydrodynamic injection of pAAV-MCS-MLC2v into the tail vein of mice confirmed that *in vivo* transgene expression from this promoter was not active in the liver (Figure 4.13A). No X-gal staining was observed in the livers of mice injected with pAAV-MCS-MLC2v, whilst high levels of staining were observed in mice injected with control plasmid pMV10 (Figure 4.13A). Quantification by β-galactosidase ELISA confirmed the lack of β-galactosidase activity in the livers of the mice hydrodynamically infused with pAAV-MCS-MLC2v (Figure 4.13B).

4.2.7 Transcriptional regulation in vivo

Our next aim was to assess the efficiency of the cardiac promoter in the SHRSP *in vivo*. We therefore injected male rats with increasing doses of rAAV6:MLC2v vectors at 6 weeks of age (2×10^{11}, 1.5×10^{12} and 3×10^{12} vp/rat). Unfortunately, X-gal staining revealed no β-galactosidase activity in any organ at either 2 or 12 weeks post-infusion, although quantification of vector genomes by Taqman™ confirmed presence of the vector *in vivo* (Figure 4.14). A dose-dependent response was observed in the heart, tibialis anterior and triceps brachii. High levels of vector accumulation in the kidney at 12 weeks post-infusion can also be observed. When compared to rAAV6:CMV*lacZ*, levels of vector genomes present in the tissues were generally less with rAAV6:MLC2v*lacZ* (Figure 4.15). In all tissues, there was an approximate 2-fold difference in vector genome accumulation. This promoter was therefore not studied further.



<u>Figure 4.11.</u> β-gal staining of cells transfected with plasmid under control of cardiac promoter.

Cells were transfected using lipofectamine with either no DNA, control plasmid pMV10 or pAAV-MCS-MLC2v (n=3/group). Forty eight hours later, cells were stained with X-gal stain to analyse β-galactosidase expression amongst the different cell types. Scale bar = 100 μm, magnification × 10.

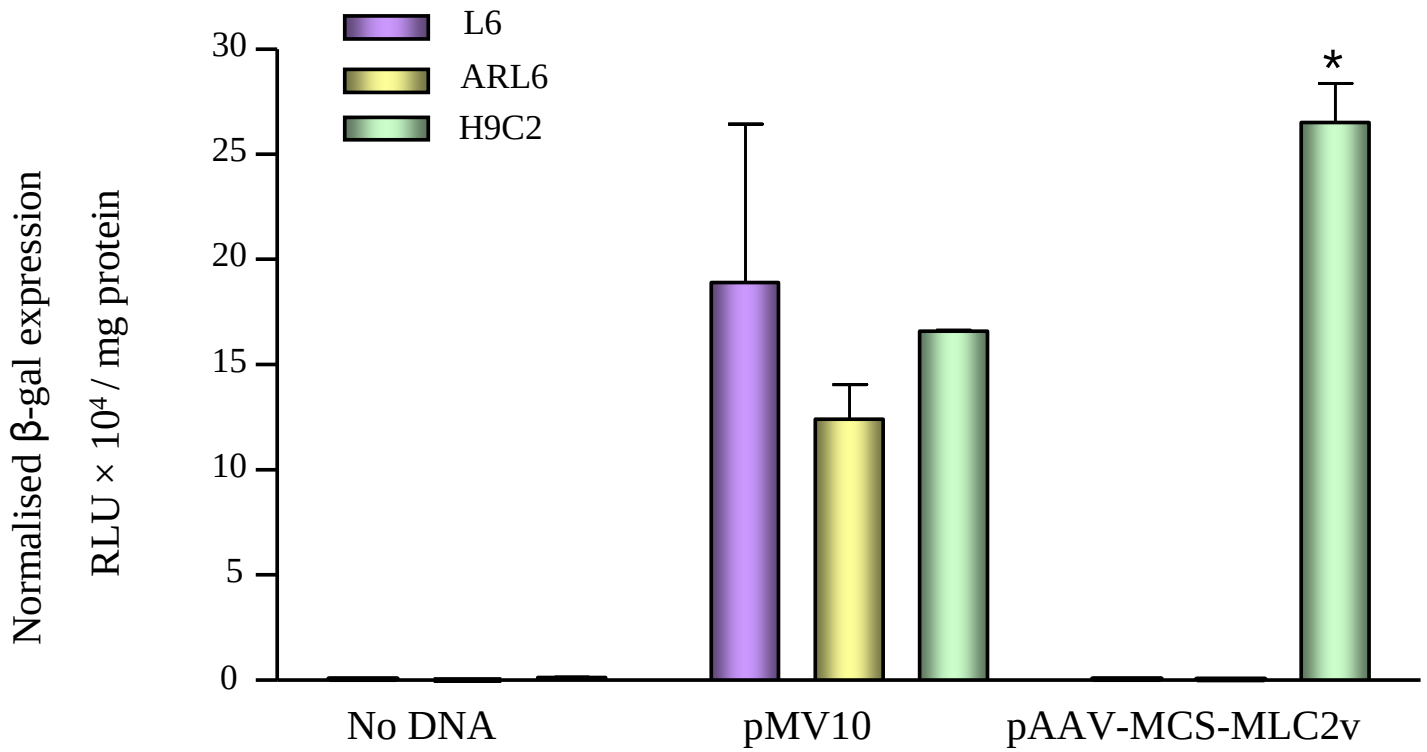
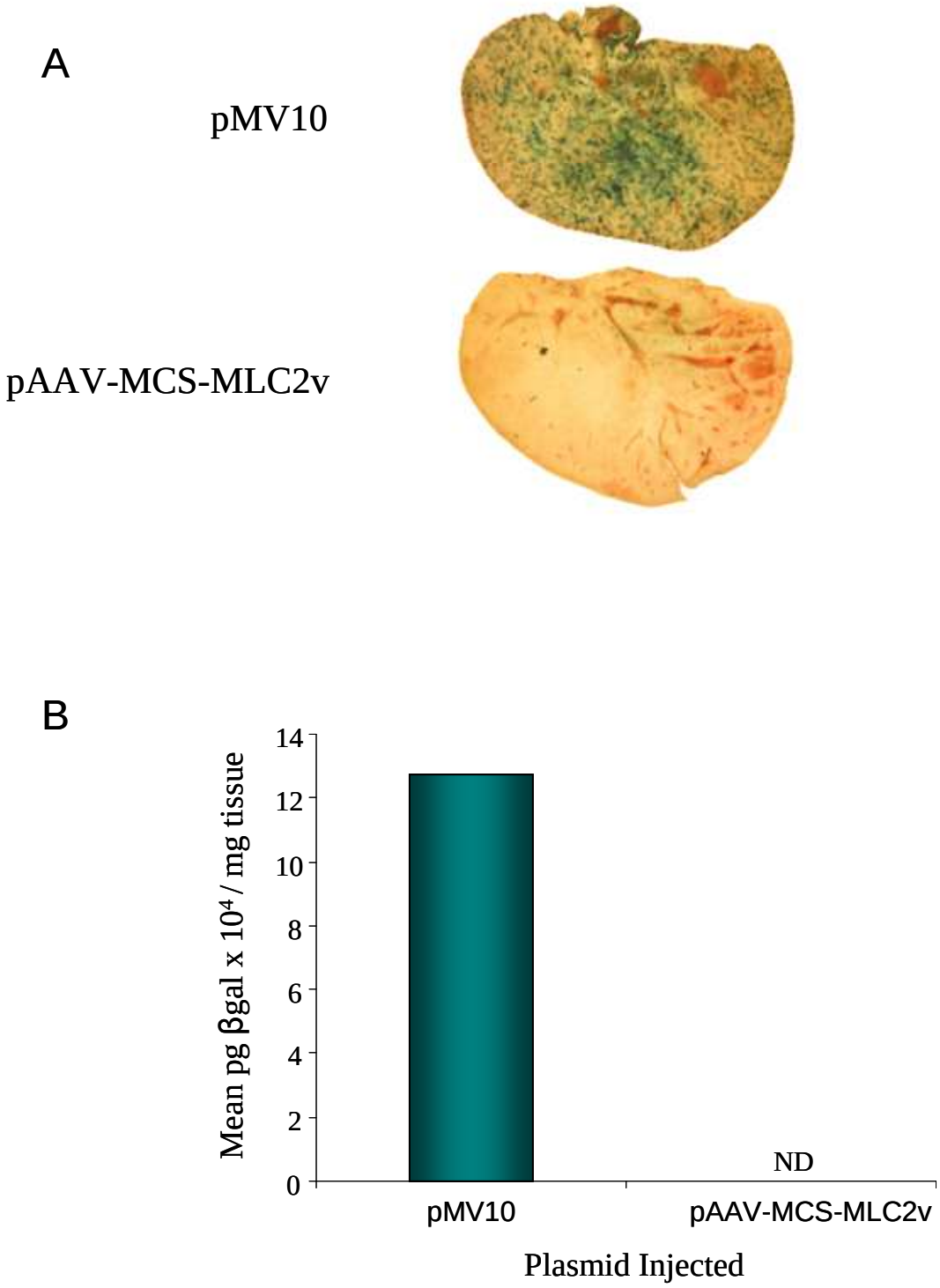


Figure 4.12. Quantification of β-gal expression mediated by pAAV-MCS-MLC2v.

Cells were transfected using lipofectamine with either no DNA, control plasmid pMV10 or pAAV-MCS-MLC2v (n=3/group). Forty eight hours later, cells were assayed to quantify β-galactosidase expression amongst the different cell types. *$p<0.01$ H9C2 pAAV-MCS-MLC2v vs. ARL-6 and L6 pAAV-MCS-MLC2v by two way ANOVA and Bonferonni posttest.



<u>Figure 4.13.</u> Liver transduction following hydrodynamic delivery of plasmids. Control plasmid pMV10 or rAAV6:MLC2v was hydrodynamically injected into the tail vein of mice (n=2/group). (A) Representative X-gal staining of liver sections and (B) Quantification of β-gal expression by Tropix Galacto-Light Plus assay.

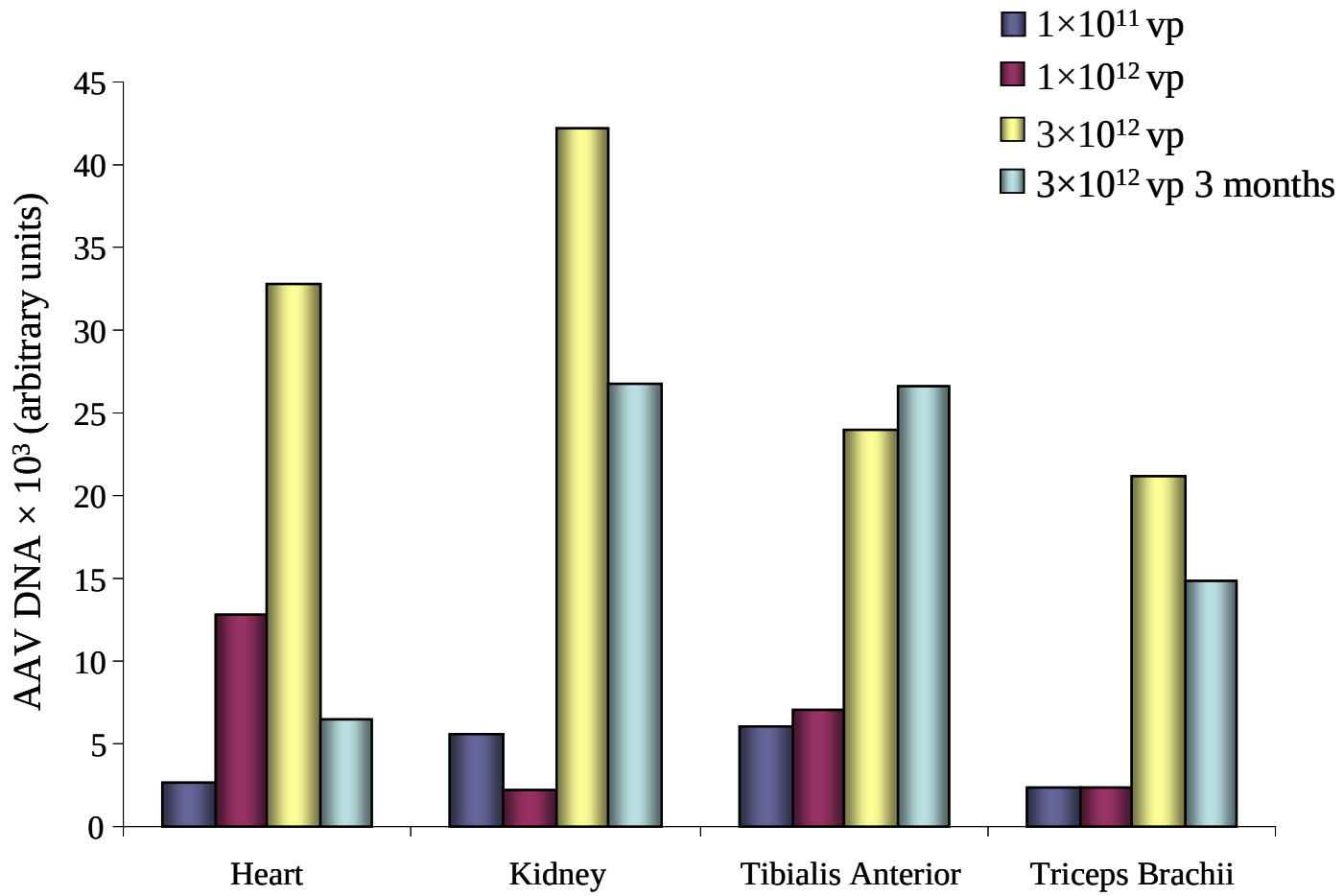
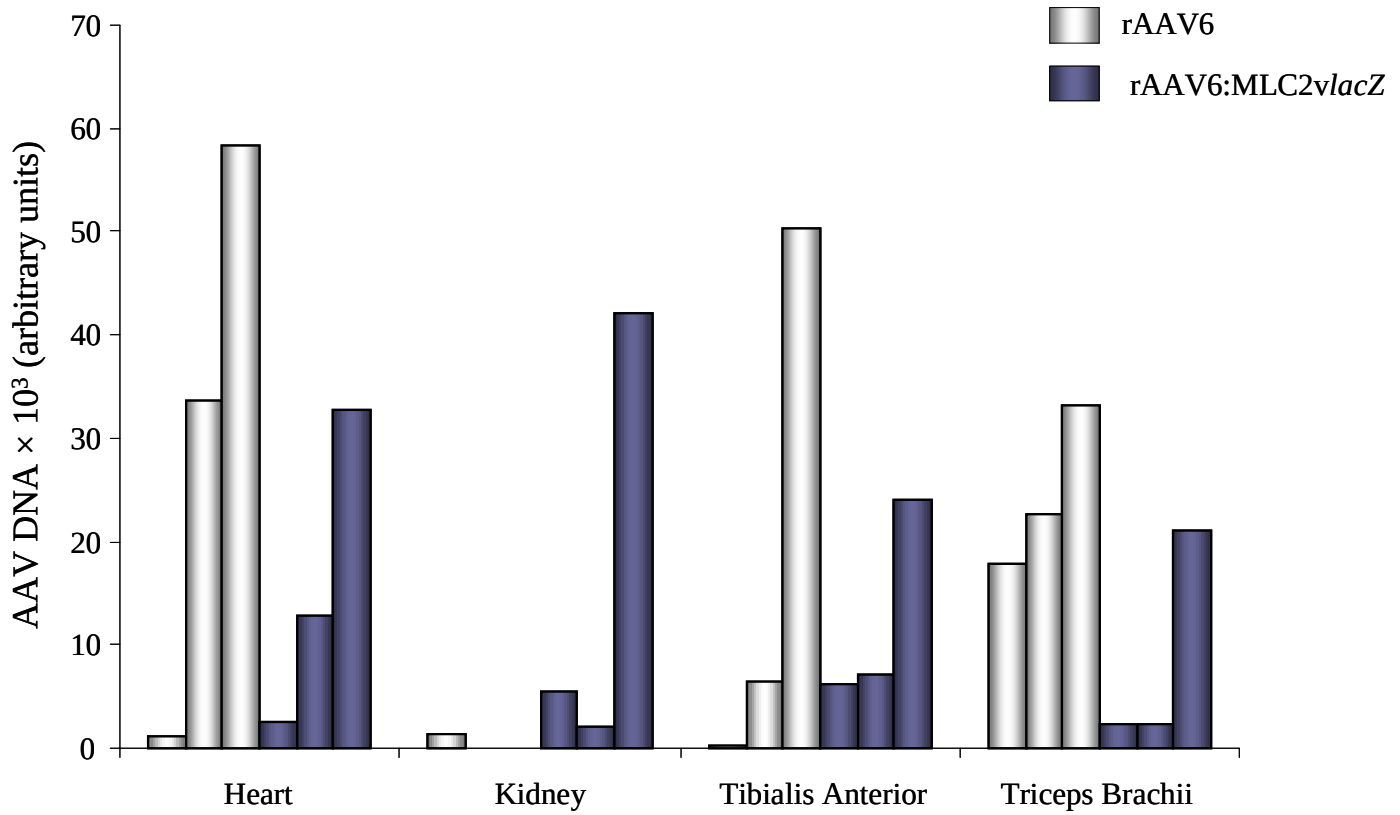


Figure 4.14. Biodistribution profile of rAAV6:MLC2v*lacZ*.

RAAV6:MLC2v*lacZ* vectors at three different doses (2×10^{11}, 1.5×10^{12} and 3×10^{12} vp/rat) were infused into 6 week old SHRSP rats (n=1/group). DNA was extracted from the tissues and Q-PCR performed.



<u>Figure 4.15.</u> Comparison of transduction of tissues by rAAV6 and rAAV6:MLC2v*lacZ*.

SHRSP were infused with 3 different doses of rAAV6:CMV*lacZ* or rAAV6:MLC2v*lacZ* (1×10^{11} vp, 1×10^{12} vp or 3×10^{12} vp). Total DNA was extracted from heart, kidney, tibialis anterior and triceps brachii and Q-PCR was performed. The three bars for each tissue with each virus represent the three different doses (left = lowest dose, right = highest dose). N=1/group.

4.3 Discussion

Efficient myocardial transduction via the intravasculature has proven challenging in the field of gene therapy. We demonstrate that intravenous administration of rAAV6 and rAAV9 vectors can be used to efficiently deliver and express genes in the heart of a rat disease model. As such, this strategy represents a useful tool to integrate and potentially treat a vast number of cardiovascular diseases. Transduction efficiency is determined by administered dose of vector, with an observed increased intensity of staining in the hearts of these animals as the dose increased. Furthermore, both these vector systems were not associated with any direct toxicity, with no observable immunological responses at 3 months post-infusion. In this study we report that gene expression is maintained for 3 months. We did not assess longer time points. Other studies have reported long term gene expression from rAAV vectors (Herzog, Yang et al. 1999; Riviere, Danos et al. 2006; Stieger, Le Meur et al. 2006) demonstrating the longevity of this approach, particularly useful for the treatment of chronic disease. RAAV6 vector-mediated transduction was not found to be dependent on vascular permeabilisation with VEGF, contrary to that previously reported in a mouse model (Gregorevic, Blankinship et al. 2004). RAAV6 vectors were found to mediate higher levels of transduction of the myocardium than rAAV9 vectors at all doses tested. The use of rAAV6 over rAAV9 vectors is thus advantageous as higher doses of rAAV9 would have to be used to reach the same levels of myocardial transduction as rAAV6.

The production of rAAV vectors has proven notoriously difficult, labour intensive and a limiting factor in the utility of these vectors. High titre stocks of rAAV were unattainable by the initial methods applied in our laboratory. Methods developed by others have allowed increased production of high titre and high purity rAAV stocks, with minimal contamination with adenoviral proteins (Halbert, Allen et al. 2001) (Blankinship, Gregorevic et al. 2004). These methods were employed to achieve high titre stocks with which to complete *in vivo* experiments. *In vitro* characterisation and comparison of the two rAAV vectors could not be carried out as no suitable cell line could be found that could be transduced by rAAV6 vectors. No previous studies have carried out *in vitro* characterisation of rAAV9 vectors. Thus all optimisation and

characterisation of rAAV6 and rAAV9 was carried out *in vivo* in a relevant disease model, the SHRSP.

There is a 17.2% amino acid difference between the capsids of clade A (includes AAV1 and AAV6) and clade F (AAV9) adeno-associated viruses (Gao, Vandenberghe et al. 2004). These differences in the area of the virion responsible for receptor binding may offer an explanation for the discrepancies in tropism between the two vectors. Single amino acid changes have previously been reported to account for the difference in liver transduction between AAV1 and AAV6 (Wu, Asokan et al. 2006). Whilst there is still a lot to discover regarding the receptors for these vectors, it is known that AAV6 uses α2-3 linked or α2-6 linked sialic acid (Wu, Miller et al. 2006), whilst AAV9 uses the 37/67-kDa laminin receptor (Akache, Grimm et al. 2006). Different distribution of these two receptors is likely associated with the differing biodistribution of these two vectors. It was previously reported that direct injection of rAAV6 vectors into the thoracic cavity of mice lead to high levels of transduction of the diaphragm (Blankinship, Gregorevic et al. 2004). Although not a direct comparison with this study, we found no transduction of the diaphragm after intravenous delivery of rAAV6 vectors. Interestingly, intravenous delivery of rAAV9 vectors resulted in high transgene expression levels throughout the diaphragm. Thus, the driving forces for transduction are different for each virus.

In the present study, rAAV9 vector genomes were also found at high levels in the kidney by quantitative PCR. However, further examination by immunohistochemical techniques revealed no detectable renal transgene expression. This could either be as a result of an inability of the rAAV9 vector to achieve transduction, or as a result of the phenomenon of the CMV promoter, which has been noted to be silenced in several organs (Loser, Jennings et al. 1998; Gregorevic, Blankinship et al. 2004). However, high β-galactosidase enzyme levels have previously been detected in the kidney following administration of rAAV9 vectors (Pacak, Mah et al. 2006; Bostick, Ghosh et al. 2007), and the CMV promoter has been shown to be transcriptionally active following the delivery of other gene transfer vectors (Wang, Li et al. 2004; Pacak, Mah et al. 2006). Several approaches could be taken to establish the cause of this. Assays to determine the methylation status of the CMV promoter would establish whether the promoter had been shut down (Brooks, Harkins et al. 2004). RT-PCR on

RNA extracted from the organ of interest could be performed to investigate whether there was a transgene translational block. Capsid labelling could also be carried out to determine if nuclear trafficking was defective and if there was a problem with capsid uncoating.

The limitation of the AAV vector system with respect to intravenous delivery to muscle is the lack of cell selectivity of non-muscle promoters. Cardiac myocyte-restricted gene transfer is highly desirable, and necessary as it already is in clinical trials. The short version of the MLC2v cardiac promoter was easily packaged into the limited packaging capacity of the AAV vector. It was shown to drive a reporter gene specifically in cardiomyocytes *in vitro*. In a previous study, rAAV2 vectors driven by the MLC2v promoter were shown to be taken up by many organs but only expressed in the heart (Phillips, Tang et al. 2002). However, in contrast to this study, we found that intravenous injection of rAAV6 vectors under the control of the MLC2v promoter led to uptake in the heart, but to no transgene expression. The lack of activity of this promoter *in vivo* is surprising, as it was previously reported to be akin to the activity of CMV promoters (Phillips, Tang et al. 2002). The presence of relatively high vector genome copy numbers in the heart and the fact that CMV driven transgene expression was high, demonstrates that the problem lies not in the transduction efficiency of the vector, but in the activity of the promoter. In this case, the activity of the CMV promoter remains superior to that of the MLC2v promoter. Comparison of a range of cardiac-specific cassettes is required to find one suitable that is functional *in vivo* when packaged into rAAV6 vectors. Recently, rAAV6 vectors under the control of a CMV-enhanced 1.5 kb MLC2v promoter were found to mediate cardiac-specific transgene expression in a porcine model (Raake, Hinkel et al. 2007). Not only does this highlight the potential of transcriptionally regulated rAAV6 vectors, it also demonstrates that rAAV6 is effective in larger animal models and is not species-dependent.

Furthermore, rAAV6 vector-mediated transgene expression under the control of a cardiac-selective enhancer/promoter element has been shown to drive chronic transgene expression in a rat heart failure model (Pleger, Most et al. 2007). A 324-bp fragment containing the α-cardiac actin myocyte-enhancer factor-2 (MEF2) domain was cloned in place of the CMV promoter to drive expression of the S100A1 gene,

which expresses a Ca^{2+}-sensing protein that plays a vital role in cardiac contractile function. Robust expression of S100A1 was achieved that was localised to the heart and led to the functional recovery of the failing rat heart (Pleger, Most et al. 2007). This demonstrates the potential of rAAV6 vectors to drive stable cardiac-specific transgene expression after intravenous delivery, which will enhance the development of cardiac gene therapies.

In conclusion, rAAV6 and rAAV9 vectors were both found to possess cardiotropic properties and as such, both will have implications in cardiovascular gene therapy. In a direct comparison of the two vectors, rAAV6 demonstrated a more favourable cardiac delivery profile than rAAV9. These results allow for the optimal choice of vector for use in future studies, and for future efficient clinical applications.

Chapter 5

RAAV6-mediated overexpression of angiotensin converting enzyme 2 (ACE2) in the myocardium of Stroke Prone Spontaneously Hypertensive Rats (SHRSP)

5.1 Introduction

In addition to its role in cardiovascular and renal homeostasis, overactivity of the RAS is implicated in the pathophysiology of hypertension and in the progression of heart failure (Zaman, Oparil et al. 2002). Inhibition of the RAS is now used as major target in the treatment of hypertension and a plethora of other cardiovascular diseases. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) type 1 (AT_1) receptor blockers are common treatments for the alleviation of high blood pressure in hypertensive patients (Stergiou and Skeva 2004). Exploration of other arms of the RAS could potentially identify and develop more specific and potentially long lasting therapeutic targets.

In 2000, a new component of the RAS was identified. Angiotensin converting enzyme 2 (ACE2) was isolated from a human heart failure ventricle cDNA library and was found to be capable of hydrolyzing circulating peptides (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000). ACE2 is known to promote Ang II degradation, and to a lesser extent Ang I degradation (Tipnis, Hooper et al. 2000), which results in the production of Ang 1-9 and Ang 1-7. Because Ang II is known to have potent vasoconstrictor functions, its degradation could result in relaxation of the blood vessels. This could occur through two main mechanisms; (1) the reduction in circulating levels of the vasoconstrictor Ang II and (2) the increase in circulating levels of the vasodilator Ang 1-7. Ang II has been shown to activate NAD(P)H oxidases in cultured vascular smooth muscle cells and vascular endothelial cells, resulting in the production of reactive oxygen species, particularly superoxide (Griendling, Minieri et al. 1994; Zhang, Schmeisser et al. 1999). Superoxide can react with NO to inactivate it, thus inhibiting its vasodilatory effects. Ang 1-7 has been shown to stimulate eNOS activation with a resultant increased NO release (Heitsch, Brovkovych et al. 2001), which causes endothelial-dependent vasodilation and an improvement in endothelial function (Faria-Silva, Duarte et al. 2005).

There are currently opposing hypotheses regarding the pathophysiology of ACE2 *in vivo*. ACE2 may play a pivotal role in the RAS by reducing concentrations of the pro-fibrotic, pro-proliferative vasoconstrictor Ang II and raising levels of the anti-fibrotic, anti-proliferative vasodilatory peptide Ang 1-7 (Ferrario, Chappell et al. 1997;

Ferrario 1998). As such, manipulation of ACE2 activity has potential utility in the treatment of cardiovascular disease, although previous studies have been conflicting. In particular, generation of ACE2 knockout mice has been reported to lead to severe contractile dysfunction and induction of hypoxia-induced genes (Crackower, Sarao et al. 2002) or, in complete contrast, to no observed effects on cardiac dimension or function (Gurley, Allred et al. 2006). The reason for these discrepancies remains unclear. In a direct assessment of genetic overexpression of ACE2, lentiviral-mediated localised overexpression of ACE2 in the hearts of spontaneously hypertensive rats (SHR) attenuated high blood pressure and perivascular fibrosis, reduced left ventricular wall thickness and increased left ventricular end systolic diameters (Diez-Freire, Vazquez et al. 2006). Again, in contrast, transgenic overexpression of ACE2 in the myocardium of mice resulted in mild interstitial fibrosis and conduction disturbances leading to ventricular fibrillation with arrest and sudden death (Donoghue, Wakimoto et al. 2003). Taken together, these findings suggest that the role of ACE2 *in vivo* is complex and may be context-dependent. It is therefore important to define the effect of ACE2 overexpression in the long term in animals predisposed to cardiovascular disease. SHRSP is a relevant model to define the effect of ACE2 overexpression and we set out to define its effects on development of LVH.

Here, the cardiac delivery profile of rAAV6 vectors (characterised in chapter 4) was exploited to overexpress ACE2 in the myocardium of SHRSP. Effects on cardiac structure and function as well as on systemic blood pressure and peripheral endothelial function were quantified.

5.2 Results

5.2.1 Characterisation of AAV6-ACE2

We first confirmed the functionality of the AAV6-ACE2 plasmid using cos cells *in vitro*. Expression of ACE2 was confirmed by Taqman™ Q-RTPCR performed on cDNA, derived from control and pAAV6-ACE2 transfected cells (Figure 5.1A). Representative Taqman traces demonstrate the expression of ACE2 (Figure 5.1B). Overexpression of ACE2 was further observed by detection of a band of the appropriate size (90 kDa) by western blot analysis following transfection of pAAV6-MCS-ACE2 into COS cells (Figure 5.1C). ACE2 function was evaluated using a fluorescence activity assay, which is based on the interaction of ACE2 with fluorogenic substrate VI. This fluorogenic substrate contains a hydrolysable group that can be hydrolysed, resulting in the cleavage of the peptide bond between the fluorophore and the quencher to produce an increase in fluorescence that can easily be detected. The ACE inhibitor captopril was used to abolish any influence of ACE. In the presence of the selective ACE2 inhibitor DX600, ACE2-specific enzymatic activity was abolished (Figure 5.2). Thus, transfection of pAAV-MCS-ACE2 resulted in efficient ACE2 production and functional activity of the enzyme.

5.2.2 Effect of ACE2 overexpression on in vivo cardiac function

Our principle aim was to document the effect of ACE2 overexpression on cardiac function over the long term and during the establishment of hypertension and LVH in the SHRSP, evident from 12 weeks of age (Davidson, Schork et al. 1995). We therefore injected male rats with rAAV6:ACE2 vectors and controls at 8 weeks of age and monitored cardiac function by ECHO and BP by tailcuff over the following 11 weeks (Figure 5.3). Left ventricular M-mode ECHO demonstrated a change in LV diameter and reduction in wall thickness over time with reduced systolic function in the rAAV6:ACE2 vector-infused animals, compared to Enalapril-treated and PBS and rAAV6:hPLAP vector-infused rats (Figure 5.4). B-mode images confirmed a reduced systolic function in rAAV6:ACE2 treated animals compared to the control groups (Figure 5.5). M-mode images were used to define wall thicknesses and internal diameters at systole and diastole. Rats treated with rAAV6:ACE2 exhibited a

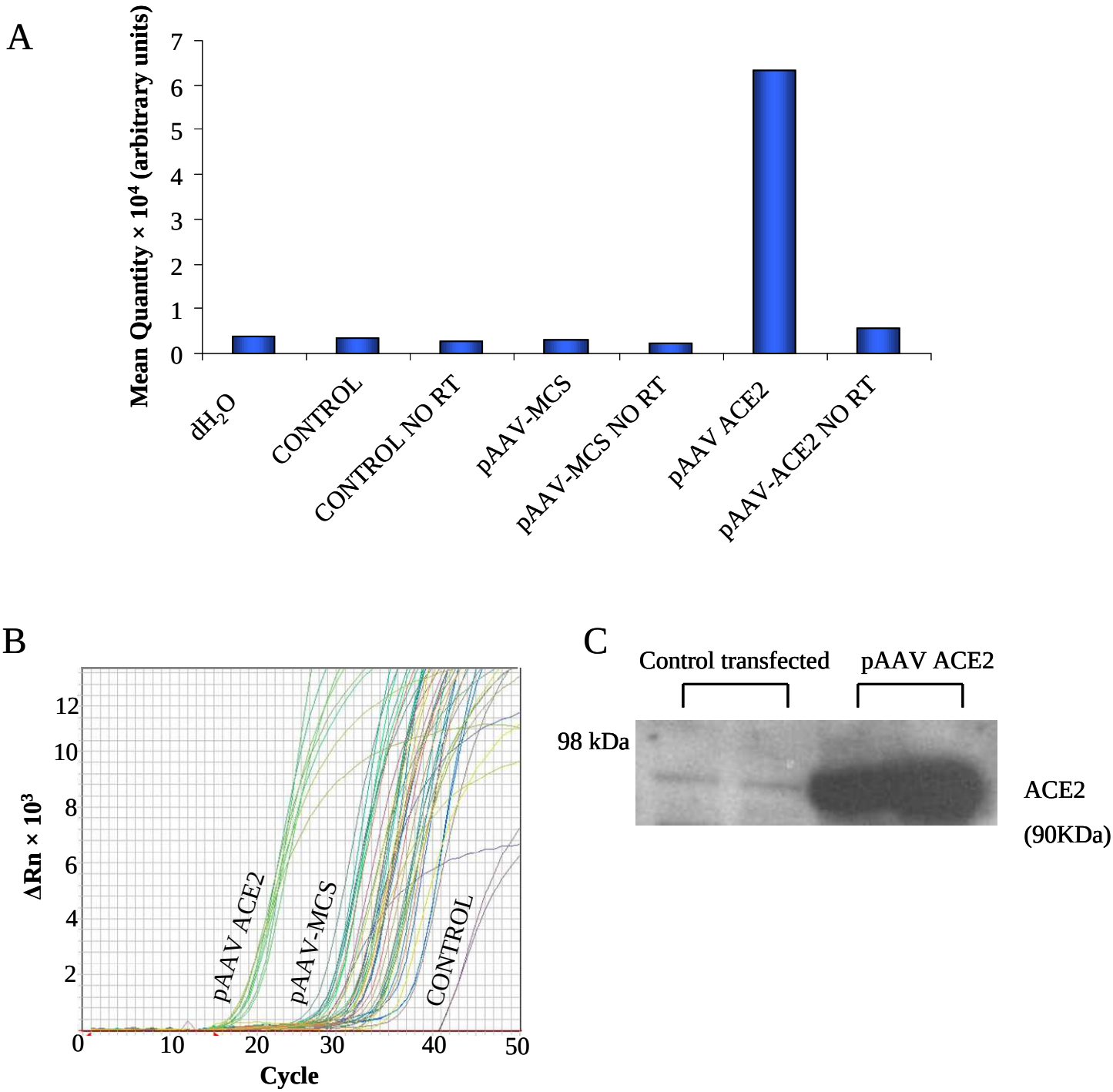


Figure 5.1. Confirmation of expression of ACE2.

Cells were transfected with pAAV-ACE2. 48 hours after lipofectamine transfection, cells were harvested and lysed. (A) RNA extracted from the cells was converted to cDNA and Taqman carried out to detect ACE2. Control samples including dH2O, no reverse transcriptase and a control plasmid expressing a different protein were included. (B) Representative Taqman traces. (C) Cell lysates were subjected to western immunoblotting and detected with an anti-ACE2 antibody on a 12% gel under reducing conditions.

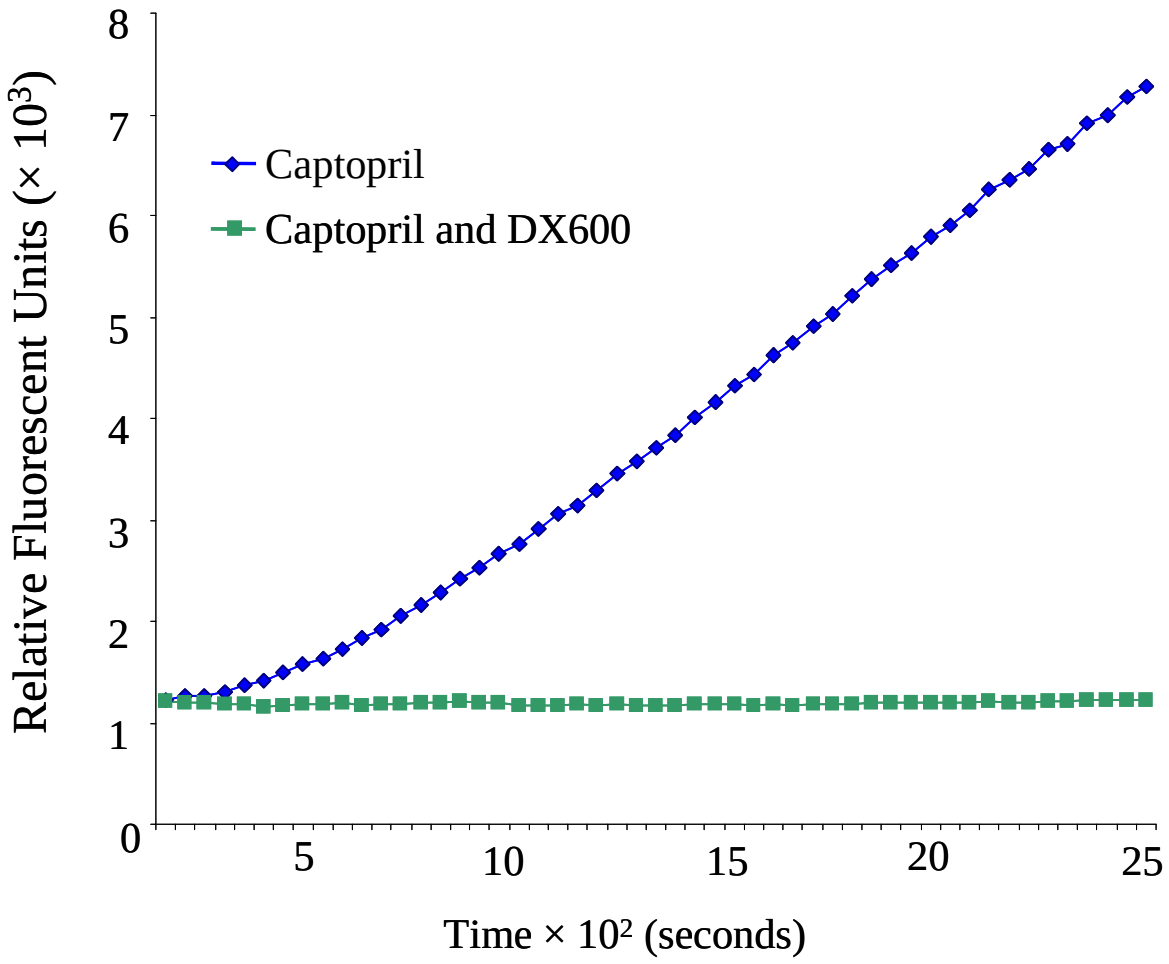
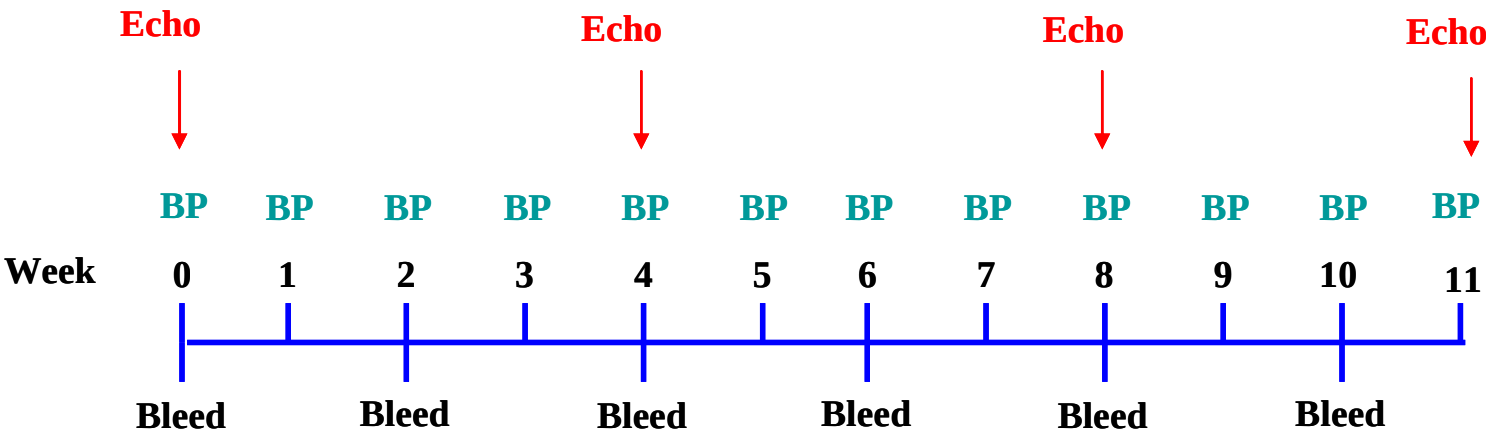


Figure 5.2. Confirmation of functional activity of ACE2.

ACE2 activity was measured in the presence of captopril and DX600, confirming that pAAV-ACE2 produces functional ACE2.



Groups :

•PBS

•rAAV6:ACE2

•rAAV6:hPLAP

•Enalapril

(N=6/group)

Figure 5.3. Overview of experimental protocol.

SHRSP were infused with 3×10^{12} vp/animal at week 0 (8 weeks of age). Blood pressure was measured weekly by tail cuff plethsymography. Blood samples were taken every second week and echocardioagraphy was carried out pre-infusion and at weeks 4, 8 and 11 post-infusion. At termination tissues were harvested for additional analysis.

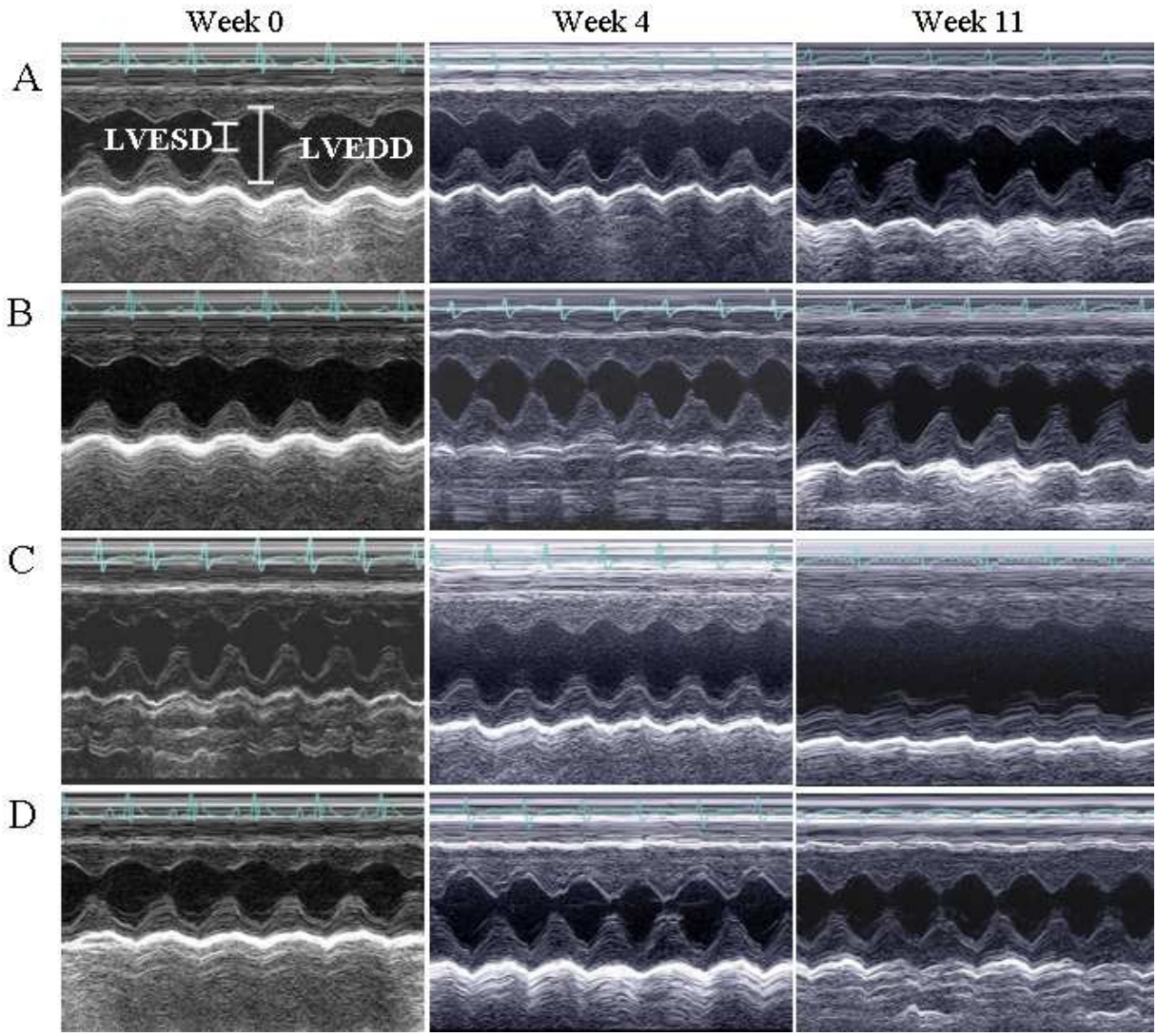


Figure 5.4. M-mode echocardiography.

Representative traces of M-mode echocardiography at pre-infusion, 4 weeks, 8 weeks and 11 weeks post-infusion in the 4 experimental groups – (A) PBS, (B) rAAV6:hPLAP, (C) rAAV6:ACE2 and (D) Enalapril treated animals.

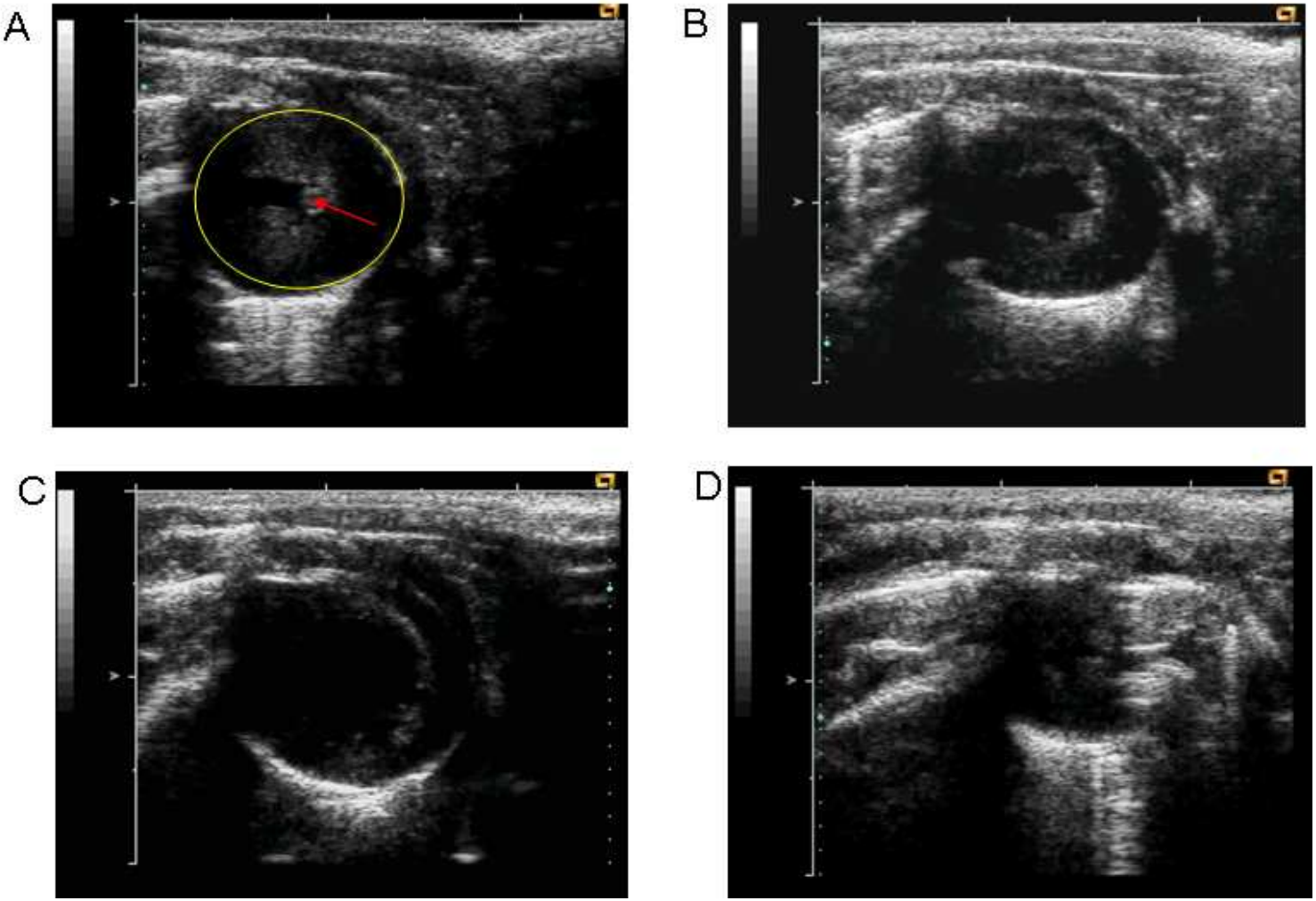


Figure 5.5. B-mode echocardiography.

Representative traces of B-mode echocardiography at 11 weeks post-infusion in the 4 experimental groups – (A) rAAV6:hPLAP, (B) PBS, (C) rAAV6:ACE2 and (D) Enalapril treatment. Images are taken at end systole. Red arrow = papillary muscles; yellow circle = outline of left ventricle. N=6/group.

significant (28%) reduction in ejection fraction, compared to controls (Table 5.1 and Figure 5.6). Fractional shortening was also significantly reduced compared to all controls (Appendix 1 and Figure 5.7). As expected, cardiac output increased with age and body mass in control SHRSP (Appendix and Figure 5.8). However, no change in cardiac output occurred in rAAV6:ACE2-infused rats (Figure 5.8). Furthermore, systolic blood pressure/end systolic volume ratio (SBP: ESV) demonstrated that the rAAV6:ACE2-infused SHRSP showed decreased left ventricular performance (Figure 5.9).

Interventricular septal wall thickness decreased by 14% in the rAAV6:ACE2 group. There was minimal effect on wall thickness in the Enalapril, rAAV6:hPLAP and PBS groups (Figure 5.10). LV mass index did not significantly differ between groups (Appendix 1). Pulse-wave and colour Doppler were used to measure the velocity of blood through the mitral valve and to qualitatively examine the valve for evidence of mitral regurgitation from the apical four-chamber. Colour doppler indicated that the blood flow through the heart of the rAAV6:ACE2 infused animals was more turbulent than in the Enalapril treated animals (Figure 5.11). This suggested a mitral valve insufficiency at systole resulting in a backflow of blood through the valves. In conclusion, ECHO studies revealed that ACE2 overexpression leads to a significant reduction in cardiac function compared to control groups.

5.2.3 Effect on blood pressure and basal NO bioavailability

A previous study has indicated a potentially beneficial effect of ACE2 overexpression on blood pressure (Diez-Freire, Vazquez et al. 2006). We therefore analysed blood pressure weekly by tail cuff. Since any blood pressure effect may also be due to potential hydrolysis of Ang II by ACE2, we also quantified endothelial function in peripheral resistance vessels (mesentery) by wire myography. Enalapril treatment was included as a positive control for BP measurements. PBS and rAAV6:hPLAP control animals show an equivalent increase in BP over time, characteristic of SHRSP (Figure 5.12). This rise in systolic BP was significantly attenuated by enalapril and by ACE2 overexpression ($p<0.001$). The effects of ACE2 overexpression were especially evident at later time points following injection (Figure 5.12). Myography demonstrated increased basal nitric oxide (NO) in the vessels from animals that had

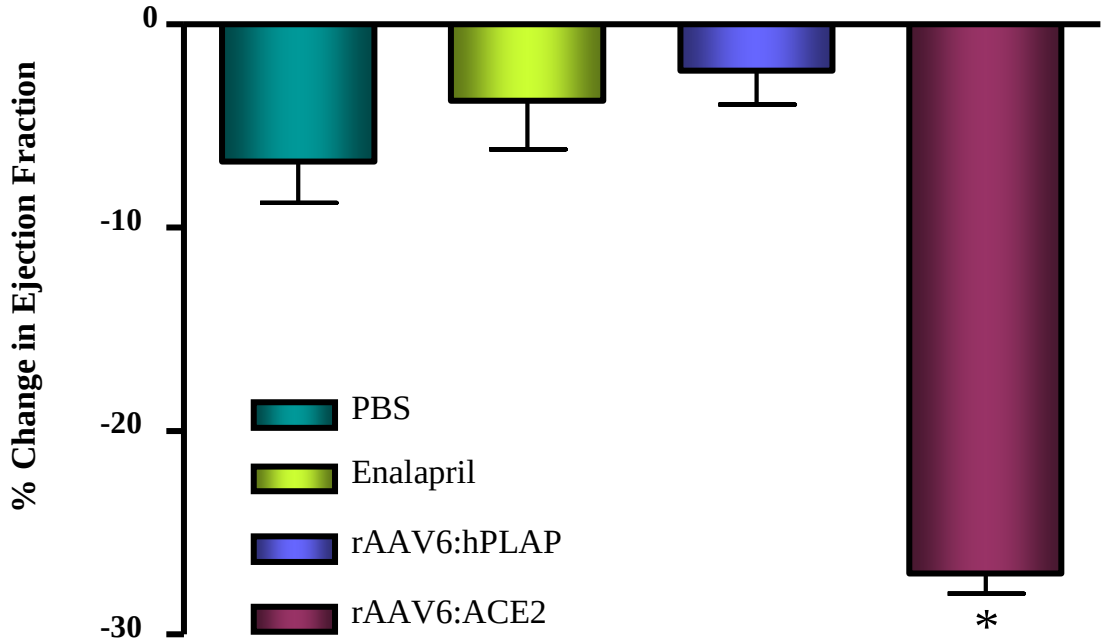


Figure 5.6. Assessment of ejection fraction.

Echocardiography was carried out pre-infusion and at 11 weeks post-infusion on rAAV6:hPLAP, PBS, rAAV6:ACE2 and Enalapril treated animals. % change in ejection fraction was calculated. *$p<0.05$ for rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:ACE2 by one way ANOVA and Tukey's post test. N=3/group.

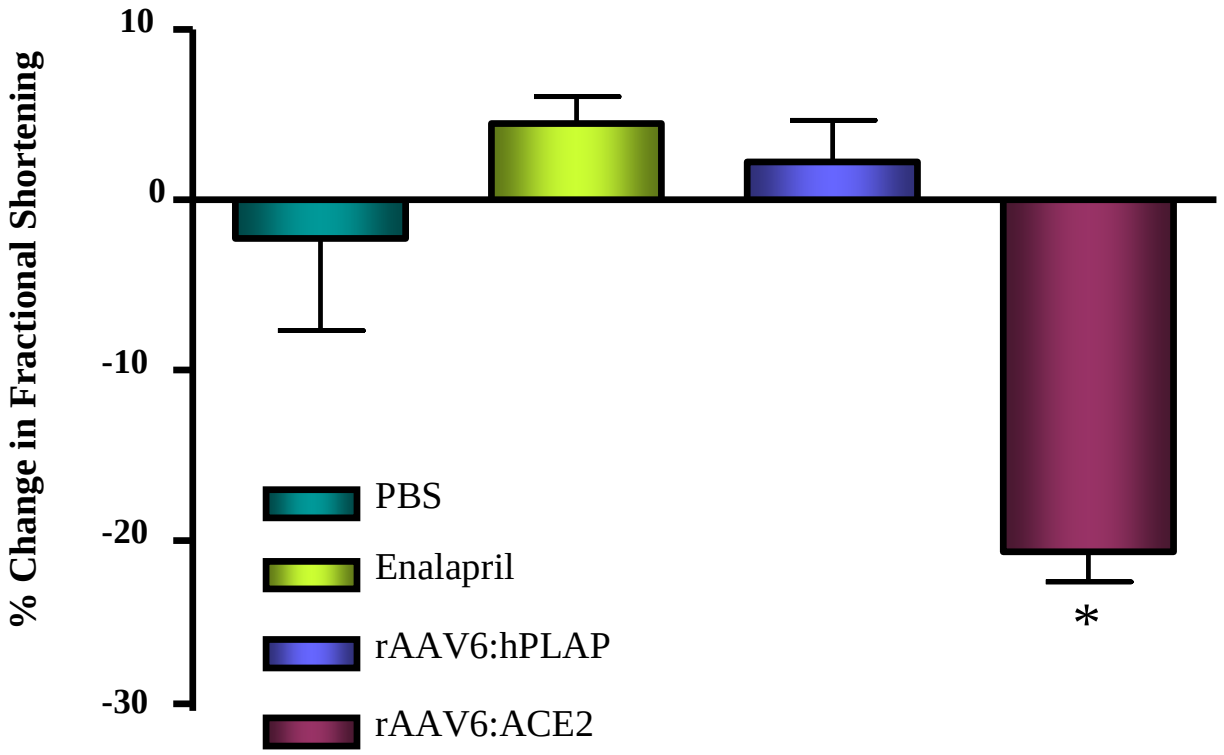


Figure 5.7. Assessment of fractional shortening.

Echocardiography was carried out pre-infusion and at 11 weeks post-infusion on rAAV6:hPLAP, PBS, rAAV6:ACE2 and Enalapril treated animals. Change in % fractional shortening was calculated. *$p<0.05$ for rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:ACE2 by one way ANOVA and Tukey's post test. N=3/group.

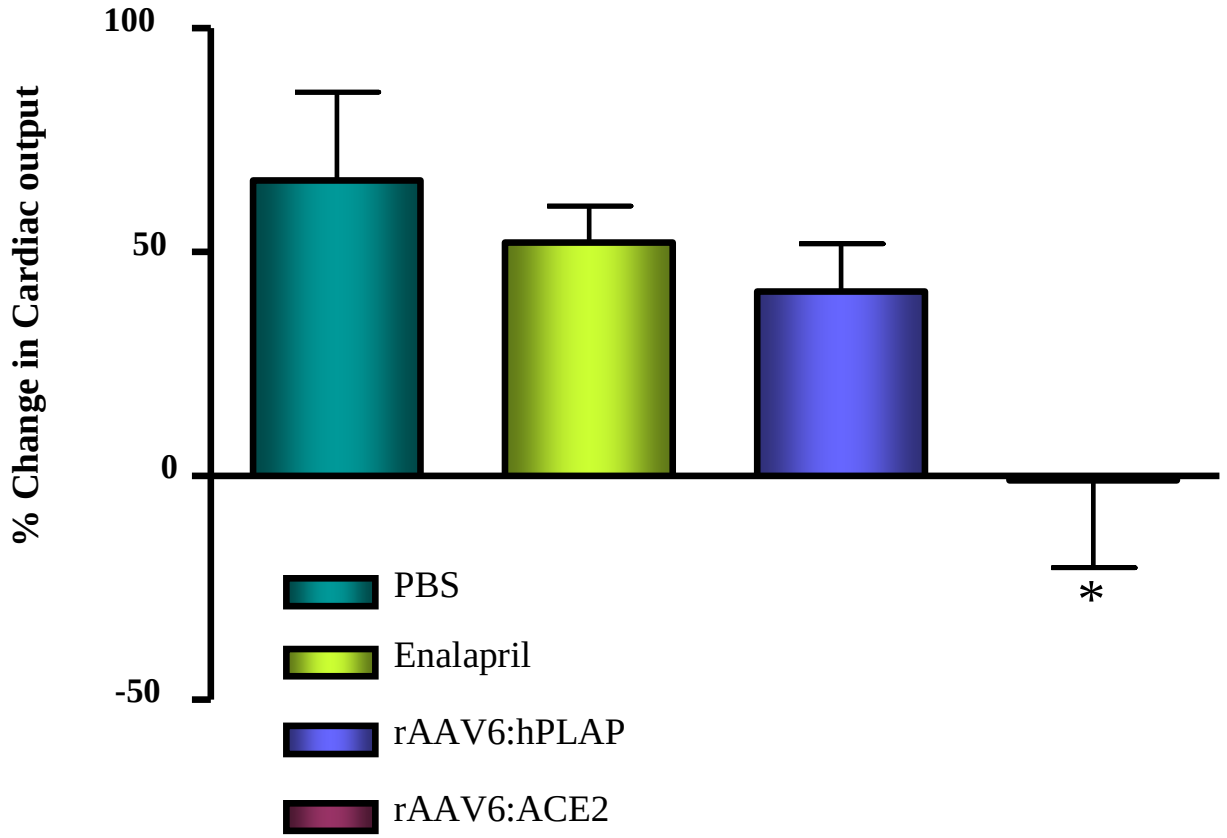


Figure 5.8. Assessment of cardiac output.

Echocardiography was carried out pre-infusion and at 11 weeks post-infusion on rAAV6:hPLAP, PBS, rAAV6:ACE2 and Enalapril treated animals. % Change in cardiac output was calculated. *$p<0.05$ for rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:ACE2 by one way ANOVA and Tukey's post test. N=3/group.

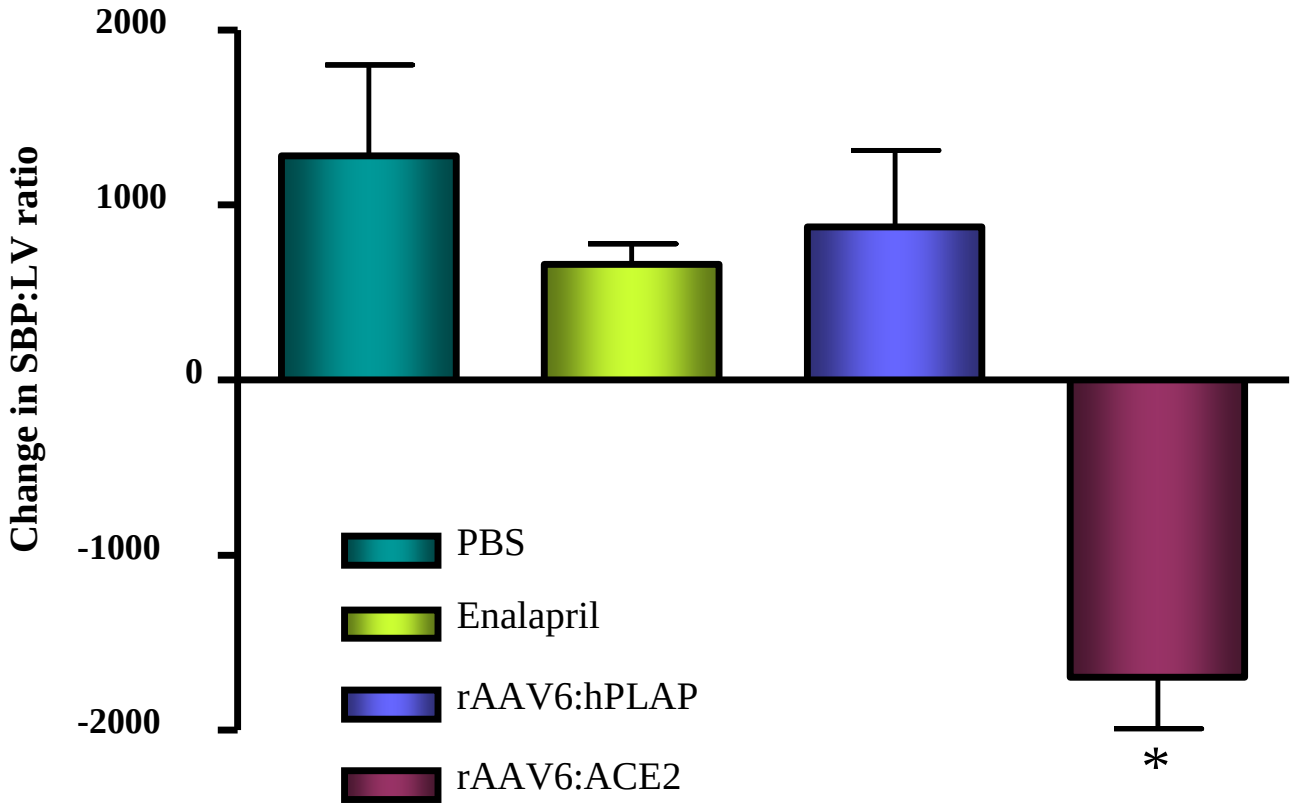


Figure 5.9.Assessment of systolic blood pressure/ end systolic volume ratio.

Echocardiography was carried out pre-infusion and at 11 weeks post-infusion on rAAV6:hPLAP, PBS, rAAV6:ACE2 and Enalapril treated animals. Change % in systolic blood pressure/ end systolic volume ratio was calculated. *$p<0.05$ for rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:ACE2 by one way ANOVA and Tukey's post test. N=3/group.

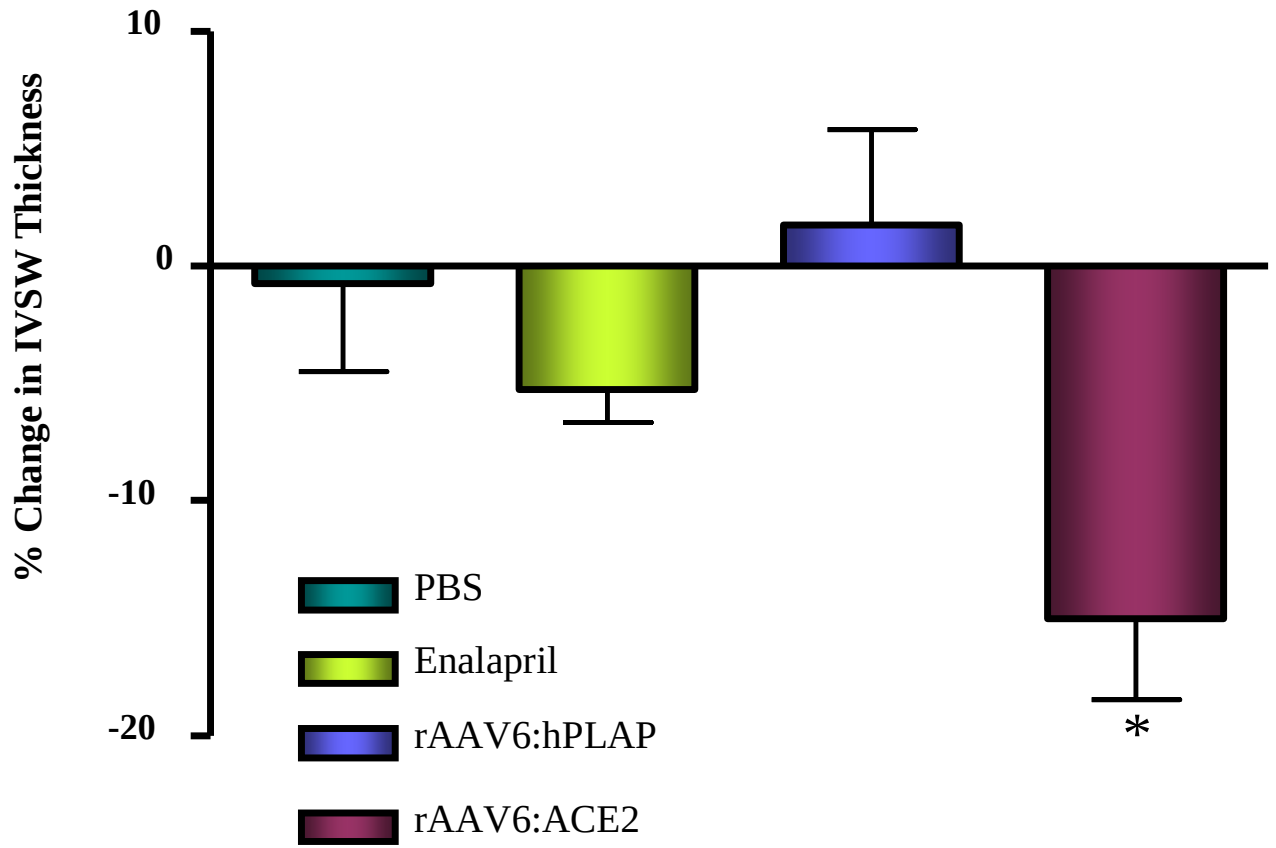


Figure 5.10. Assessment of interventricular septal wall thickness.

Echocardiography was carried out pre-infusion and at 11 weeks post-infusion on rAAV6:hPLAP, PBS, rAAV6:ACE2 and Enalapril treated animals. % change in interventricular septal wall thickness was calculated. *$p<0.05$ for rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:ACE2 by one way ANOVA and Tukey's post test. N=3/group.

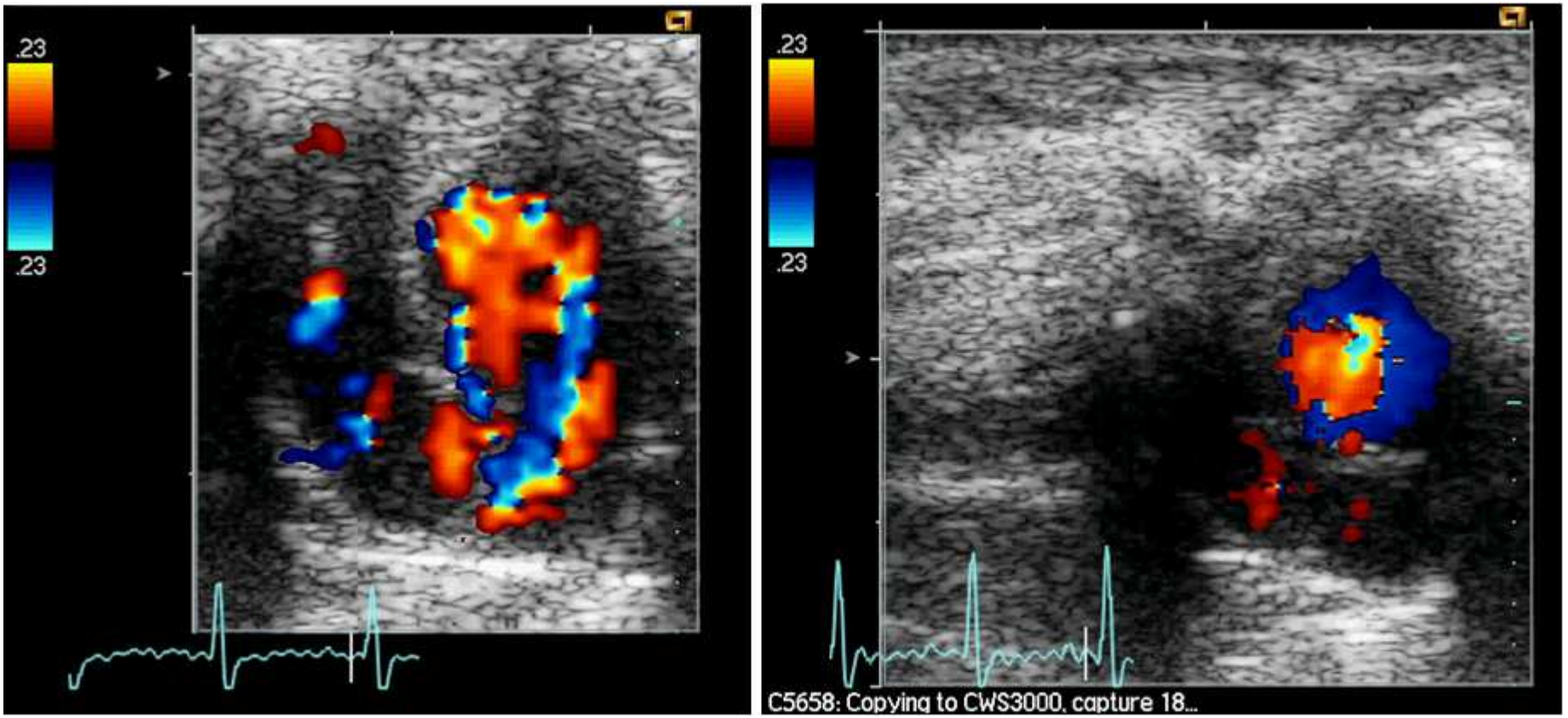


Figure 5.11. Evaluation of blood flow.

Colour doppler sonography carried out 11 weeks post-infusion on (A) rAAV6:ACE2 infused and (B) Enalapril treated animals. 4 chamber view of the heart showing left venticle (yellow arrow), right ventricle (white arrow), left atria (yellow arrow head), right atria (white arrow head) and mitral valve (green arrow). Colours are representative of blood flow direction; blue represents blood flow away from the transducer and red represents blood flow towards the transducer. A mix of colour (yellow/orange) indicates a mixed direction of blood flow, seen at the mitral valve level in systole is recognised as mitral valve regurgitation. N=1/group.

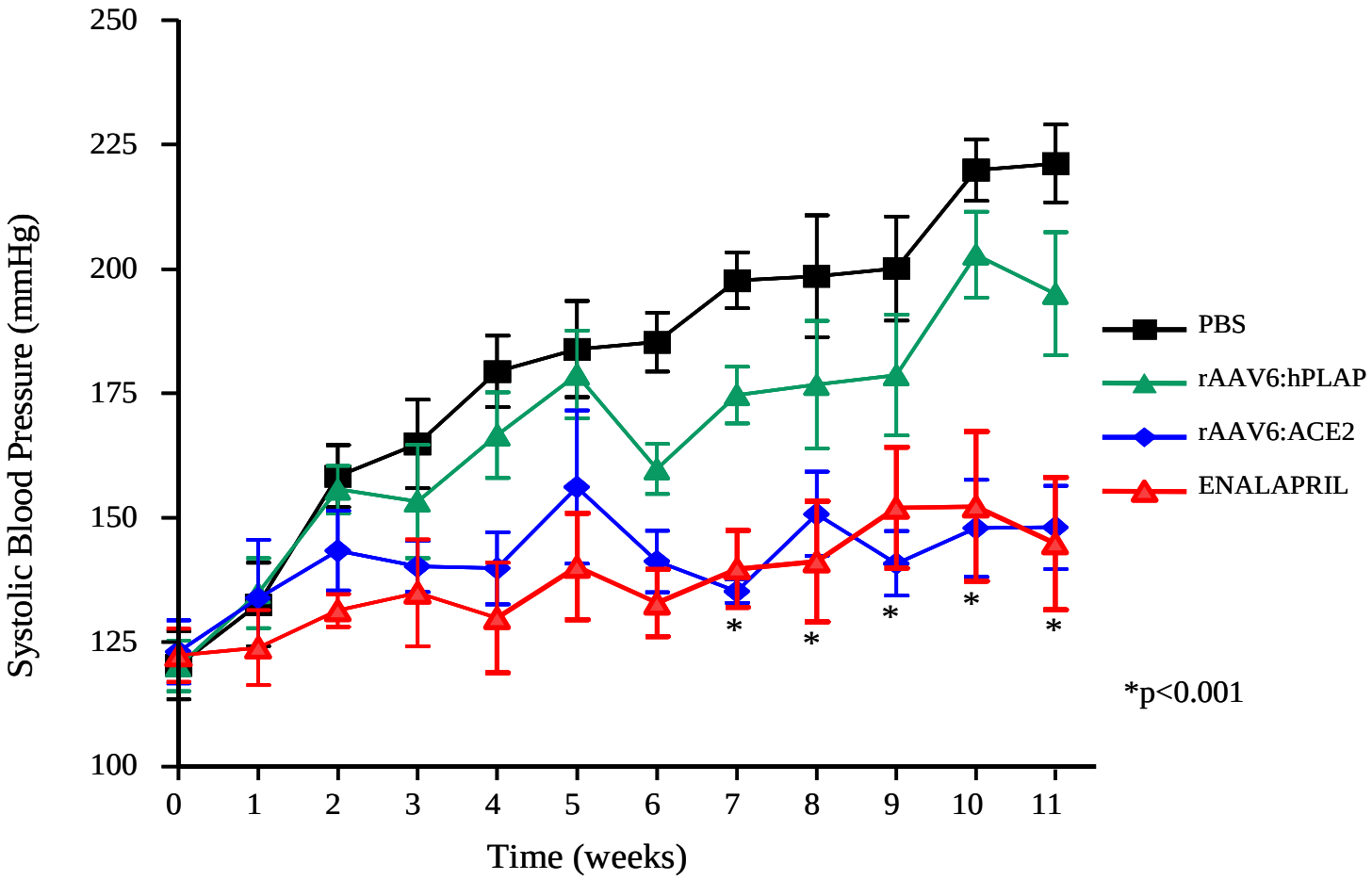


Figure 5.12. **Effect of overexpression of ACE2 on systolic blood pressure.**

Animals were infused with either rAAV6:hPLAP, rAAV6:ACE2, PBS or treated with Enalapril (n=6/group). Systolic blood pressure was measured weekly by tail cuff. Data are presented as mean ± SE. * p<0.001 rAAV6:ACE2 and Enalapril vs. PBS and rAAV6:hPLAP, as determined by two way ANOVA analysis and Bonferroni test.

received rAAV6:ACE2 compared to the rAAV6:hPLAP group, as determined by the area under the curve of concentration-response curves to contractile agonists in the presence and absence of L-NAME (Figure 5.13). Thus, whilst ACE2 had a severe effect on cardiac function, overexpression also resulted in improved peripheral endothelial function.

5.2.4 Histological evaluation of cardiac structure

We first confirmed sustained overexpression of ACE2 in rAAV6:ACE2 injected rats at 11 weeks post-injection (Figure 5.14A). Clear evidence of cardiac dysregulation in rAAV6:ACE2 transduced hearts can be seen with irregular myocyte shape and cell infiltration (Figure 5.14A & 5.14B). The effect of ACE2 on cardiac fibrosis was assessed by picrosirius red (Figure 5.15A) and Masson's trichrome stain (Figure 5.15B). Severe myocardial interstitial fibrosis was only observed in the rAAV6:ACE2 transduced SHRSP (Figure 5.15).

5.2.5 Histological evaluation of other tissues

The kidney and skeletal muscle were examined to assess any effects of ACE2 overexpression in non-target areas. No abnormal kidney structure was observed (Figure 5.16A), and no signs of renal fibrosis were found (Figure 5.16B). We also confirmed that overexpression of ACE2 was not present in the kidney of rAAV6:ACE2 injected rats (Figure 5.16C). To check that the effects of ACE2 overexpression were limited to the heart, muscle tissue was examined for structure (Figure 5.17A) and fibrosis (Figure 4.17B). This confirmed that the negative effects of ACE2 overexpression were not seen in either non-targeted areas, such as the kidney, nor in other muscle beds which are known to be transduced by rAAV6 vectors.

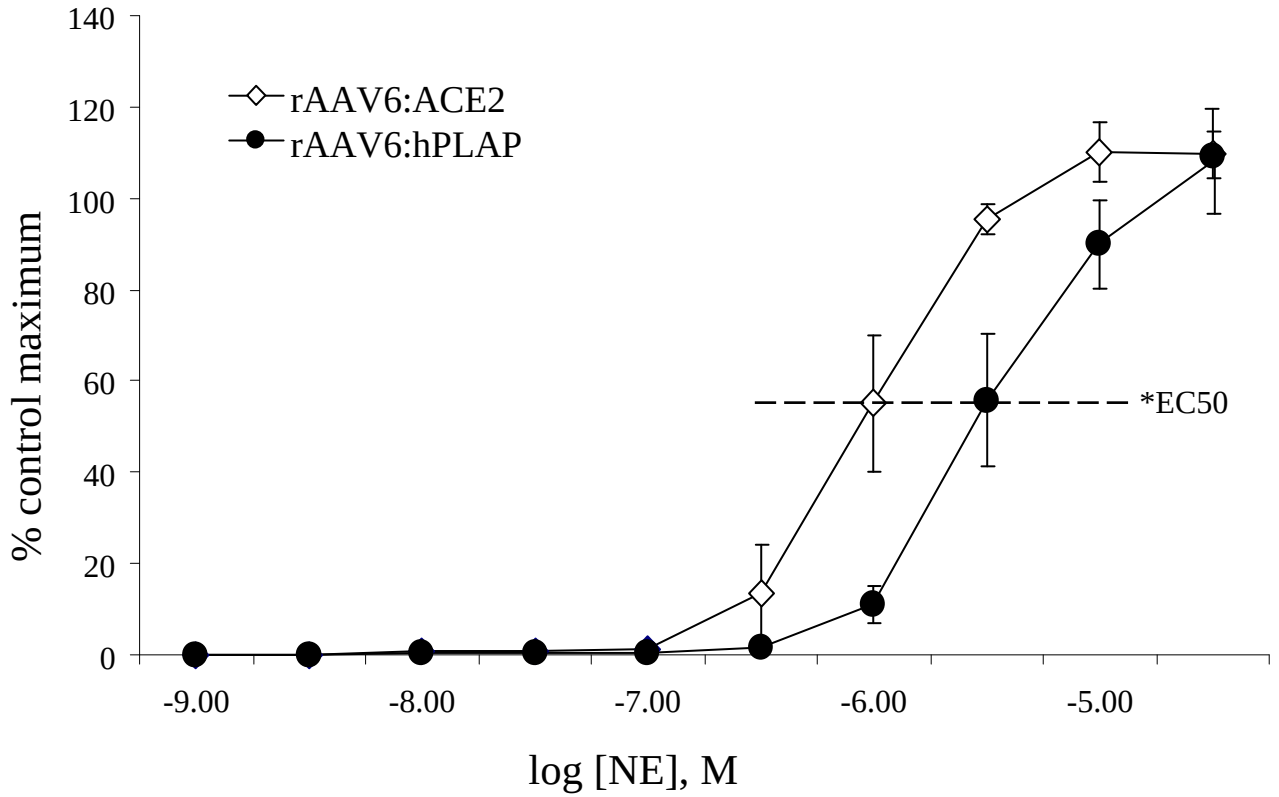
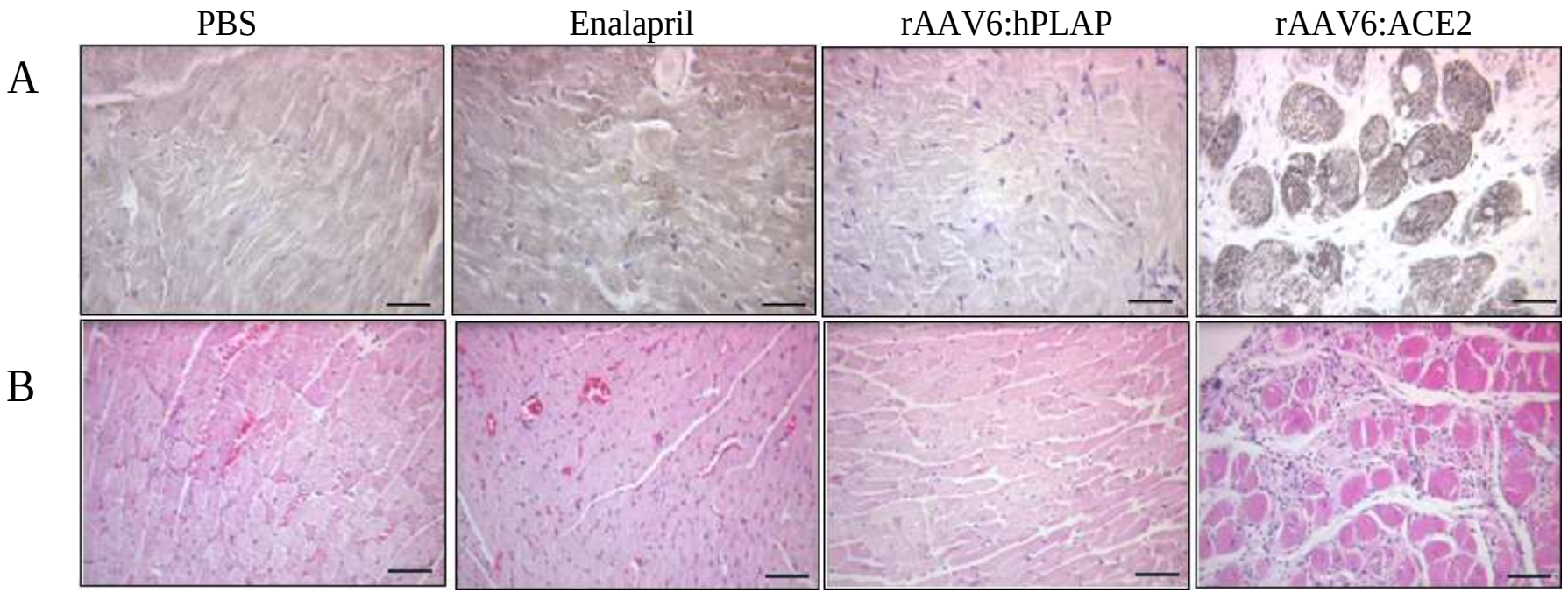


Figure 5.13 Effect of overexpression of ACE2 on basal NO bioavailability.

Small vessel myography was carried out on mesenteric arteries from rAAV6:ACE2 and rAAV6:hPLAP infused animals at termination (n=4/group). Basal NO bioavailability was determined as the magnitude of augmentation of contractile responses in presence of NOS inhibitor, L-NAME (1 × 10-4M). *P<0.05 at EC50 by students t test.



<u>Figure 5.14.</u> Histological analysis of cardiac structure.

Histological analysis was carried out on sections of heart from animals in all 4 groups at termination (n=4/group). (A) Immunohistochemistry with an anti-ACE2 antibody. Scale bar = 100 µm, magnification × 40. (B) H&E staining in all groups. Scale bar = 30 µm, magnification × 20.

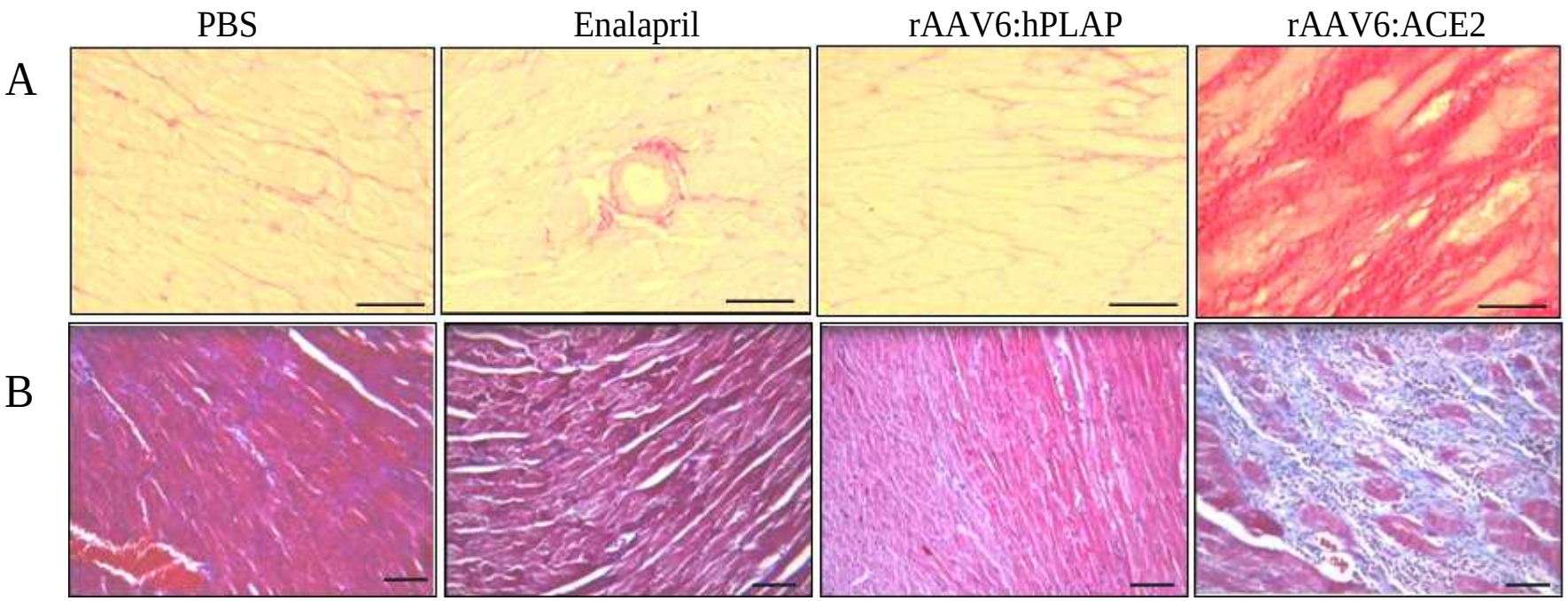


Figure 5.15. Assessment of cardiac fibrosis.

Heart sections from rAAV6:ACE2, rAAV6:hPLAP, PBS and Enalapril treated animals were analysed for fibrosis and collagen content (n=4/group). (A) Picrosirius red staining. Scale bar = 100 µm, magnification × 25. (B) Masson's trichrome staining. Scale bar = 100 µm, magnification × 40.

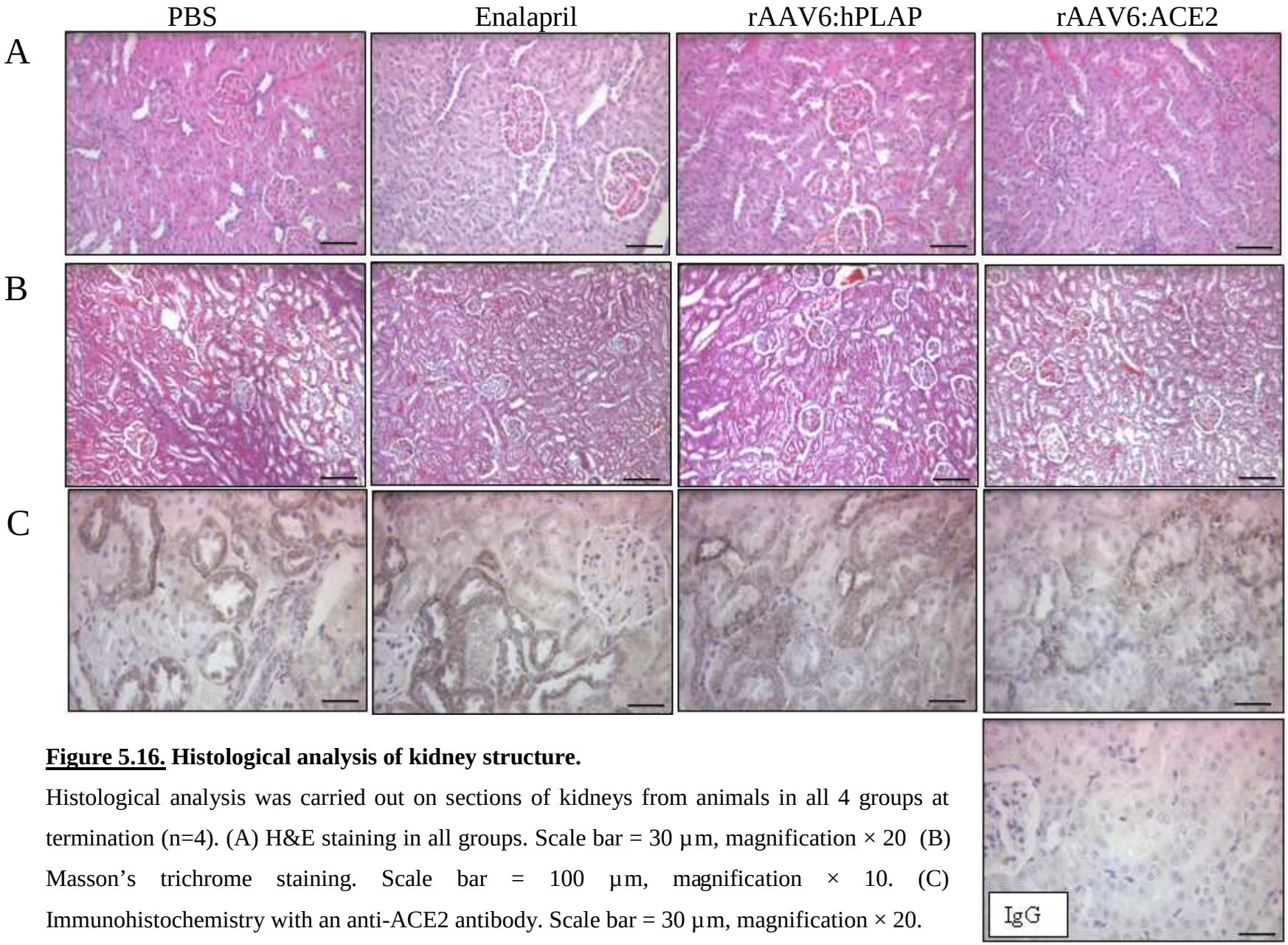
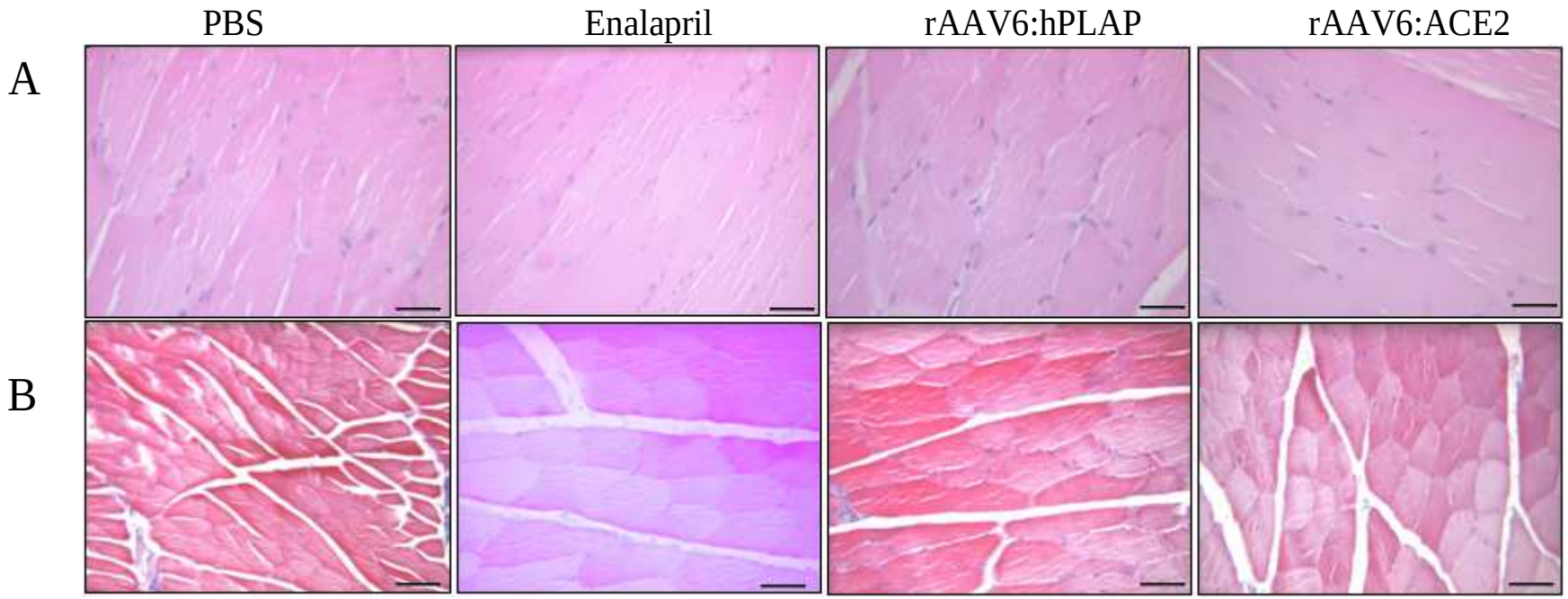


Figure 5.16. Histological analysis of kidney structure.

Histological analysis was carried out on sections of kidneys from animals in all 4 groups at termination (n=4). (A) H&E staining in all groups. Scale bar = 30 µm, magnification × 20 (B) Masson's trichrome staining. Scale bar = 100 µm, magnification × 10. (C) Immunohistochemistry with an anti-ACE2 antibody. Scale bar = 30 µm, magnification × 20.



<u>Figure 5.17.</u> Histological assessment of muscle.

Muscle sections from rAAV6:ACE2, rAAV6:AP, PBS and Enalapril treated animals were analysed for fibrosis. (A) H&E staining in all groups. Scale bar = 100 µm, magnification x 40. (B) Masson's trichrome staining. Scale bar = 30 µm, magnification x 20.

5.3 Discussion

ACE2 has been proposed as a critical component of the RAS, acting in opposition to ACE by hydrolysis of Ang II. In the present study, we demonstrated that sustained (11 week) rAAV6-mediated ACE2 overexpression in the SHRSP exerts detrimental effects on cardiac structure and function whilst increasing basal NO bioavailability, suggesting both positive and negative effects of overt ACE2 overexpression *in vivo*. Myocardial changes were characterised by morphological adaptations including severe myocardial interstitial and perivascular fibrosis, an increase in collagen content and abnormal myocardial organisation. We also demonstrate, for the first time, that intravenous administration of rAAV6 vectors can be used to efficiently deliver and express genes in the heart of a rat cardiovascular disease model. Importantly, rAAV6 expressing the reporter gene hPLAP was found to have no deleterious effects on cardiac structure, function or any significant effect on BP – potentially important findings for clinical trials using rAAV6 vectors.

At present, the role of ACE2 in the RAS remains ambiguous. Previous ACE2 intervention studies either in mice (Donoghue, Hsieh et al. 2000; Crackower, Sarao et al. 2002; Gurley, Allred et al. 2006), or rats (Diez-Freire, Vazquez et al. 2006), have shown conflicting results. Crackower et al (Crackower, Sarao et al. 2002) proposed ACE2 as an essential regulator of heart function through work with ACE2 knockout mice. The loss of ACE2 resulted in severe cardiac contractility defects and also an increase in Ang II levels, indicating that ACE2 controls levels of Ang II *in vivo* (Crackower, Sarao et al. 2002). The same study revealed ACE2 as a strong candidate gene linked to a hypertensive quantitative trait locus on the X chromosome. In a separate study, ACE2 gene transfer resulted in significant attenuation of high BP and cardiac fibrosis in the SHR (Diez-Freire, Vazquez et al. 2006). However, Donoghue et al (Donoghue, Wakimoto et al. 2003) showed that transgenic mice with increased cardiac ACE2 expression displayed high incidence of sudden death, showing that the role of ACE2 is more complex than originally thought.

Ang II is well documented in playing a role in hypertension and LVH. Studies in ACE2-deficient mice (Gurley, Allred et al. 2006) found that these mice showed an

increase in systolic blood pressure and elevated plasma levels of Ang II, indicating that ACE2 is a key pathway for the metabolism of Ang II.

The blood pressure data in this present study correlates with that published in another study using lentiviral overexpression of ACE2 following intracardiac injection (Diez-Freire, Vazquez et al. 2006). However, it is unclear as to whether this occurs via the same mechanism. In the lentiviral study (Diez-Freire, Vazquez et al. 2006), expression of ACE2 after intracardiac injection was also shown to occur in the kidney through systemic leakage of the vector, whilst the biodistribution patterns of our vector shows no evidence of substantial expression in the kidney. We also confirmed by immunohistochemical techniques that there was no overexpression of ACE2 in the kidneys. Therefore attenuation of high blood pressure in the SHR study (Diez-Freire, Vazquez et al. 2006) could be due to beneficial effects of ACE2 acting directly to metabolise Ang II into Ang 1-7 in the kidney. The high blood pressure attenuation in the present study could be explained by a beneficial effect of ACE2 acting on the peripheral circulation, as the expression of ACE2 in our study is not limited to the myocardium. Accordingly, here we have demonstrated greater basal NO bioavailability in the mesenteric arteries in rAAV6:ACE2 infused animals at 3 months post gene delivery than in rAAV6:hPLAP controls, suggesting that ACE2 overexpression directly improves endothelial function. SHRSP have been shown to have reduced NO bioavailability in comparison to the WKY reference strain (McIntyre, Hamilton et al. 1997). ACE2 overexpression may have a beneficial peripheral action and detrimental effects in the myocardium. Indeed evidence of a tissue-localized RAS is well established (Campbell 1987; Paul, Wagner et al. 1993) including a cardiac RAS (Dzau 1988), with every component of the RAS having been identified in cardiac and vascular myocytes and fibroblasts. Although the improvement in endothelial function may have reduced systolic blood pressure, the severe cardiac dysfunction and fibrosis may have lowered blood pressure as a result of the development of diastolic and systolic abnormalities generating cardiac decompensation.

In the study most akin to that described here, local delivery of a lentiviral vector overexpressing ACE2 into the heart resulted in transduction levels of >50% in some areas of the myocardium but to <5% in other areas (Diez-Freire, Vazquez et al. 2006).

Whilst the lentivirus study reported reduced blood pressure and beneficial effects on cardiac fibrosis, it is plausible that ACE2 expression levels were not as high as in the present study. Thus lower ACE2 levels may produce cardioprotective effects whilst avoiding the induction of the detrimental effects on the heart. The hearts of the rAAV6:ACE2 treated animals in the present study showed a reduction in ejection fraction and fractional shortening, a decrease in interventricular wall thickness, a decrease in SBP: LV ratio and no increase in cardiac output overtime along with histological evidence of heart failure. All these are consistent with severe cardiac dysfunction progressing towards heart failure. This is in agreement with the transgenic mouse study, which also found that overexpression of ACE2 resulted in profound cardiac dysfunction and mild cardiac fibrosis (Donoghue, Wakimoto et al. 2003). Cardiac fibrosis is a marker of cardiac failure and contributes to ventricular wall stiffness, impairing cardiac relaxation so that the ventricles do not fill properly thus resulting in abnormal diastolic function (Doering, Jalil et al. 1988; Jalil, Doering et al. 1989; Brilla, Janicki et al. 1991). The pathogenesis of heart failure inevitably proceeds to dilated cardiomyopathy, in which heart chambers become markedly enlarged and contractile function deteriorates. In early stages, cardiac enlargement is an adaptive process to help the heart maintain cardiac output. Past a certain point, however, the heart overcompensates for declining systolic performance, and dilation becomes a pathologic process. Dilation in the present study along with decreased wall thickness would account for the lack of change in LVMI in the ACE2 transduced SHRSP. The mechanisms behind this are poorly understood, although recently a causal relationship between myocyte death and cardiomyopathy has been established (Wencker, Chandra et al. 2003). Wall thinning is consistent with loss of myocytes, which in heart failure is known to be associated with fibrosis and apoptosis.

It is plausible that although Ang II has deleterious effects, it may have a necessary function in a signalling pathway to enable the heart to proceed through adaptive remodelling and progressing to failure. For example, ERK1/2 activation is stimulated by Ang II and in experimental models, blocking ERK1/2 activation rendered the heart more susceptible to failure, suggesting that ERK1/2 had a role in protecting the heart from cell death and failure (Purcell, Wilkins et al. 2007).

Additionally, increased ACE2 overexpression without corresponding ACE upregulation could shift the balance from the production of Ang 1-7 into Ang 1-9, as ACE is required to convert Ang 1-9 into Ang 1-7. As yet, a role for Ang 1-9 remains unidentified and so further study into the function of Ang 1-9 could clarify whether it is having any deleterious effect on cardiac function. To ascertain if Ang 1-9 has any activity, stimulation of cardaic cells with Ang II to induce hypetrophy could be carried out in the presence of Ang 1-9 peptide and an ACE inhibitor. The ACE inhibitor would ensure that any blocking of hypertophic effects were as a result of the activity of Ang 1-9 and not as a result of the converted product, Ang 1-7.

It is also important to consider the role of ACE2 in hydrolysing several other peptides, most notably apelin-13 (Vickers, Hales et al. 2002). Apelins have been implicated as regulators of cardiovascular function (Lee, Cheng et al. 2000) and have been shown to exert cardioprotective effects through the activation of pathways that are associated with myocardial preservation (Simpkin, Yellon et al. 2007). Apelin has been shown to cause vasodilatation, reduced ventricular preload and afterload, and increased cardiac contractility (Japp and Newby 2008). Thus the hydrolysation of apelins-13 may prove to be physiologically relevant. To test whether the apelins played a role in the pathophysiology of our model, an assay to measure the levels of apelin present in the hearts of our animals at termination could be carried out. The downregulation of apelins may have had adverse effects on our model.

In conclusion, the data in this chapter demonstrates the development of severe cardiac abnormalities associated with sustained ACE2 overexpression *in vivo*. Further work should address the extent to which these effects are correlated to the ACE2 expression levels to determine if beneficial effects can be obtained with reduced expression levels, or if the increased expression of ACE2 at any level is deleterious for cardiac morphology and function.

Chapter 6

General Discussion

This thesis has focused on the development and use of viral vectors that are targeted to the heart, in order to achieve efficient cardiac overexpression of ACE2. As the renin angiotensin system is pivotal in the control of sodium balance, volume homeostasis and therefore long-term blood pressure, it is targeted clinically to treat hypertension and heart failure. Overactivity of the RAS plays a fundamental role in the pathophysiology of hypertension and progression of heart failure (Zaman, Oparil et al. 2002). However, this pathway can be manipulated and blocked at several levels. In hypertension, the reduction of blood pressure leads to a sequence of events that ultimately results in a reduced ventricular afterload and preload, a reduction in blood volume and even in the inhibition and reversal of cardiac and vascular hypertrophy. This highlights that the RAS is an important pathway to be targeted in achieving therapeutically beneficial outcomes. The relatively recent discovery of new enzymes, such as ACE2, has brought into question the current classical view of the RAS. Currently, the role of ACE2 in the RAS remains ambiguous. It is undetermined as to whether ACE2 contributes to the regulation of cardiac structure and function and whether, through its actions to metabolize Ang II, it has a role in the regulation of blood pressure by attenuation of the hypertensive actions of Ang II. This added level of complexity to the RAS has provided a novel opportunity to modulate ACE2 expression.

A major hurdle in the development of cardiovascular gene therapy is the limited availability of vectors that are tissue or cell selective and that can be delivered intravenously. Another issue to be addressed is the need for long-term transgene expression, depending on the application. Non-viral vectors tend to be limited by their low transfection efficiencies and transient gene expression. Viral vectors are more promising vectors as they can mediate higher levels of transgene expression and are being developed to be more selective for their target organ or cell type. Strategies to improve targeting and reduce immunogenicity have been applied to adenovirus and adeno-associated viral vectors, which is very applicable to cardiovascular gene therapy. The discovery of new serotypes of AAV that have a natural tropism for muscle, including cardiac muscle, has greatly aided the development of non-invasive cardiac gene delivery. This thesis investigated the use of targeted viral vectors and of the development of novel vectors, with the aim of producing a viral vector that is highly efficient and selective at transducing cells of the cardiovascular system. The

primary focuses were on myocyte targeting with rAAV6 vectors and vascular endothelial cell targeting by vector engineering strategies. Whilst the initial results from the heart targeting peptides were encouraging, these peptides were unable to succesfully retarget the vectors that they were tested in. Finding a suitable vector for these peptides may not be possible or could prove to be time consuming. In light of this, rAAV6 represents a far more useful vector for future developments, provided that an efficient element of transcriptional regulation can be accomodated.

For targeting the cardiac vasculature, I evaluated, both *in vitro* and *in vivo*, candidate heart targeting peptides identified through phage display in which the heart vasculature was probed for heart-specific endothelial markers. Limitations of phage display for the isolation of targeting peptides potentially include high non-specific binding of phage and lower levels of phage available to interact with the target, as much phage will be taken up by the liver and spleen immediately proceeding intravascular injection (Balestrieri and Napoli 2007). However, despite these challenges, four potential vascular targeting candidates were identified as CRPPR, CSGMARTKC, CRSTRANPC and CPKTRRVPC. All four showed an increase in ability to home to the heart when compared to insertless phage. Once shown to be selective for the heart tissues, these four peptides were used to modify Ad5, Ad19p and AAV2 vectors to assess if they increased the selectivity of these vectors to endothelial cells of the vasculature. Whilst AdKO1 peptide-modified vectors were promising *in vitro*, phage-display derived targeting peptides generally proved disappointing in the context of Ad19p and AAV2 retargeting, with the tropism of peptide modified vectors remaining unchanged. It is important to note that the high specificity of these peptides for the heart can be exploited not only for viral vector retargeting, but potentially also for the conjugation of therapeutic agents to the peptides. Most work in this area so far has focused on the delivery of cancer therapies (Ellerby, Arap et al. 1999), but this application could be translated to the delivery of cardiovascular therapies.

Sequestration in the liver is a major limitation of Ad vectors (Huard, Lochmuller et al. 1995). To enable Ad vectors to mediate transduction of alternative organs, the abolition of binding to CAR and the incorporation of targeting peptides into the capsid of the vector were proposed to re-route the vector. CAR-binding ablated vectors were

generated after the discovery of the key sites on the vectors that mediate binding (Roelvink, Mi Lee et al. 1999). However, it was soon discovered that ablating CAR binding alone did not change the hepatic tropism of these vectors *in vivo*, despite promising results *in vitro* (Alemany and Curiel 2001; Mizuguchi, Koizumi et al. 2002; Smith, Idamakanti et al. 2003), suggesting the use of alternate receptor pathways *in vivo* by Ad5 vectors. Ad5 vectors harbouring mutations in both CAR and HSPG binding regions have demonstrated reduced hepatic tropism *in vivo* (Smith, Idamakanti et al. 2003; Nicol, Graham et al. 2004). Regardless of this fact, the incorporation of targeting peptides into the capsid of these vectors did not lead to the transduction of the target tissue (Kritz, Nicol et al. 2007). Triple mutant Ad vectors that are ablated of CAR, integrin and HSPG binding have been generated, and mediate lower levels of liver transduction than unmodified vectors (Koizumi, Mizuguchi et al. 2003; Koizumi, Kawabata et al. 2006). Whilst AdKO1 vectors may not be effective *in vivo*, they are useful *in vitro* tools to assess the targeting capacity of the selected peptides in the context of a viral vector (Mizuguchi, Koizumi et al. 2002). Clearly, the four identified peptides are highly selective, and have demonstrated that they can be incorporated into viral vectors with successful retargeting. With the selection of an appropriate vector, the incorporation of these peptides could result in the generation of highly selective vectors, but this remains to be tested.

It has recently been documented that blood coagulation factors, especially factor X, play a major role in targeting Ad5 vectors to hepatocytes *in vivo* (Shayakhmetov, Gaggar et al. 2005; Parker, Waddington et al. 2006), suggesting that coagulation factors in the blood would need to be eliminated for efficient retargeting of vectors based on this serotype. It has been shown that factor X binds directly to the adenovirus, resulting in high levels of liver transduction mediated through HSPGs (Parker, Waddington et al. 2006; Waddington, Parker et al. 2007). Recently, it has been discovered that interaction between the virus and factor X is not mediated through the fiber protein as originally suggested (Shayakhmetov, Gaggar et al. 2005), but is instead mediated through the hexon protein (Waddington, McVey et al. 2008). This finding has major implications on both vector infectivity biology and on vector design. By disrupting the interaction between factor X and Ad5 hexon using a snake-venom derived factor X blocking protein (X-bp), liver transduction was significantly reduced in mice and rats (Waddington, McVey et al. 2008). Pre-treatment with X-bp

used in combination with our peptide-modified AdKO1 vectors may allow for the efficient retargeting of these vectors.

Alternative serotypes of Ad vectors that differ in their fibers are being explored as potential gene delivery vectors to identify those with reduced coagulation factor binding (Denby, Work et al. 2004). When pseudotyped onto Ad5 vectors, the fiber of serotype 19p was shown to mediate greatly reduced liver tropism *in vivo* and to display a reduced capacity for the binding of blood coagulation factors (Denby, Work et al. 2004). However, despite the great potential of Ad19p vectors, the insertion of heart targeting peptides into the HI loop on the fiber did not retarget the vectors accordingly. A reduced liver tropism was maintained however, confirming the natural lack of hepatic tropism previously reported (Denby, Work et al. 2004). The screening of a panel of vectors, including alternative virus types may lead to the identification of a suitable platform vector into which targeting peptides can be inserted.

AAV vectors have emerged as promising alternative vectors to adenoviral vectors for cardiovascular gene therapy, as a result of their inability to mediate sustained transgene expression in non-dividing cells. However, AAV2 exhibits poor tropism for human vascular endothelial cells (Nicklin, Buening et al. 2001) and so the development of rAAV2 vectors requires either retargeting, or the use of alternate serotypes. Recently, it has been shown that rAAV1 and rAAV5 vectors are more efficient than rAAV2 vectors at transducing endothelial cells *in vitro* and *in vivo* when used at low titres (Sen, Conroy et al. 2007). However, previous studies have demonstrated that AAV serotypes -2 through -8 are all poor transducers of vascular cells (Dishart, Denby et al. 2003; Denby, Nicklin et al. 2005), and our present data with rAAV6 and rAAV9 vectors would agree with these findings. Genetic engineering of rAAV2 vectors through the insertion of a targeting peptide has previously been shown to be a viable retargeting technique, with rAAV2 vectors being retargeted to the human luteinizing hormone receptor (Shi, Arnold et al. 2001), the vasculature the lung and brain *in vivo* (Work, Buening et al. 2006) and to atherosclerotic lesions (White, Buening et al. 2007). This study characterised AAV2 based vectors containing the heart targeting peptides CPKTRRVPC, CRPPR and CSGMARTKC and found no evidence of retargeting. The net charge of the peptides is thought to be important in determining whether HSPG binding will be ablated by

the insertion of the peptide into the HSPG binding site (Opie, Warrington et al. 2003; Perabo, Goldnau et al. 2006). Positively charged peptides are thought to maintain the HSPG binding abilities of the vectors (Perabo, Goldnau et al. 2006). As the net charge of all three peptides is positive, this could explain the lack of retargeting observed from the peptide-modified vectors, although the hepatic cell line HepG2 remained untransduced, suggesting a contributory rather than a causative role. Potentially the insertion of these peptides into the rAAV2 capsid has resulted in impaired intracellular trafficking of AAV to the nucleus.

Whilst alternate AAV serotypes may not have improved endothelial transduction (Denby, Nicklin et al. 2005), they have been shown to efficiently target other organs, including the myocardium, liver and brain (Wang, Zhu et al. 2005; Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006). In particular, recombinant vectors derived using the capsid genes of AAV serotypes including AAV6, -8 and -9 have demonstrated powerful tropism for skeletal and cardiac muscle (Kawamoto, Shi et al. 2005; Wang, Zhu et al. 2005; Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006), achieving high cardiac transduction rates following systemic injection (Wang, Zhu et al. 2005; Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006). In a direct comparison of AAV serotypes 1-8, direct injection into the myocardial wall lead to high levels of myocardial transduction mediated by AAV-1, -6 and -8 (Palomeque, Chemaly et al. 2007). Prior studies have shown the efficiency of both rAAV6 (Blankinship, Gregorevic et al. 2004; Gregorevic, Blankinship et al. 2004) and rAAV9 (Inagaki, Fuess et al. 2006; Pacak, Mah et al. 2006) vectors in achieving myocardial gene transfer. It is difficult to compare the serotypes across studies; route of administration, virus dose administered, ages of animals and use of vascular permeablising agents have differed between studies. We demonstrate that intravenous administration of rAAV6 and rAAV9 vectors can be used to efficiently deliver and express genes in the heart of SHRSP, a relevant rodent CVD model. Furthermore, we show that rAAV6 vectors posses a more favourable cardiac gene delivery profile and as such are used for the ultimate aim of this thesis – to overexpress ACE2 in the myocardium of the SHRSP. The low numbers of animals used in the comparsion of these two vectors is a limitation of this study, but was necessary due to a lack of virus which has a laborious production method. The repetition of these results cannot be assured; however, the dose dependent results that were seen from both vectors demonstrate that these

vectors behave as expected in the SHRSP. RAAV6 vectors are a useful tool to study the molecular mechanisms of cardiovascular disease, and have recently been approved for use in patient clinical trials. RAAV6 vectors expressing sarco-endoplasmic reticulum calcium ATPase pump (SERCA2a), driven by the CMV promoter, have been approved for a human clinical trial to patients in chronic heart failure in the UK (www.clinicaltrials.gov) and USA (www.wiley.co.uk/genetherapy). SERCA2a plays a fundamental role in lowering cytoplasmic calcium levels during relaxation and the observed decline in contractile function in failing hearts is often associated with lowered protein levels of SERCA2a (Del Monte, Dalal et al. 2004). This vector has been developed to improve systolic and diastolic function of the failing ventricle through restoration of SERCA2a levels, as it has been shown that levels of SERCA2a are reduced in the failing ventricle (Pleger, Most et al. 2007).

Wide spread dissemination of vectors following intravenous administration raises the issue of potential transduction of non-target tissues. To increase specificity of the vector, an element of transcriptional control was incorporated into rAAV6 vectors. This approach has been successful in achieving muscle specific transgene expression (Sun, Zhang et al. 2005; Salva, Himeda et al. 2007), and has been used to develop tissue-specific regulatory cassettes that mediate high levels of transgene expression in both skeletal and cardiac muscle (Salva, Himeda et al. 2007). Despite being shown to successfully drive myocardial specific expression from rAAV2 vectors (Phillips, Tang et al. 2002), the incorporation of the rat cardiac-specific promoter MLC2v into our rAAV6 vectors was unsuccessful *in vivo*. The activity of the MLC2v promoter was clearly not strong enough to induce the expression of reporter gene *lacZ* following intravenous administration, despite evidence of efficiency and selectivity *in vitro*. This does not rule out the possibility of achieving transcriptional control with rAAV6 vectors however. To increase the strength of the promoter, a CMV element could be fused to the MLC2v promoter, as has recently been demonstrated with the longer 1.5 kb version of the promoter (Muller, Leuchs et al. 2006; Raake, Hinkel et al. 2007). Alternative cardiac-specific enhancer/promoter elements could be investigated further to identify one that successfully drives myocardial specific transgene expression. The α-cardiac actin enhancer/elongation factor 1α (EF1α) promoter has recently been used to drive cardiac-specific expression of the transgene S100A1 from rAAV6 vectors

(Pleger, Most et al. 2007). It is important that the regulatory element is small enough so as to maximise the capacity available for the insertion of the transgene.

We have used cardiac gene delivery, mediated by recombinant AAV6 vectors, to examine the physiological role of ACE2 *in vivo* in established cardiovascular disease model, the SHRSP, which is a model of genetic hypertension with susceptibility to concentric left ventricular hypertrophy (LVH). Previous studies into the actions of ACE2 have generated conflicting results (Crackower, Sarao et al. 2002; Donoghue, Wakimoto et al. 2003; Diez-Freire, Vazquez et al. 2006; Gurley, Allred et al. 2006). Because ACE2 is known to hydrolyse Ang I and Ang II (Tipnis, Hooper et al. 2000), it was considered that ACE2 may play a pivotal role in the RAS by reducing concentrations of the pro-fibrotic, pro-proliferative vasoconstrictor Ang II and raising levels of the anti-fibrotic, anti-proliferative vasodilatory peptide Ang 1-7. Ang 1-7 has been found to exert beneficial effects on CVD models (Santos, Ferreira et al. 2004; Benter, Yousif et al. 2007), establishing a potentially cardioprotective role for this circulating peptide. Thus ACE2 would be deemed as a negative regulator of the RAS. However, our findings are contrary to the expected hypothesis that ACE2 could function to improve cardiac function in hypertensive animals. We found that sustained and high-level ACE2 overexpression exerts detrimental effects on cardiac structure as characterised by severe myocardial interstitial fibrosis and abnormal myocardial organisation. Severe cardiac dysfunction was characterised by a marked and significant reduction in ejection fraction and fractional shortening. We also found that basal NO bioavailability was increased, suggesting both positive and negative effects of ACE2 overexpression *in vivo*.

Clearly there are limitations with our approach to the assessment of overexpression of ACE2. One of the most interesting aspects of this work is that in a study that was very similar in design to our own, differing in choice of vector and in delivery method, very different results were attained (Diez-Freire, Vazquez et al. 2006). Potentially there is a dose-dependent response occurring, as suggested by Donoghue et al (Donoghue, Wakimoto et al. 2003), who found that higher ACE2 expressing transgenic mice had a higher severity of symptoms including a higher incidence of death than the lower expressing line (Donoghue, Wakimoto et al. 2003). Thus in the study by Diez-Freire et al (Diez-Freire, Vazquez et al. 2006), the lentiviral vector

potentially expressed ACE2 at low enough levels to display cardioprotective effects whilst avoiding the induction of the detrimental effects on the heart (Diez-Freire, Vazquez et al. 2006). Further investigation into the dosing effects of ACE2 could establish such a correlation, enhance our knowledge of the RAS and enable the development of improved therapeutic strategies.

It is also important to consider that ACE2 functions as a multifunctional enzyme. In particularly, it has been shown that ACE2 efficiently cleaves apelin (Vickers, Hales et al. 2002). There is increasing evidence that apelins play crucial roles in the maintenance of cardiac function (Berry, Pirolli et al. 2004). In myocardial I/R models, apelin administered at pharmacological doses produced cardioprotective effects as evidenced by reduced infarct sizes (Simpkin, Yellon et al. 2007). Aged apelin knockout mice were found to develop impaired cardiac contractility, systolic dysfunction and progressive heart failure (Kuba, Zhang et al. 2007). Through the cleavage of apelins, it would appear that ACE2 may be able to potentially knock out crucial cardioprotective functions of other systems other than the RAS.

No studies to date have identified a mechanistic pathway of the interactions of ACE2. It is unclear as to exactly which peptides ACE2 interacts with to mediate its effects *in vivo*. It also remains ambiguous as to whether ACE2 is acting locally or systemically. Based on the findings of this study, we have performed global gene expression profiling to examine potential pathways that are perturbed upon the onset of the actions of ACE2 (assessed at 4 weeks post-infusion). Illumina gene expression analysis revealed the upregulation of several fibrosis-associated genes including collagen type III alpha 1 (COL3A1), fibronectin 1 (FN1) and lysyl oxidase (LOX). Furthermore, genes including apelin, myosin heavy chain 11 (MYH11) and GATA binding protein 6 (GATA6) genes were downregulated (unpublished data). Thus gene expression analysis revealed activation of a pro-fibrotic phenotype at the transcriptional level. Further investigation of these pathways is required to fully understand the mechanistic basis of these findings.

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Appendix 1 Echocardiography findings

Group	Weeks Post-infusion	%EF	%FS	CO (ml/min)	LVMI (mg/g)	RWT	% IVSWT	SBP:ESV	HR (b/min)
PBS	0	88.0 ± 1.5	53.0 ± 2.3	168.6 ± 1.6	2.48 ± 0.1	0.54 ± 0.02	45.0 ± 1.9	2314 ± 433	405.8 ± 1.9
	11	81.3 ± 1.1	50.8 ± 4.1	234.5 ± 18.5	2.96 ± 0.1	0.60 ± 0.01	44.3 ± 3.1	3600 ± 382	375.7 ± 4.0
rAAV6:hPLAP	0	84.5 ± 1.3	49.0 ± 1.7	183.5 ± 7.8	2.51 ± 0.1	0.55 ± 0.02	41.5 ± 3.5	1965 ± 126	428.9 ± 19.9
	11	82.3 ± 1.3	51.3 ± 2.5	233.2 ± 17.8	2.86 ± 0.1	0.60 ± 0.03	43.3 ± 3.5	2842 ± 458	377.2 ± 7.5
rAAV6:ACE2	0	89.0 ± 1.7	55.0 ± 3.2	225.6 ± 2.2	2.84 ± 0.3	0.49 ± 0.01	48.7 ± 1.8	2277 ± 341	393.4 ± 2.6
	11	62.0 ± 2.7*	34.3 ± 4.9*	204.9 ± 17.6*	2.62 ± 0.2	0.49 ± 0.01	33.7 ± 4.4*	582 ± 57*	372.2 ± 9.2
Enalapril	0	84.3 ± 1.3	51.5 ± 0.3	179.1 ± 6.7	2.37 ± 0.1	0.52 ± 0.02	47.5 ± 1.9	2343 ± 159	385 ± 8.7
	11	80.5 ± 2.1	56.0 ± 1.5	231.3 ± 10.1	2.31 ± 0.1	0.57 ± 0.01	42.25 ± 1.3	3003 ± 257	411 ± 4.0

EF, ejection fraction; FS, fractional shortening; CO, cardiac output; LVMI, left ventricular mass index; RWT, relative wall thickness; IVSWT, interventricular septal wall thickness; SBP:ESV, ratio of systolic blood pressure to end systolic volume; HR, heart rate.*$p<0.05$ rAAV6:ACE2 vs. PBS, Enalapril and rAAV6:hPLAP.

Peptide	Tissue	Recovery (PFU)/ mg tissue
INSERTLESS	Liver	$1.0 \times 10^5 \pm 2.3 \times 10^3$
	Spleen	$3.2 \times 10^4 \pm 7.4 \times 10^3$
	Brain	41 ± 8
	Kidney	60 ± 20
	Lung	$2.3 \times 10^2 \pm 44$
	Heart	3.4 ± 0.8
CRPPR	Liver	$8.2 \times 10^5 \pm 2.0 \times 10^5$
	Spleen	$2.7 \times 10^5 \pm 8.1 \times 10^4$
	Brain	$4.2 \times 10^2 \pm 2.7 \times 10^2$
	Kidney	$7.9 \times 10^2 \pm 1.2 \times 10^2$
	Lung	$3.2 \times 10^3 \pm 6.8 \times 10^2$
	Heart	$1.5 \times 10^3 \pm 6.1 \times 10^2$
CPKTRRVPC	Liver	$2.3 \times 10^5 \pm 1.0 \times 10^5$
	Spleen	$3.0 \times 10^4 \pm 9.5 \times 10^3$
	Brain	23 ± 4
	Kidney	16 ± 2
	Lung	$3.3 \times 10^3 \pm 1.4 \times 10^3$
	Heart	43 ± 9
CRSTRANPC	Liver	$1.5 \times 10^6 \pm 3.0 \times 10^5$
	Spleen	$2.1 \times 10^6 \pm 2.2 \times 10^5$
	Brain	$8.0 \times 10^2 \pm 2.9 \times 10^2$
	Kidney	$3.7 \times 10^3 \pm 5.0 \times 10^2$
	Lung	$1.2 \times 10^4 \pm 6.1 \times 10^3$
	Heart	$7.4 \times 10^3 \pm 1.0 \times 10^3$
CSGMARTKC	Liver	$8.3 \times 10^4 \pm 2.2 \times 10^4$
	Spleen	$4.1 \times 10^4 \pm 1.0 \times 10^4$
	Brain	37 ± 15
	Kidney	$2.1 \times 10^2 \pm 21$
	Lung	$1.2 \times 10^2 \pm 76$
	Heart	$2.8 \times 10^2 \pm 83$
CPKRPR	Liver	$4.0 \times 10^5 \pm 5.3 \times 10^4$
	Spleen	$7.6 \times 10^5 \pm 3.8 \times 10^5$
	Brain	97 ± 48
	Kidney	83 ± 81
	Lung	$1.8 \times 10^3 \pm 1.5 \times 10^3$
	Heart	29 ± 14
CGRSKTVC	Liver	$6.3 \times 10^5 \pm 1.7 \times 10^5$
	Spleen	$1.3 \times 10^6 \pm 3.9 \times 10^5$
	Brain	$1.7 \times 10^2 \pm 99$
	Kidney	$1.4 \times 10^3 \pm 7.2 \times 10^2$
	Lung	$2.3 \times 10^4 \pm 1.6 \times 10^4$
	Heart	81 ± 7.6

Appendix 2 Phage recovery data.

Phage recovery data shown as mean recovery (PFU)/ mg tissue ± SEM (n=3/group).