The Development of Viral Vectors for Targeted Gene Delivery to Atherosclerotic Plaques.

By

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Summary

Cardiovascular disease is one of the leading causes of death in the Western world. One of the most common causes is the rupture of unstable atherosclerotic plaques, which can lead to thrombus formation, occlusion of the artery and myocardial infarction. Therefore there is a need for treatments that stabilise vulnerable plaques. Gene therapy has the potential to provide a novel treatment for this. To maximise therapeutic gene expression and minimise any potential adverse effects due to unwanted transgene expression in non-target tissues, a gene delivery vector specifically targeted to areas of atherosclerotic vasculature is required. The vector is also required to efficiently infect cells to produce relatively long term transgene expression, be stable in blood, non-toxic, non-immunogenic and producible at high titres. Viral vectors, particularly those based on adenovirus (Ad) and adeno-associated virus (AAV) have many of the desired features, but transduce vascular cells relatively inefficiently and in a non-selective manner. Methods of altering their tropism have been established and could be utilised to develop vectors with a high degree of selectivity for atherosclerotic plaques. Detargeting of vectors can be achieved by mutating regions of the virus capsid that are thought to bind the native cellular receptors and retargeting to novel cell types is achieved by inserting peptide ligands into the virus capsid. The aim of this study was to develop atherosclerotic plaque targeted vectors and to characterise Ad and AAV vector platforms in this regard. Two approaches were taken. In the first approach three previously identified plaque targeting peptides were tested for their ability to target viral vectors to atherosclerotic plaques. The second approach involved performing phage display in a mouse model of plaque rupture to identify novel peptides that specifically target unstable plaques. Further work was carried out to characterise and develop methods for using an AAV2 based peptide library as a novel tool for biopanning.

The atherosclerotic plaque targeting peptides CAPGPSKSC, CNHRYMQMC and CQEPTRLKC were incorporated into the fiber capsid protein of the Ad5 based vector AdKO1, which contains mutations that block binding to the primary receptor, the coxsackie and adenovirus receptor (CAR). *In vitro*, the peptide-modified AdKO1

vectors produced higher levels of transduction of several endothelial cell lines, compared to unmodified AdKO1, suggesting the vectors target vascular cells, but further testing was required *in vivo*. It has previously been demonstrated that following systemic administration AdKO1 vectors are not efficiently detargeted from the liver, so the peptides were incorporated into alternative vector platforms. The AdKO1S* vector platform contains mutations that block binding to CAR and the putative co-receptor heparin sulphate proteoglycans (HSPG) and it has been shown to be detargeted *in vivo*. However, all the peptide-modified AdKO1S* vectors were found to be non-infective. Pseudotyping of Ad vectors involves replacing the fiber of one serotype with that of another, to produce a vector with a novel tropism. The plaque targeting peptides were inserted into an Ad5 vector pseudotyped with the Ad19p fiber (Ad5/19p). Although the peptide-modified Ad5/19p vectors transduced vascular cells more efficiently than unmodified Ad5/19p, the increased transduction was not specific to vascular cells. Therefore, in this study, none of the Ad vectors tested provided a suitable platform. The CAPGPSKSC and CNHRYMQMC peptides were also incorporated into the receptor binding site of the AAV2 capsid. Promising results were achieved both *in vitro* and *in vivo*. In an ApoE-/- mouse model of atherosclerosis the peptide-modified AAV2 vectors were found to be targeted to the brachiocephalic artery and aorta (sites where plaques are known to develop) and detargeted from all other tissues examined, which suggests that the vectors are retargeted to areas of atherosclerotic vasculature with a high degree of selectivity. Further testing of these vectors is required to determine if they are plaque specific or specific for vascular cells.

Currently there are no effective ways to diagnose and locate the presence of unstable plaques and there are no treatments aimed specifically at inducing stabilisation or regression of unstable plaques. In parallel with the testing of the previously identified plaque targeting peptides, biopanning with phage display libraries was performed in a mouse model of atherosclerotic plaque rupture that has been shown to reproduce unstable plaques. Targeted pools of peptides were identified and BLAST searching identified many peptides with homology to proteins known to be associated with atherosclerosis

and plaque rupture. Further testing of the individual candidate peptides is now required to analyse their targeting capacity.

HSPG is known to be the primary receptor for AAV2. Insertion of targeting peptides into the HSPG binding site on the AAV2 capsid has been shown to be an effective way of detargeting and retargeting the vector with some peptides, but for other peptides the vector maintains the ability to bind HSPG. Characterisation of an AAV2 library with random 7-mer peptides inserted into the HSPG binding site has shown that in general positively charged peptides enable AAV to retain the capacity to bind HSPG. This is thought to be because AAV2 binds HSPG through an electrostatic interaction between negatively charged HSPG and the positive charge of the binding site. This interaction is blocked by insertion of negative or neutral peptides but can be maintained by the insertion of positively charged peptides. This information can be used for the development of AAV2 based vectors using peptides identified by phage display. *In vitro* biopanning with the AAV library was also used to identify endothelial cell targeted peptides that are thought to infect cells independently of an interaction with HSPG. Methods were also developed to use the library for *in vivo* biopanning.

This study has made progress towards the development of atherosclerotic plaque targeted vectors by identifying and characterising peptides that have the potential to target vectors specifically to areas of atherosclerosis. These vectors could potentially be developed as tools to enable gene delivery selectively to atherosclerotic plaques or could be utilised with imaging agents to detect plaques. This work has also provided further characterisation of Ad and AAV platform vectors that may be utilised in the development of vectors with a highly selective tropism.

Declaration

I declare that this thesis has been written entirely by myself and has not previously been submitted for a higher degree. This thesis is a record of work performed by myself with the exception of the production of the AAV vectors and AAV libraries (Dr H. Buening and Dr L.Perabo, University of Cologne, Germany), surface plasmon resonance (Dr J. McVey, Imperial Collage London), intravenous injections for phage display (Dr S. White, University of Bristol) and plaque histology (Dr R. MacDonald, University of Bristol). The research was carried out in the BHF Glasgow Cardiovascular Research Centre, University of Glasgow, under the supervision of Professor Andrew H. Baker.

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Table of Contents

List of figures

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Production and Characterisation of Atherosclerotic Plaque Targeted Viral Vectors

Chapter 5: Development and Characterisation of an AAV2 based peptide library

List of tables

List of abbreviations

aa	amino acid
AAV	adeno associated virus
ABC	avidin and biotinylated horseradish peroxidase complex
ACE	angiotensin converting enzyme
Ad	adenovirus
ApoA-I	apolipoprotein A-I
ApoE-/-	apolipoprotein E knockout
APS	ammonium persulphate
ASTEROID	A Study to Evaluate the Effect of Rouvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden
β-gal	β-galactosidase
BCA	bicinchoininc acid
BCA	brachiocephalic artery
B-CLL	B-cell chronic lymphocytic leukemia
bp	base pairs
BSA	bovine serum albumin
BLAST	basic local alignment search tool
BRCA2	breast cancer 2, early onset gene
C4BP	complement component C4-binding protein
CABG	coronary artery bypass grafting
CAP	CAPGPSKSC peptide
CAR	coxsackie and adenovirus receptor
CAV9	coxsackie virus A9
CHD	coronary heart disease
CMH	CNHRYMQMC peptide
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide

COX	cyclo-oxygenase
CQE	CQEPTRLKC peptide
CR	complement receptor
CsCl	caesium chloride
Ct	cycle threshold
CTL	cytotoxic T lymphocytes
CVD	cardiovascular disease
CX8C	constrained 8-mer library
DAB	3,3' diaminobenzidine
DC	dendritic cells
DMEM	Dulbecco's minimal essential media
DMEM-PI	DMEM supplemented with 1% (w/v) BSA, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EC	endothelial cell
EC-SOD	extracellular superoxide dismutase
EDTA	ethylendiamine tetra-acetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
EPCR	endothelial cell protein C receptor
ER	endoplasmic reticulum
EVG	Elastic von Gieson stain
Fab	antibody fragment
FAM	6-carboxyfluorescein
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR1	fibroblast growth factor receptor type 1

FITC	fluorescein-5-isothiocyanate
FIX	factor IX
Flt-1	fms-like tyrosine kinase-1
FVII	factor VII
FX	factor X
g	centrifugal force
g	gram
g3p	minor coat protein gene III protein (of M13 phage)
GMCSF	granulocyte macrophage-colony stimulating factor
gp	genomic particles
Grp78	glucose-regulated protein 78
H and E	haematoxylin and eosin stain
HCAEC	human coronary artery endothelial cells
HCl	hydrochloric acid
HdAd	helper dependent adenovirus
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
HMG-CoA reductase	3-hydroxy-3 methylglutaryl coenzyme A reductase
HO-1	heme-oxygenase 1
HRP	horseradish peroxidase
HSGs	heparin sulphate glycosaminoglycans
hsp	heat shock protein
HSPG	heparin sulphate proteoglycan
HSV-1	herpes simplex virus -- 1
HSVEC	human saphenous vein endothelial cells
HTNV	hantavirus
ICAM	intracellular adhesion molecules
ID	infectivity dose
IHC	immunohistochemistry
IL	interleukin

INF-γ	interferon γ
IPTG	isopropyl-beta-D-thiogalactopyranoside
ITR	inverted terminal repeat
IU	international units
kb	kilo base
KCl	potassium chloride
kDa	kilo Daltons
kg	kilogram
L	litre
lamR	laminin receptor
LB	Luria Bertani
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LFA-1	leukocyte function-associated antigen-1
LID	lipofectin, integrin and DNA vector
LnLL	N-acetyl-L-leucyl-L-leucyl-norleucine
LOX-1	lectin-like oxidized LDL receptor
LRP	low density lipoprotein receptor related protein
M	molar
M	cell media
MCP-1	monocyte chemoattractant protein –1
M-CSF	macrophage colony stimulating factor
MEM	minimal essential media
mins	minutes
mg	milli gram
$MgCl_2$	magnesium chloride
MHC	major histocompatability complex
MIEmCMV	major immediate early murine cytomegalovirus enhancer/promoter
mJ	milli Joules
ml	milli litre

mM	milli molar
MMP	matrix metalloproteinases
MOI	multiplicity of infection
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MT1-MMP	membrane type 1 matrix metalloproteinase
mw	molecular weight
NaCl	sodium chloride
ng	nano gram
NKR-P1B	natural killer cell receptor protein 1B
nm	nano meter
NO	nitric oxide
NOS	nitric oxide synthase
NP-40	non-idet P40
NR	non-recombinant phage
OD	optical density
OSBP	oxysterol-binding protein
OTC	ornithine transcarbamylase
oxLDL	oxidized low density lipoproteins
p	pico
P	cell pellet
PAF-AH	platelet-activating factor acetylhydrolase
PAGE	polacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEG	polyethylene glycol
pfu	plaque forming unit
pHPMA	poly[N-(2-hydroxy-propyl)methylacrylamide]
pl	plasmid
PLA_2	phospholipase A_2 domain

PMSF	phenylmethanesulfonylfluoride
PTCA	percutaneous transluminal coronary angioplasty
RES	reticuloendothelial system
RGD	arginine, glycine, aspartic acid. Integrin targeting motif
RGE	rat glomerular endothelial cells
RLU	relative light units
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute media
RSV	rous sarcoma virus
RU	relative units
s	seconds
S	cell supernatant
SAP	shrimp alkaline phosphatase
scAAV	self-complementary AAV
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SHRSP	stroke-prone spontaneously hypertensive rats
SMC	smooth muscle cell
SMMHC	smooth muscle myosin heavy chain
sMSR	soluble macrophage scavenger receptor
SOD	superoxide dismutase
SPR	surface plasmon resonance
SVT	single virus tracing
TAIP2	transforming growth factor β induced apoptosis protein 2
TAMRA	6-carboxytetramethylrhodamine
TCID	tissue culture infectivity dose
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor β

TIMP	tissue inhibitors of matrix metalloproteinases
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
µg	micro gram
µl	micro litre
µM	micro molar
U	units
UNG	uracil–N-glycoylase
UV	ultra-violet
V	volts
v/v	volume per volume
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
VKORC1	vitamin K epoxide reductase complex, subunit 1-like 1 isoform 1
VLA-4	very late antigen-4
VLDLR	very low density lipoprotein receptor
vp	virus particles
VSV-G	vesicular stomatis virus glycoprotein
vWF	von Willebrand factor
w/v	weight per volume
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
X8	linear 8-mer library
X-SCID	X-linked severe combined immune deficiency

List of publications

Appendix 1

Endel J*, Goldnau, D*, Perabo L*, **White K*,** Humme S, Work L, Janicki H, Hallek M, Baker AH, Buening H. HSPG binding properties of adeno-associated virus retargeting mutants and consequences for their *in vivo* tropism. *Journal of Virology* (2006) **80**, 7265-7269 *Authors contributed equally.

White K, Büning H, Work L, Hallek M, Nicklin S, Baker A. Development and characterisation of viral vectors with high specificity for atherosclerotic plaques. American Society of Gene Therapy Abstract. *Molecular Therapy* (2006) **13**, S11

Goldnau, D., Perabo, L., **White, K.**, Endell, J., Humme, S., Work, L., Jannicki, H., Hallek, M., Baker, A.H., Buening, H. HSPG binding properties of adeno-associated virus retargeting mutants and consequences for their *in vivo* tropism. American Society of Gene Therapy Abstract. *Molecular Therapy* (2006) **13**, S48

White K, Büning H, Work L, Hallek M, Nicklin S, Baker A. *In vitro* and *in vivo* characterization of atherosclerotic plaque-targeting vectors. British Society Gene Therapy (2006)

White K, Buening, H, McVey, J, Work, L, Nicklin, SA, Baker, AH. Comparing adenovirus and adeno-associated virus vector platforms for atherosclerotic plaque targeted gene therapy. *Journal of Virology* (in preparation).

White K. Nicklin, SA, Baker AH. Novel vectors for *in vivo* gene delivery for cardiovascular disorders. *Expert Opinion on Biological Therapy* (in preparation).

Chapter 1:

Introduction

1.1 Cardiovascular disease

The term cardiovascular disease (CVD) is used to describe all diseases of the heart and circulatory system. It encompasses monogenic disorders such as familial hypercholesterolemia and more complex conditions such as hypertension and coronary heart disease (CHD), which are caused by a combination of multiple genetic and environmental factors. CVD is the leading cause of death in both men and women in the majority of Western countries and by 2020 it is predicted to be the main cause of death worldwide (Murray and Lopez, 1997). In the UK more than a third of people now die from CVD and over 20% of the population is thought to have some form of CVD (www.heartstats.org). Nearly half of all CVD related deaths are caused by CHD and a further 28% of deaths are due to stroke (caused by atherosclerosis in the cerebral artery) (www.heartstats.org). CHD caused by atherosclerosis in the coronary artery, leads to narrowing and obstruction of the artery, resulting in angina or myocardial infarction. Atherosclerosis is therefore a major health concern as it is one of the leading causes of death worldwide.

1.2 Atherosclerosis

Atherosclerosis is caused by the deposition of cholesterol in the artery wall in combination with an inflammatory immune response. Atherogenesis (the process by which atherosclerosis develops) is a complex process that normally begins at an early age and remains asymptomatic for decades.

The arterial wall consists of 3 layers, the intima, the media and the adventitia (Figure 1.1). The intima is the inner most layer of the vessel surrounding the vessel lumen. On the lumenal side is a layer of endothelial cells (EC) that are involved in regulating haemostatic processes in the lumen and anti-inflammatory, mitogenic and contractile processes in the vessel wall. The media is made up of layers of smooth muscle cells (SMC) and extracellular matrix proteins (mainly elastin). The adventitia mainly consists of connective tissue and fibroblasts.

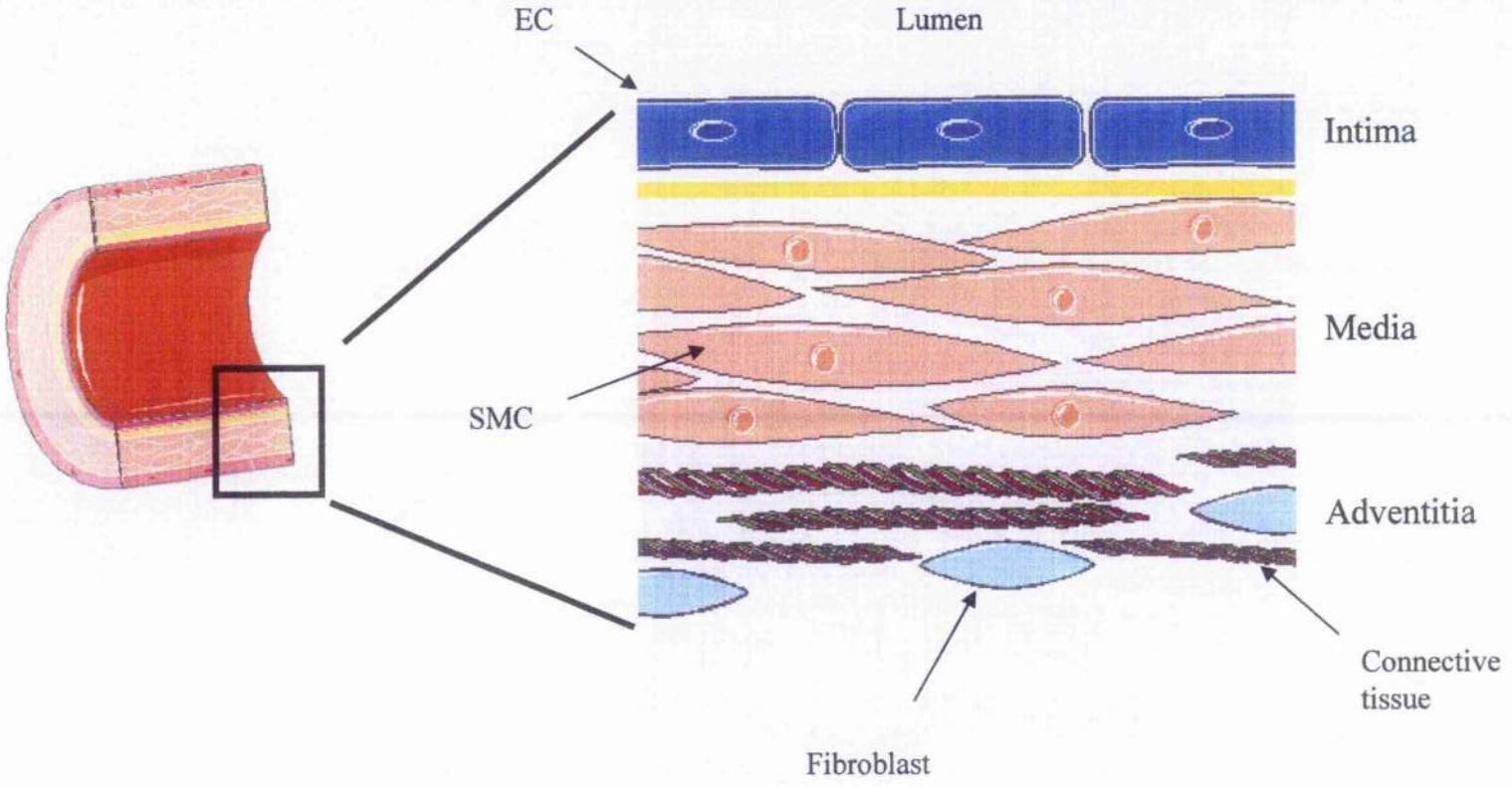


Figure 1.1 Structure of arteries. The intima is made up of endothelial cells (EC), the media mainly consists of smooth muscle cells (SMC) and the adventitia mainly consists of connective tissue and fibroblasts.

Damage to the endothelium and endothelial dysfunction are thought to initiate atherogenesis. Endothelial dysfunction, a situation in which ECs no longer carry out their normal biochemical processes can be caused by many factors including hypertension, hypercholesterolemia, diabetes, toxins found in tobacco smoke and disturbances in blood flow (Nerem, 1992, Fuster *et al.*, 2005). Endothelial dysfunction causes a decrease in nitric oxide (NO) synthesis, an increase in oxidation and uptake of circulating lipoproteins, monocyte entry into the vessel wall, SMC proliferation, extracellular matrix deposition and vasoconstriction (Fuster *et al.*, 2005). NO availability is important for maintaining the vessel as it is involved in vasodilation, inhibition of vascular SMC proliferation and migration and down-regulation of inflammatory and adhesion molecules (Gewaltig and Kojda, 2002). Reactive oxygen species (ROS) decrease NO bioavailability and upregulate the transcription of many pro-inflammatory genes, chemokines, adhesion molecules and pro-thrombotic factors in the endothelium, causing endothelial dysfunction (Melo *et al.*, 2004).

One of the main determinants of where atherosclerosis develops is the haemodynamic strain caused by blood flow. Atherosclerosis most commonly occurs at points where blood flow causes low average shear stress but high oscillatory shear stress e.g. vessel bifurcation points (Dai *et al.*, 2004, Malek *et al.*, 1999). Endothelial dysfunction and shear stress caused by changes in blood flow can cause inflammatory activation of ECs, which stimulates them to express leukocyte adhesion molecules such as P-selectin, E-selectin, intracellular adhesion molecules (ICAMs), vascular cell adhesion molecule-1 (VCAM-1), and integrins. Leukocytes loosely adhere to selectins before forming stable attachments to adhesion molecules (Blakenberg *et al.*, 2003). In response to chemokines such as monocyte-chemoattractant protein-1 (MCP-1), monocytes and T-cells migrate into the intima (Boring et al., 1998, Gu *et al.*, 1998). In the intima monocytes differentiate into macrophages in response to macrophage colony stimulating factor (M-CSF) (Ishibashi *et al*, 1990, Clinton *et al.*, 1992). Oxidised low density lipoprotein (oxLDL) is produced by LDL cholesterol reacting with free radicals produced by macrophages, SMC and EC. Modified lipoproteins such as oxLDL cannot bind to the normal LDL receptor, but as part of the innate immune response, macrophages express

scavenger receptors that bind to and take up modified lipoproteins, and apoptotic cell fragments. This causes them to develop into foam cells (Hansson, 2001). At this stage fatty streaks are said to have formed.

Foam cells and lymphocytes within the vessel wall secrete cytokines that stimulate SMC proliferation, extracellular matrix production (Libby, 2002) and enhance the inflammatory process. SMC proliferation and migration from the media into the intima in conjunction with increased uptake of lipoproteins and inflammatory cells and production of extracellular matrix proteins leads to the formation of a fibrous cap covering a lipid rich core, resulting in the production of an atherosclerotic plaque (Figure 1.2A).

The fibrous cap consisting of SMC and extracellular matrix proteins has an important protective role in maintaining plaque stability and preventing the pro-thrombotic molecules in the lipid core coming into contact with the blood (Stary, 1990). Plaques continue to develop, with changes occurring in their size and cellular and molecular composition. Activated T-cells in the intima produce inflammatory cytokines such as interferon-γ (INF-γ), that further stimulate macrophages. Foam cells produce more pro-inflammatory cytokines and reactive oxygen species, propagating the inflammatory response and signaling macrophages to die by apoptosis. This leads to the formation of a necrotic core within the plaque. In established plaques tissue factors, proinflammatory cytokines and matrix metalloproteinases (MMPs) are secreted. MMPs degrade the extracellular matrix, weakening the fibrous cap and as the lipid core continues to grow an unstable plaque forms.

Plaque growth and remodeling can cause narrowing of the lumen or outward growth. Plaque growth into the lumen is associated with the formation of large stable plaques that cause angina, whereas outward remodeling is more commonly a feature of unstable plaques (Schoenhagen *et al.*, 2000).

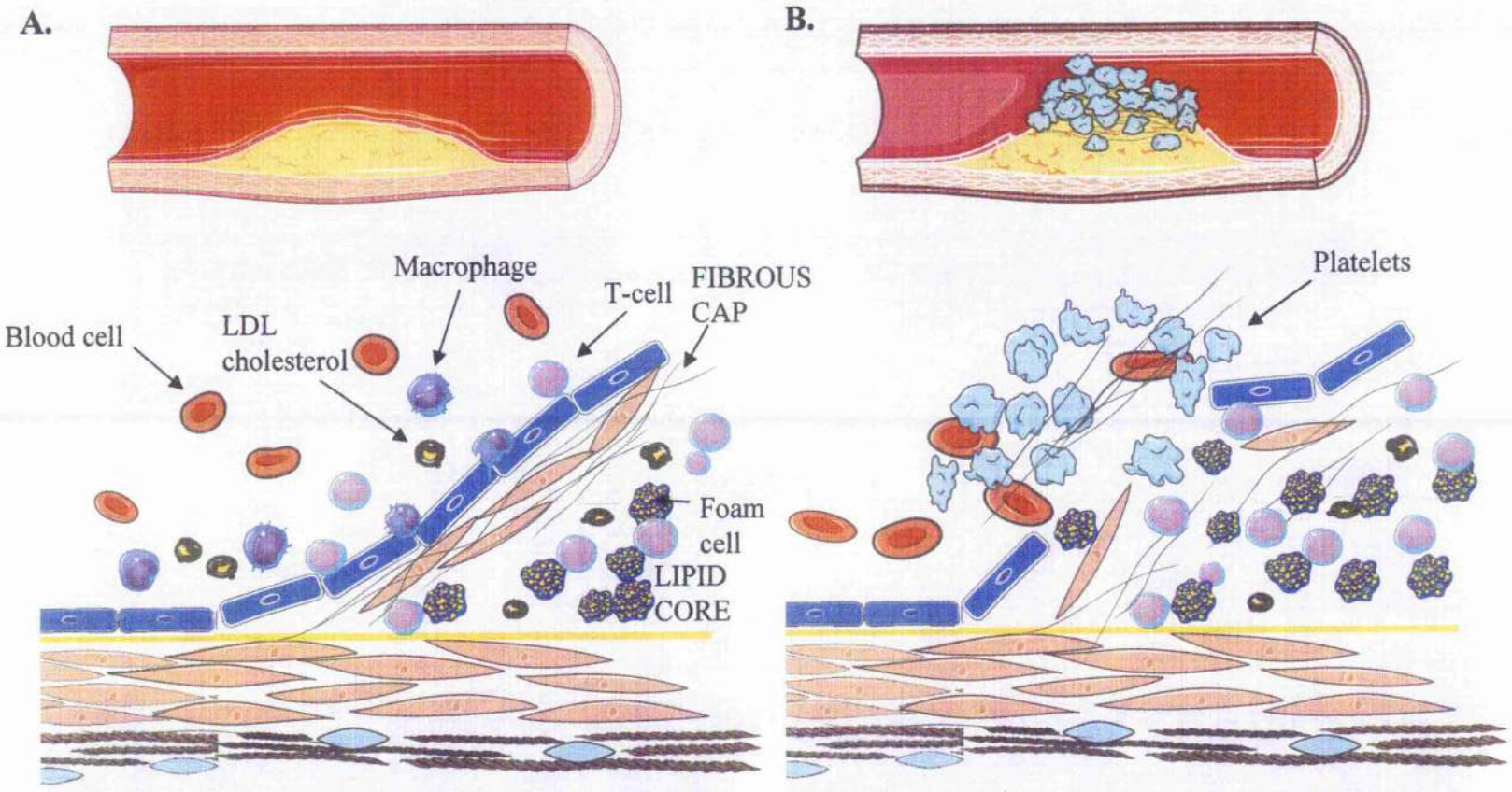


Figure 1.2 Development of unstable atherosclerotic plaques. **A.** Complex plaques consist of a lipid core made up of phospholipids, free cholesterol, crystalline cholesterol and cholesteryl esters, cells of the immune system including macrophages and T-lymphocytes, extracellular matrix proteins including collagen, proteoglycans, fibronectin and elastin fibers and smooth muscle cells (Fuster et al., 2005). **B.** When plaque rupture occurs platelets and fibrin form a thrombus which can cause occlusion of the artery.

1.2.1 Plaque rupture

Until recently it was thought that the risk of death due to atherosclerosis was increased by the extent of stenosis and lumenal narrowing. However in the last 15 years it has been noted that the major risk factor is actually plaque rupture, which leads to clot formation (thrombus) and blockage of arteries (Falk *et al.*, 1995, Davies, 1996). Approximately 70% of all fatal myocardial infarctions are caused by plaque rupture (Falk *et al.*, 1995) and the remaining 30% are due to plaque erosion, where the endothelial cells are removed (Virmani *et al.*, 2000, Farb *et al.*, 1996). The cause of plaque erosion is poorly understood but eroded plaques generally have lower macrophage infiltration, less calcification (Farb *et al.*, 1996) and contain higher levels of mast cells than rupture prone plaques (Kovanen *et al.*, 1995). It has been proposed that mast cells produce proteases that disrupt endothelial cell adhesions, which leads to plaque erosion (Mayranpaa *et al.*, 2006). More is known about what causes plaques to become unstable and prone to rupture. Unstable plaques are generally described as those that have a thin fibrous cap and a large lipid core. Autopsy studies have been used to define their features more precisely (Table 1.1). Although unstable plaques are relatively well classified the final trigger that causes plaque rupture to occur is not normally known, although its probable that external factors acting on the plaque such as an increase in blood pressure can act as the trigger.

Plaque rupture enables contact between blood and the thrombogenic and pro-coagulant proteins from the plaque such as tissue factor from apoptosed macrophages (Mallat and Tedgui, 2000) and oxidized lipids (Toschi *et al.*, 1997, Fernández-Ortiz *et al.*, 1994) (Figure 1.2B). This results in thrombus formation that can occlude the vessel and is a common cause of myocardial infarction (Virmani *et al.*, 2000). However, not all ruptures result in occlusion of arteries, many are asymptomatic and result in the formation of smaller clots that are then incorporated into the plaque following repair by collagen synthesis. This tends to accelerate plaque progression and increase plaque instability (Mann and Davies, 1999). The extent of thrombus formation following plaque rupture is dependent on a number of factors including composition of the plaque, rate of blood flow

Feature	Comment	Reference
Plaque size	Large plaques are generally more stable and cause angina, whereas plaques that cause less than 50% narrowing of the lumen are unstable. Outward remodeling and medial atrophy that does not decrease the lumen size are more commonly associated with unstable plaques.	(Schoenhagen *et al.*, 2000, Ambrose *et al.*, 1988, Rao *et al.*, 2005)
Large lipid core	Plaques with a lipid rich core that takes up more than 50% of the plaque volume are more unstable.	(Davies, 1996, Rao *et al.*, 2005)
Thin fibrous cap	Plaques with thin fibrous caps (less than 65μm thick) depleted in collagen and SMC are defined as unstable.	(Burke *et al.*, 1997)
Cholesterol levels	A high total/HDL cholesterol ratio is associated with plaque instability. HDL cholesterol is protective as it promotes reverse cholesterol transport from tissues to the liver. LDL cholesterol is proatherogenic as it mediates uptake of cholesterol into macrophages.	(Burke *et al.*, 1997, Barter, 2004)
Red blood cell accumulation	Neo-vascularisation and intra-plaque hemorrhages increase plaque vulnerability as red blood cells enhance necrotic core expansion and increase the immune response.	(Kolodgie *et al.*, 2003, Rao *et al.*, 2005, Mann and Davies, 1999)

Table 1.1 Characteristics of unstable plaques.

in the area and systemic procoagulant activity (Fuster *et al.*, 2005). Lipid-rich plaques are the most thrombogenic and high levels of tissue factor found in macrophage rich areas of the plaque also contribute to thrombus formation (Toschi *et al.*, 1997) (Fernández-Ortiz *et al.*, 1994). Factors which increase systemic procoagulant activity include high levels of plasma LDL cholesterol, smoking and hyperglycemia in diabetics (Rauch *et al.*, 2001).

1.2.2 Current treatments to reduce risk factors for atherosclerosis

There are a number of ways of treating atherosclerosis depending on the underlying cause of the condition. The majority of treatments focus on reducing risk factors that are known to contribute to the progression of atherosclerosis and major cardiovascular events. Lifestyle changes such as ceasing smoking, increasing exercise, improving diet and weight loss are recommended to reduce environmental risk factors. Management of other conditions associated with an increased risk of atherosclerosis, such as diabetes and hypertension is also important.

The main focus of the current treatment for atherosclerosis is to try and reduce the incidence of plaque rupture and thrombus formation by controlling the risk factors involved. Therefore drugs focus on reducing hypertension, reducing the total/HDL cholesterol ratio and administering anticoagulants to prevent thrombus formation occurring. There are many anti-hypertensive drugs, for example beta-blockers that can be used to slow down the heart rate and lower blood pressure. Angiotensin converting enzyme (ACE) inhibitors lower blood pressure by reducing the generation of angiotensin II and can also reduce inflammation caused by macrophages secreting interleukin-6 (IL-6) (Schieffer *et al.*, 2000). Reducing blood pressure can reduce the stress exerted on plaques and therefore reduce the likelihood of plaque rupture occurring. Aspirin can also be prescribed to reduce platelet aggregation, thrombus formation and it has an anti-inflammatory effect.

The development of statins was a major advance in the treatment of atherosclerosis. Their main mechanism of action is to lower cholesterol by inhibiting 3-hydroxy-3

methylglutaryl coenzyme A (HMG-CoA) reductase, the key regulatory enzyme in the production of cholesterol. However, patients with normal serum cholesterol levels also benefit from them, probably due to their anti-inflammatory action as they have been shown to down regulate T-cell activation of macrophages (Sposito and Chapman, 2002) and inhibit the expression of the pro-inflammatory CD40/CD40L (Semb *et al.*, 2003) and MCP-1 (Morikawa *et al.*, 2002). There is also evidence from both humans and animal models, that statins down regulate tissue factor and MMP expression in plaque macrophages (Aikawa *et al.*, 2001, Cortellaro *et al.*, 2002). Additionally some statins are thought to stabilise plaques (Kennedy *et al.*, 2005) by increasing expression of tissue inhibitors of matrix metalloproteinases (TIMPs), which results in an increase in plaque collagen content (Crisby *et al.*, 2001).

There are many different types of statins available, which have slightly different properties, but despite their wide ranging mode of action, not everyone responds to or can tolerate statins so other forms of treatment are required (Oka and Chan, 2005). Other drugs that can improve the lipoprotein balance include niacin (Vitamin B3), drugs that inhibit the absorption of dietary cholesterol such as ezetimibe and fibrates that increase HDL levels, and lower LDL cholesterol. Although there is now evidence that some statins might mediate some plaque regression, there are no treatments aimed directly at altering the plaque composition.

In advanced cases of atherosclerosis, surgical procedures such as angioplasty and coronary artery bypass grafting (CABG) are used. Percutaneous transluminal coronary angioplasty (PTCA) is a technique that involves the insertion of a balloon into the artery at the site of the plaque. Inflating the balloon breaks up the plaque and stretches the artery. However, surgical manipulation of the artery results in damage to the endothelium that causes inflammation, smooth muscle cell proliferation and thrombus formation. This results in neointimal hyperplasia and narrowing of the vessel. A stent (a hollow metal mesh) can be deployed into the artery to provide support and reduce the subsequent narrowing of the vessel. However stents themselves can be proinflammatory and this can result in tissue growth around the stent and instent restenosis. This occurs in

30-60% of patients with complex lesions that receive a bare metal stent (Fattori and Piva, 2003). To reduce the rate of instent restenosis stents can be coated in drugs that have an immunosuppressive, anti-proliferative, anti-inflammatory, anti-thrombotic and/or prohealing effect. For example, the first drug eluting stents were coated in sirolimus (rapamycin), which blocks cell cycle progression, reducing SMC, T-cell and B-cell proliferation and so reduces tissue growth and the immune response (McKeage *et al.*, 2003). Even with the use of drug eluting stents restenosis remains a problem (Brasen *et al.*, 2001) and additionally they have been associated with an increase in late stent thrombosis (occurring more than 12 months after procedure) (McFadden *et al.*, 2004). Further long-term studies are required but it seems that even with recent advances in stent development the procedure still has some limitations.

CABG involves taking an artery or vein from another anatomical site of the patient and grafting it from the aorta to the coronary artery to bypass the narrow section of the artery to improve blood flow. Damage caused to the vessel during the operation can cause neointima formation due to smooth muscle cell migration, proliferation and extracellular matrix production. This results in occlusion of the bypass vessel. Where possible, arteries such as the internal mammary or radial artery are used for the graft as they have lower rates of failure than vein grafts, due to the anatomical differences between veins and arteries (Nežic *et al.*, 2006). However, for patients that require multiple vessel grafts veins are commonly used. Vein graft failure due to neointima formation occurs in about 50% of patients between 5-10 years after the procedure (Tsui and Dashwood, 2002).

1.2.3 Plaque stabilisation and regression

Atherosclerosis has traditionally been thought of as a progressive and irreversible disease; however there is now evidence from both animal models and clinical trials that shows that plaque stabilisation and regression is possible by reducing lipid content and inflammation in the plaque. Lipid lowering is thought to decrease macrophage accumulation, reduce inflammation and increase collagen content, resulting in remodeling of the arterial wall and plaque stabilisation (Libby and Aikawa, 2002). For example, transplantation of a section of atherosclerotic aorta from apolipoprotein E

knockout (ApoE-/-) mice into healthy C57/BL6 mice caused a reduction in the size of lesions by about 50% in just 3 weeks (Reis *et al.*, 2001). This dramatic regression was thought to be due to a 90% decrease in plasma cholesterol levels (Reis *et al.*, 2001). The number of foam cells was also significantly reduced, as they migrated from the lesions to the draining lymph node (Llodra *et al.*, 2004). Aikawa *et al.* (Aikawa *et al.*, 1998) have also shown that lowering plasma cholesterol levels can increase plaque stability. Rabbits were fed a high fat diet and had plaque formation induced by balloon injury to the carotid artery. Half were then fed a normal diet and half were maintained on the high fat diet. 16 months later lesions of animals that were fed a normal diet contained fewer macrophages, had decreased MMP activity and contained more collagen (Aikawa *et al.*, 1998), suggesting they were more stable. A similar study in rabbits showed that after 6 months of cholesterol withdrawal the plaques contained fewer macrophages, less apoptotic cells and more collagen, however the size of plaques were unaffected by cholesterol lowering (Kockx *et al.*, 1998). Pravastatin is thought to cause plaque stabilisation in humans by decreasing plaque lipid content, lipid oxidation and inflammation and increasing TIMP1 and collagen content (Crisby *et al.*, 2001). It has recently been shown to have a similar effect in ApoE-/- mice without affecting plasma cholesterol levels (Johnson *et al.*, 2005b). A recent clinical trial (A Study to Evaluate the Effect of Rouvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden, ASTEROID) has shown that high doses of rouvastatin can cause plaque regression after 2 years of treatment (Nissen *et al.*, 2006). A mean reduction in LDL cholesterol of 53% was achieved in combination with a significant increase in HDL cholesterol (Nissen *et al.*, 2006). This resulted in an average decrease in plaque volume of 6.8% and is the first trial in humans that has been able to show that a decrease in plaque volume can be achieved. However the trial design was not sufficiently powered to show significance at the level of plaque regression, so further studies are in progress (Nissen *et al.*, 2006).

1.2.4 Future forms of treatment

Due to the high incidence of cardiovascular disease it has been suggested that everyone over the age of 55 should be treated prophylactically with a combination of drugs that has become known as the Polypill (Wald and Law, 2003). The Polypill would consist of a

statin, blood pressure lowering drugs (e.g. a Beta-blocker, an ACE inhibitor and a thiazide (a diuretic)), folic acid and aspirin (Wald and Law, 2003). It is predicted that this would reduce the incidence of CVD events by more than 80% (Wald and Law, 2003). However there are likely to be problems with patient compliance of seemingly healthy people.

There are also many novel treatments for CHD that are under investigation. Many of these focus on reducing the immune response that occurs. For example, the pro-inflammatory cytokine tumour necrosis factor α (TNFα) has been implicated in plaque development as ApoE-/- and TNFα-/- double knock-out mice develop smaller lesions that express significantly lower amounts of other inflammatory molecules including ICAM-1, VCAM-1 and MCP-1 compared to ApoE-/- mice (Ohta *et al.*, 2005). Also their macrophages have a lower expression level of scavenger receptor so take up less oxLDL, (Ohta *et al.*, 2005). In ApoE-/- mice inhibition of TNF with a TNF-antagonist also decreased plaque formation (Laurat *et al.*, 2001). Inhibition of other cytokines implicated in plaque development, such as INF-γ {Whitman, 2000 #6081}, could also be of benefit.

Another approach has investigated vaccinating with oxLDL, or heat shock protein 60. In animal models this has reduced atherosclerosis development, possibly by activating a protective B- or T-cell response (Palinski *et al.*, 1995, Zhou *et al.*, 2001, Maron *et al.*, 2002).

Other ways of improving the HDL/total cholesterol ratio by increasing the amount of circulating HDL are being developed. HDL is required for reverse lipid transport to remove cholesterol from peripheral tissues including the artery wall and take it to the liver for excretion. HDL also has anti-oxidative, anti-inflammatory and anti-thrombotic properties (Shah and Chyu, 2005). The first evidence that HDL has a protective effect was from cholesterol fed rabbits that were given purified HDL, which caused a decrease in atherosclerosis (Badmion *et al.*, 1990). Evidence suggests that increasing HDL cholesterol has a higher atheroprotective effect than a decrease in LDL cholesterol (Lee and Choudhury, 2006). There are several methods of increasing HDL levels that are

currently undergoing pre-clinical and clinical trials. Some promising results have been achieved using ApoA-I Milano, a rare naturally occurring mutant of the ApoA-I protein. ApoA-I is the major protein component of HDL cholesterol. The mutant contains a single amino acid substitution that provides increased resistance to the development of atherosclerosis (Franceschini *et al.*, 1980). A small proof of concept trial demonstrated a significant regression in plaques in patients receiving recombinant ApoA-I Milano (Nissen *et al.*, 2003). But weekly injections were required so it may be more effective to use gene transfer techniques to enable the patient to synthesise the protein. Wang *et al.* (Wang *et al.*, 2006a) recently reported some success with *ex vivo* gene therapy using a retroviral vector expressing ApoA-I from a macrophage specific promoter. Infected bone marrow cells were transplanted into ApoE, ApoA-I double knock-out mice. After 24 weeks of fat feeding the degree of aortic atherosclerosis was reduced by 65% (Wang *et al.*, 2006a). Gene therapy therefore has the potential to treat CHD.

1.3 Animal models of atherosclerosis

To develop new treatments for atherosclerosis an accurate animal model of the human disease is required. Many different animal models of atherosclerosis have been developed, but it has proven very difficult to reproduce many of the features of the human condition to produce a model that accurately reflects the molecular and phenotypic changes that occur in the development of unstable plaques. There are several models which use artificial methods to cause plaque rupture, such as inflating balloon catheters in plaques (Rekhter *et al.*, 1998a), using forceps to damage mouse aortas (Reddick *et al.*, 1998) and administration of Russell viper venom (a procoagulant and endothelial toxin) and histamine to cholesterol fed rabbits (Abela *et al.*, 1995). However for this study these models are unsuitable as they do not reflect the natural molecular changes that occur in unstable plaques.

Another study produced plaques in the mouse carotid artery by placing a silastic collar around the artery (der Thusen *et al.*, 2002). Luminal overexpression of p53 from an adenovirus (Ad) vector caused apoptosis, which led to cap thinning (der Thusen *et al.*, 2002). 3/16 animals showed evidence of plaque rupture 14 days after Ad administration.

To increase the occurrence of this, mice were treated with phenylephrine to make them hypertensive, resulting in plaque rupture in 40% of the animals. (der Thusen *et al.*, 2002). A recent study with ApoE-/- fed a normal diet used carotid artery ligation to induce plaque formation, before a cuff was placed around the artery to cause cracks in the plaque cap (Sasaki *et al.*, 2006). Four days after cuff insertion, cracks in the neointima and thrombus formation were consistently observed and by 7 days occlusive thrombi were found in all mice (Sasaki *et al.*, 2006). This provides a fast and reliable method of inducing plaque development and rupture that lead to the formation of occlusive thrombi, which are not commonly seen in many other models. However, these models require a lot of manipulation of the plaque to induce rupture, so they provide relatively limited information about the natural progression of unstable plaques and plaque rupture. The main advantage of these models is the short time period required for plaque development and rupture and that the timing of plaque rupture can be controlled.

Recently a new approach has been taken to developing a mouse model of plaque rupture. ApoE-/- transgenic mice with vascular SMC that express the human diphtheria toxin receptor (SM22α-hDTR ApoE-/-) were created (Clarke *et al.*, 2006). Mice were fat fed for 12 weeks from the age of six weeks and then were given diphtheria toxin, which induced vascular SMC apoptosis. Although this had no effect on plaque size it caused thinning of the plaque cap, with a loss of SMC and extracellular matrix proteins and an increase in necrotic core size (Clarke *et al.*, 2006). Treatment with diphtheria toxin also caused a significant increase in the amount of macrophages and the proinflammatory cytokine IL-6 (Clarke *et al.*, 2006). Although these plaques had many of the features of unstable plaques no thrombus formation was detected (Clarke *et al.*, 2006). However, the mice were culled 3 weeks after diphtheria toxin administration, so if this was extended plaque rupture may occur. This method may provide a rapid and reliable model for generating unstable plaques with many features of human plaques, in a relatively short time period.

One of the first reports of spontaneous plaque rupture was seen in 42-60 week old ApoE-/- mice that developed lesions in their brachiocephalic artery (BCA) which showed

evidence of intraplaque hemorrhages possibly due to plaque rupture (Rosenfeld *et al.*, 2000). The mice had layered lesions suggesting that multiple thrombotic events could have occurred (Rosenfeld *et al.*, 2000). This had not been noticed previously as most studies focused on the aorta and did not examine the vasculature at these longer time points. The BCA (or innominate artery) is a small artery that connects the aortic arch to the right subclavian and right carotid artery (Figure 1.3). The occurrence of plaque rupture specifically in the BCA has been proposed to be due to a particularly high degree of tension in the wall of this artery. Another study using ApoE and LDL receptor (LDLR) knockout mice provided evidence of a low level (3 of 82 cholesterol fed mice) of plaque rupture in mice monitored for up to 12 months (Calara *et al.*, 2001).

A reliable model of plaque rupture seems to have been developed in C57/Bl6/129SvJ ApoE-/- mice by feeding a diet of 21% beef lard and 0.15% cholesterol (Johnson and Jackson, 2001, Williams *et al.*, 2002). After 14 months of fat feeding many mice had plaque rupture events with associated thrombus formation in the BCA. Analysis of the lesions showed many similarities with unstable human plaques including a thin fibrous cap with reduced amounts of SMC and elastin overlying a lipid rich core. Many of the plaques had a layered appearance, which has also been seen in human plaques and was proposed to be due to previous silent ruptures (Burke *et al.*, 2001). Alternatively it could be due to periods of rapid plaque growth, but either way it is further evidence that the plaques produced in this model have a similar phenotype to those seen in humans. It was thought that 79% of plaques analysed had features consistent with earlier plaque ruptures having occurred. Of 98 mice, 51 showed evidence of plaque rupture in the BCA. This level of plaque rupture had not previously been reported in ApoE-/- mice, and it may be due to the strain background used in this case as 129Sv mice absorb cholesterol more effectively than C57/Bl6 (Jolley *et al.*, 1999). Also, C57/Bl6 mice express a higher level of transforming growth factor β1 (TGF-β1) than 129Sv mice, which may decrease inflammation within the plaque and so have a protective role (Williams *et al.*, 2002). Other studies have also shown the strain background is important, for example, C57/Bl6 mice develop 7-9 fold more atherosclerosis than FVB mice that have higher plasma cholesterol levels. The main problems with both this and the study by Rosenfield *et al.*

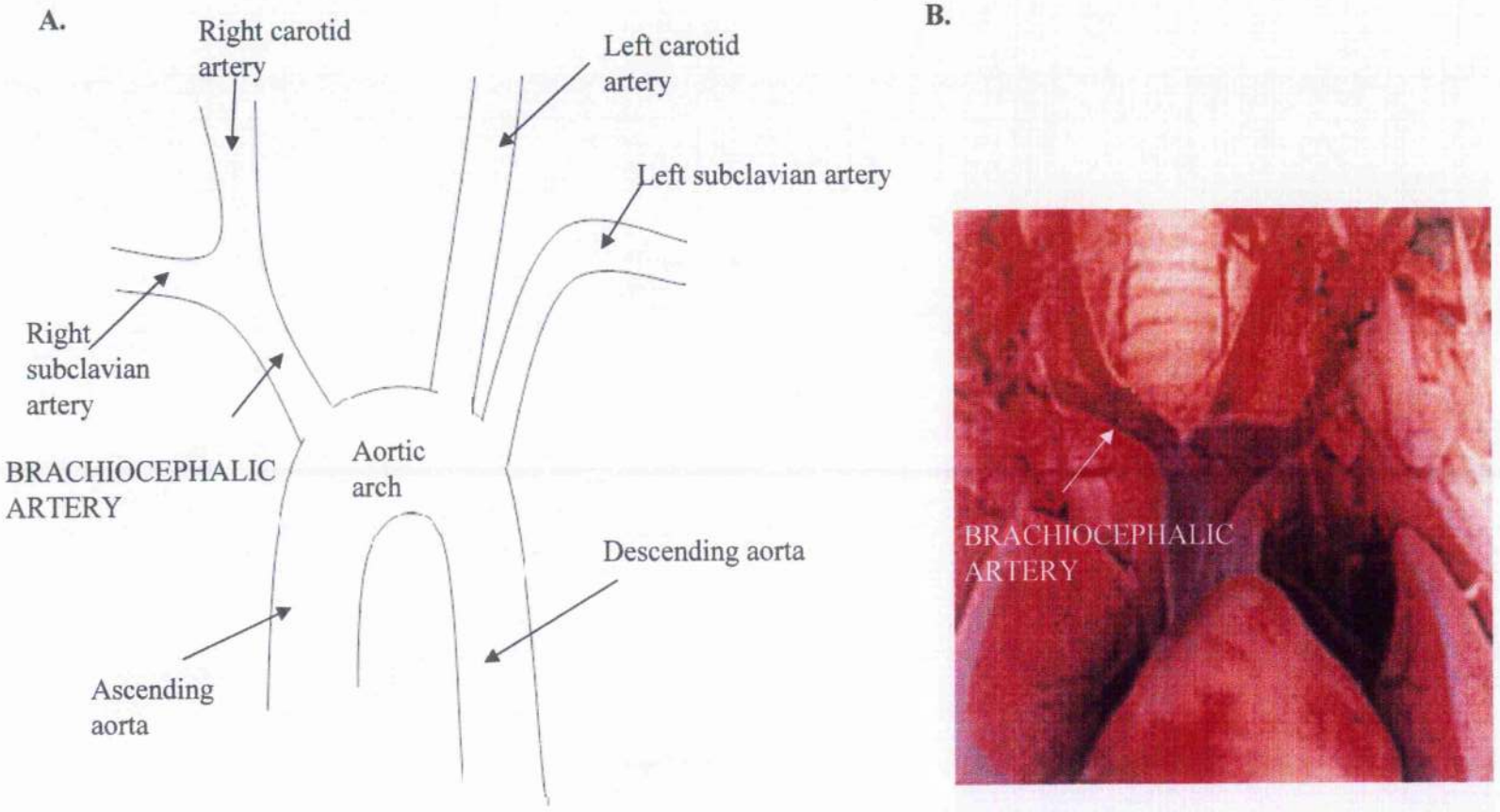


Figure 1.3 Location of the BCA. A. Schematic diagram showing the location of the brachiocephalic artery. **B.** Image of the BCA (obtained from C. Jackson, University of Bristol)

(Rosenfeld *et al.*, 2000) is the length of time required to develop the phenotype and the fact that plaque rupture did not cause the development of fibrin-rich platelet clots that can occlude the artery after plaque rupture, which is commonly seen in humans.

Further work using this model has shown that these mice can develop unstable plaques after only 8 weeks of fat feeding (Johnson *et al.*, 2005b). After just 4 weeks of fat feeding, fatty streaks were visible in the BCA and by 6 weeks plaques had developed (Johnson *et al.*, 2005b). After 8 weeks these were complex lesions with a layered appearance and in 62% of 173 mice plaque rupture was seen (Johnson *et al.*, 2005b). Pravastatin, which is thought to stabilise atherosclerotic plaques in humans (Crisby *et al.*, 2001), was administered to these mice in conjunction with fat feeding. After 9 weeks this was found to have no affect on the incidence of plaque rupture, however when the study was extended to 40 weeks, mice treated with pravastatin had a lower incidence of plaque rupture (Johnson *et al.*, 2005b). At both time points control animals had a significantly higher incidence of buried fibrous layers indicative of previous plaque ruptures (Johnson *et al.*, 2005b). Treatment with pravastatin also reduced the occurrence of sudden death (Johnson *et al.*, 2005b). When treatment was delayed until after the plaques had developed, the incidence of plaque rupture was decreased probably due to a stabilising effect of pravastatin (Johnson *et al.*, 2005b). These results suggest that this provides a relatively quick and reliable model of atherosclerotic plaque rupture. However, the presence of fibrous layers and buried caps within the plaques cannot be assumed to be due to previous plaque rupture, but may be due to intraplaque haemorrhages from plaque microvessels (Lutgens *et al.*, 2003), but no neovascularization has been seen in over 700 ApoE-/- mice (Johnson *et al.*, 2005b). Therefore this model is one of the most reliable models of spontaneous plaque rupture that has many of the features of the human disease and the disease state is achieved in a relatively short period of time.

1.4 Gene therapy

Gene therapy can simply be defined as the delivery of a gene to host cells to express a protein that has a therapeutic effect. The gene can replace a missing or defective gene, produce a protein that inhibits the undesirable effects of another protein, or introduce a

new cellular function such as a protein with metabolic, structural or signaling functions (Dzau *et al.*, 2003). Initially it was proposed as a form of treatment for monogenic disorders such as cystic fibrosis and Duchenne's muscular dystrophy, where supplying the correct form of the gene should cure the condition, but gene therapy treatments are now being developed for a wide range of diseases, including highly complex conditions such as CHD and cancer.

1.4.1 Current status of gene therapy

In 1990 the first gene therapy clinical trial was approved for treating adenosine deaminase deficiency using a retrovirus vector (Kohn *et al.*, 1998), since then over 1000 gene therapy trials have been carried out. About 2/3 of these are for cancers and less than 10% are for vascular disorders (http://www.wiley.co.uk/wileychi/genmed/clinical/). The majority of these focused on inducing angiogenesis to treat ischemia (Melo *et al.*, 2005). As yet no serious side effects have been reported in any cardiovascular gene therapy clinical trials.

The first government approved gene therapy treatment was Gendicine (SiBono, Shenzen, China), which was approved in 2004 in China for treatment of head and neck squamous cell carcinomas. It is a replication deficient Ad vector that expresses tumour suppressor p53 from a Rous Sarcoma Virus (RSV) promoter. In over 2500 patients no significant side effects have been seen and the vector seems to be well tolerated (Peng, 2005). In combination with radiotherapy, Gendicine caused complete regression of cancers in 64% of patients and partial regression in 29% of patients in clinical trials for head and neck squamous cell carcinomas (Peng, 2005). Gendicine has also been shown to have a beneficial affect in the treatment of advanced liver and lung cancers (Peng, 2005). There have currently been over 50 cancer clinical trials using Ad vectors expressing p53 (Peng, 2005). These have been carried out for a range of cancers including bladder (Pagliaro *et al.*, 2003) and lung cancers (Swisher *et al.*, 2003). No serious side effects have been seen but some patients suffer from minor problems such as pain at the site of injection, fatigue and fever (Peng, 2005).

Currently one of the main challenges of gene therapy research is the development of vectors that can be administered systemically and deliver the therapeutic genes selectively to the target tissue and cell type so that the gene is expressed at a high enough level to have a therapeutic effect without either the vector or the gene product causing any significant side effects.

1.5 Gene therapy vectors

The requirements of an efficient gene delivery vector vary depending on the disease to be treated, but generally they are required to be selective for the target cell type or organ, non-toxic, non-immunogenic, stable *in vivo* and producible on a large scale. Due to differences in the mode of delivery, target cell type and required duration of transgene expression, many gene therapy treatments are likely to require the development of a unique vector tailored for that application. Therefore there are many different types of vectors being researched, but these can broadly be divided into non-viral and viral vectors.

1.5.1 Non-viral vectors

Non-viral vectors are less immunogenic and easier to produce than viral vectors, but the level of transgene expression they can provide is significantly lower than can be achieved with viral vectors. Several strategies are being developed to try and overcome this deficiency.

The simplest form of vector is naked plasmid DNA. When in contact with the cell membrane, a small amount is taken up by the cell resulting in a very low level of transduction. To improve the efficiency of cellular uptake, plasmid DNA can be incorporated into cationic liposomes or cationic proteins e.g. poly-lysine (McKenzie *et al.*, 2000). Alternatively, histones can be used to compact the DNA into complexes which enable cellular entry via an interaction with sulphated membrane bound proteoglycans (Mislick and Baldeschwieler, 1996). *In vitro* these vectors do produce some improvement in transduction, but *in vivo* transgene expression levels remain low due to their poor transfection efficiency, and because they are rapidly cleared from

plasma (Filon and Phillips, 1997, Johnson-Saliba and Jans, 2001). Although liposomes improve cellular uptake of the DNA, once inside the cell the majority of the DNA remains in the cytoplasm where it is degraded as the vectors are inefficient in escaping the liposome/endosome complex, avoiding degradation in the cytoplasm, trafficking to the nucleus and crossing the nuclear membrane (Johnson-Saliba and Jans, 2001, Jans *et al.*, 2000). Plasmids that reach the nucleus remain extrachromosomal and are rapidly degraded so they provide only transient gene expression (Niidome and Huang, 2002). To try and overcome these deficiencies, proteins that enable receptor-mediated endocytosis, endosomal disruption, and nuclear import sequences have been incorporated into the vector complex (Glover *et al.*, 2005, Cartier and Reszka, 2002). For example a novel vector known as GD5 contains the DNA-binding domain of the yeast transcription factor GAL4 (to form the complex with the DNA), an antibody that binds the tumour-associated cell surface protein ErbB2 (to target the vector), and the diphtheria toxin translocation domain to disrupt the endosome (Uherek *et al.*, 1998). To overcome the transient nature of the gene expression, site-specific recombinases commonly found in viruses that infect prokaryotes are being incorporated into vectors to mediate recombination into the host genome (Glover *et al.*, 2005).

In an attempt to target liposomes to specific cell types and increase their cellular uptake, a system has been developed to form a complex of a liposome (Lipofectin), an integrin targeting peptide and plasmid DNA (LID system) (Hart *et al.*, 1998). This has been used to increase transduction of vascular EC and SMC *in vitro*, but transgene levels remain far lower than that achieved with Ad vectors (Parkes *et al.*, 2002). Recently it has also been used *in vivo* in a rat carotid angioplasty model (Meng *et al.*, 2006). A LID vector expressing TIMP1 was applied to the adventia of balloon injured carotid arteries. 28 days later the intima to media ratio was 46% lower in treated arteries compared to controls, suggesting the vector produced therapeutic levels of transgene expression. This demonstrates that the LID system can be used to enhance non-viral gene delivery.

Many physical methods of gene delivery have also been developed to improve the efficiency of non-viral gene therapy. Hydrodynamic pressure created by injecting large

volumes of DNA can be used to force more of the plasmid from the blood into tissues. This has been shown to be particularly effective in improving gene transfer to hepatocytes, following tail vein injection in mice (Niidome and Huang, 2002). However, it has limited application in patients as it can only be used in tissues where it is possible to create high pressure e.g. by using a blood pressure cuff on limbs (Glover *et al.*, 2005).

Electroporation is commonly used for *in vitro* transfection, as it increases the efficiency with which DNA passes through the cell membrane. It has also been shown to function *in vivo* in many tissues including skin, lung, liver, kidney, heart, brain, muscle and tumours (Magin-Lachmann *et al.*, 2004, Somiari *et al.*, 2000, Wells, 2004). However the technique has many disadvantages as it requires a highly invasive procedure, is limited to accessible tissues, causes considerable cell death and the level of gene expression achieved is still lower than that from viral vectors (Li and Huang, 2000). Another method known as the gene gun involves firing gold particles coated with DNA at the target tissue. This enables delivery of the DNA directly into the cytoplasm. Due to its limited tissue penetration it has mainly been used for gene delivery to skin. Little success has been achieved with this technique, for example in a mouse tumour model, delivery of cytokines was found to have no affect on tumour development (Kitagawa *et al.*, 2003).

One of the more successful methods for increasing the efficiency of non-viral gene delivery is to apply ultrasound to the target tissue at the time of injection. This increases the permeability of the cell membrane and therefore increases uptake of DNA. Gas filled microbubbles can be used as a contrast agent to increase the efficiency of the technique (Niidome and Huang, 2002). It has been shown to increase transduction of vascular cells about 300-fold compared to naked DNA alone (Lawrie *et al.*, 1999). *In vivo* it has been demonstrated to increase plasmid uptake into the carotid artery of rabbits and rats following intra luminal delivery (Huber *et al.*, 2003, Taniyama *et al.*, 2002). A therapeutic effect of ultrasound enhanced gene delivery was demonstrated in a porcine model of saphenous vein grafting (Akowuah *et al.*, 2005). Veins were transfected *ex vivo* with a plasmid expressing TIMP3. 28 days after grafting the luminal area was

significantly larger than controls (Akowuah *et al.*, 2005). Unlike the other techniques described, ultrasound is less invasive and can be focused on inaccessible tissues deep within the body and it can be used to increase the selectivity of a vector as the ultrasound can be focused on a specific tissue (Newman *et al.*, 2001). The use of ultrasound in other procedures has shown it has a good clinical safety profile, so it is a promising technique for improving non-viral gene delivery to the vasculature.

Although these technologies can improve transgene expression, they still remain in general, less efficient than viral vectors.

1.5.2 Viral vectors

There are many different types of viruses that are being investigated as potential vectors for gene therapy. The type of viral vector used for a particular gene therapy depends on the cell type to be targeted and whether transient or long-term transgene expression is required. The important features of the main types of viral vectors compared to non-viral vectors are summarised in Table 1.2. Historically, the most commonly used are retroviruses and adenoviruses. This is highlighted by clinical trial data (Figure 1.4) that show that these are used in 50% of all clinical trials. Although there are disadvantages associated with each type of vector, our understanding of virus biology enables them to be altered to create a vector with the desired properties that expresses the transgene in the target tissue type. The work in this study focuses on the development of adenovirus and adeno-associated virus vectors (AAV), which are described in detail in sections 1.6 and 1.7.

1.5.2.1 Retroviral vectors

Retroviruses are enveloped viruses with an RNA genome. They can be used to efficiently infect proliferating cells to provide long term transgene expression as their RNA genome is reverse transcribed and integrated into the host DNA (Daly and Chernajivski, 2000). However, they cannot infect non-dividing cells as they cannot cross the nuclear membrane, so infection can only occur in cells undergoing mitosis as the nuclear envelope is broken down (Miller *et al.*, 1990, Lewis and Emerman, 1994). This

Vector	Tropism	Packaging Capacity	Length of gene expression	Safety concerns?	Production
Liposomes	Broad but transduction is inefficient	>20 kb	Weeks to months	Good safety profile	Easy to produce at required concentrations
Retroviruses	Dividing cells	8 kb	Years	Randomly integrate into host DNA so are potentially oncogenic	Have relatively low titres
Lentiviruses	Can be pseudotyped to produce vectors with broad tropism for dividing and non-dividing cells	8 kb	Years	Randomly integrate into host DNA so are potentially oncogenic but non-integrating vectors overcome this	Have relatively low titres
Adenovirus	Broad, but have strong liver tropism	Up to 8 kb for first generation vectors but up to 36 kb for HdAds	Weeks with first generation vectors but up to approximately 2.5 years with HdAds	Can cause strong immune response	Produced at high titres
Adeno-associated virus	Broad, but have strong liver tropism	4.6 kb	Months to years	Integrate at low frequency but no firm evidence of oncogenicity	Produced at relatively high titres

Table 1.2 Features of commonly used gene therapy vectors.

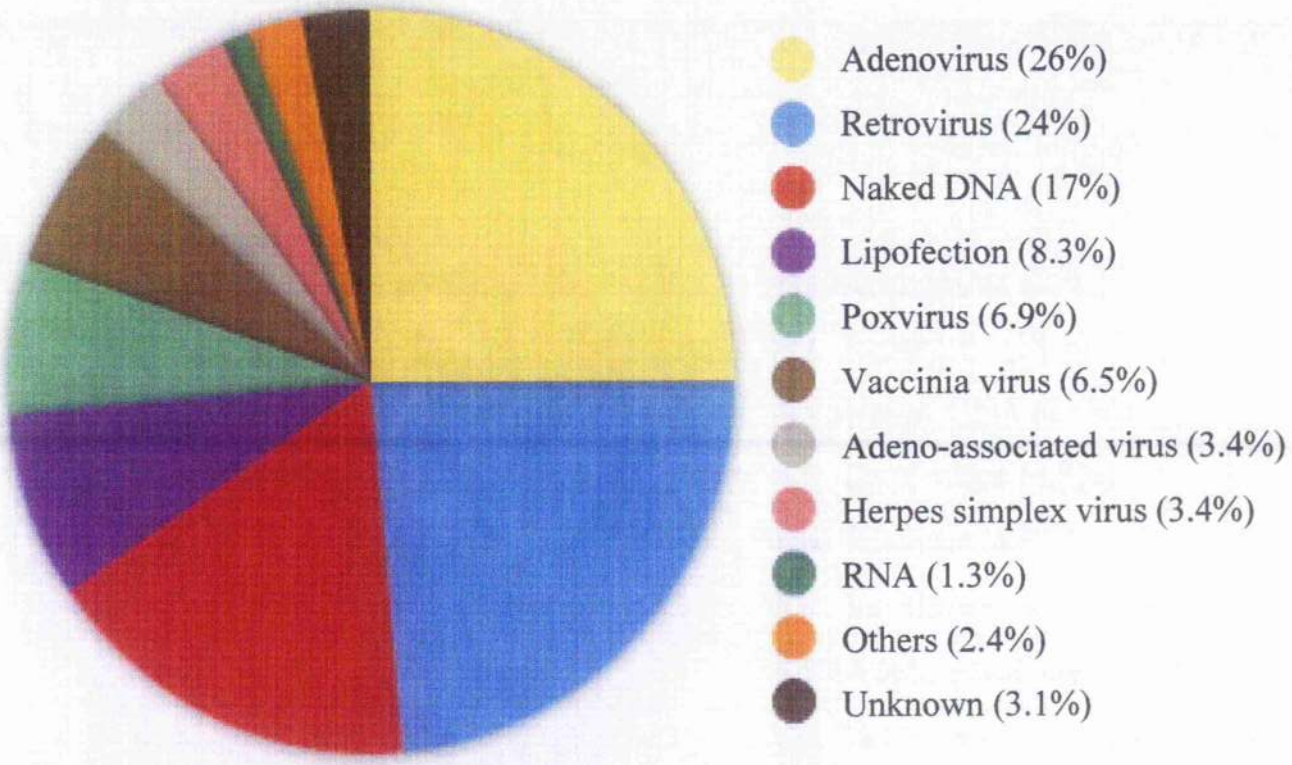


Figure 1.4 Types of vectors used in gene therapy clinical trials. The data is based on clinical trials taking place worldwide before July 2006 (http://www.wiley.co.uk/genmed/clinical/).

is a particular problem for vascular gene therapy as most vascular cells are quiescent at the time of gene delivery. Unlike other retroviruses, lentiviruses (a subclass of retroviruses) can transduce both dividing and non-dividing cells and have been shown to efficiently transduce both EC and SMC (Dishart *et al.*, 2003).

To use retroviruses as gene therapy vectors, all viral genes are removed leaving the long terminal repeats, which are required for reverse transcription, integration and virus packaging (Young *et al.*, 2006). This enables the production of virus that can carry expression cassettes of up to 8 kb and prevents the occurrence of an immune response against viral gene products. It also improves the safety of these vectors as it prevents recombination with wild type virus occurring (Dull *et al.*, 1998).

There are also concerns about the safety of using retroviruses as integration into host DNA is potentially oncogenic. The site of integration does not seem to be sequence-specific but it does seem to occur more frequently in gene rich regions (Wu *et al.*, 2003b) (Schroder *et al.*, 2002). This problem has been highlighted by recent data from a clinical trial for X-linked severe combined immune deficiency (X-SCID) that used *ex vivo* gene transfer of an oncoretroviral vector expressing the common cytokine receptor gamma chain (γc) into autologous bone marrow cells. Although a sustained clinical benefit was seen in nearly all patients (Hacein-Bey-Abina *et al.*, 2002, Cavazzana-Calvo *et al.*, 2005), three children have developed leukemia within three years of the initial treatment due to insertional mutagenesis of the vector causing up-regulation of expression of the proto-oncogene LMO2 (Marshall, 2002, Hacein-Bey-Abina *et al.*, 2003a, Hacein-Bey-Abina *et al.*, 2003b, Cavazzana-Calvo *et al.*, 2005). This has been fatal in one of the cases. These trials have highlighted both the exceptional potential of gene therapy and the associated safety concerns.

Recently a major advance was made in the safety of lentivirus vectors by the production of non-integrating vectors that provide stable transgene expression *in vivo* (Philippe *et al.*, 2006, Yanez-Munoz *et al.*, 2006). Previously non-integrating lentiviruses have been shown to provide prolonged transgene expression in a range of non-dividing cell types *in*

vitro (Sanez *et al.*, 2004, Lu *et al.*, 2004, Vargas Jr. *et al.*, 2004). Two studies have now produced HIV based vectors with a mutated integrase gene, that produce transient gene expression in dividing cells and long-term gene expression in quiescent cells, as the viral genome remains episomal (Philippe *et al.*, 2006, Yanez-Munoz *et al.*, 2006). One study showed transgene expression in the mouse eye lasted at least 9 months and the level of expression achieved was equivalent to that seen with integrating vectors (Yanez-Munoz *et al.*, 2006). In both a mouse and rat model of retinitis pigmentosa, the level of therapeutic gene expression achieved following subretinal injection caused a significant improvement in the condition of the rodents (Yanez-Munoz *et al.*, 2006). Another study demonstrated stable gene expression lasting at least 6 months in the dog eye following subretinal injection of the vector (Philippe *et al.*, 2006). It was estimated that vector integration occurred 500-1250 times less than with unmodified viruses (Philippe *et al.*, 2006), suggesting that the mutated virus would have a greatly improved safety profile. However the mutation affected transgene expression, causing an approximately 10-fold decrease in expression possibly due to the integrase mutation affecting another stage of gene expression such as virion maturation, uncoating or nuclear import (Philippe *et al.*, 2006). With further improvements in the expression cassette it may be possible to enhance this. These vectors therefore have great potential for gene therapy with less safety concerns.

1.5.2.2 Other types of viral vectors

Herpes Simplex Virus-1 (HSV-1) has also been investigated for use as a gene therapy vector. It is a large, double stranded DNA virus with a broad host cell range that includes non-dividing cells. As it is particularly efficient at transducing neuronal cells and hepatocytes it has been tested for treating gliomas (tumours of glial cells in the central nervous system), with some encouraging results from initial clinical trials (Papanastassiou *et al.*, 2002, Brown, 2005).

For cancer gene therapy there are several other oncolytic viruses such as vaccinia virus (Zeh and Bartlett, 2002), polio virus (Gromeier *et al.*, 2000) and measles virus (Hallak *et al.*, 2005), which are being investigated. For example, a measles virus targeted to $\alpha_v\beta_3$

integrin on activated endothelial cells has been shown to cause regression of myeloma tumours in a mouse model (Hallak *et al.*, 2005).

1.6 Adenovirus

Ad based vectors are now the most commonly used type of vector in clinical trials (Figure 1.4) and they are the most widely used agent for vascular gene transfer. This is because Ads have many characteristics that can be exploited to develop highly efficient gene delivery vectors. As they are one of the more established gene therapy vectors their natural biology has been more extensively studied than most, which has enabled the development of efficient methods for modifying the virus capsid and genome to produce replication deficient, tropism-modified vectors at relatively high titres. They can have a large packaging capacity for expression cassettes (up to 36 kb) so they can be produced to contain a wide range of expression cassettes and transgenes. They efficiently transduce both non-dividing and actively dividing cells without incorporation of viral genes into the host genome, so there are fewer concerns about the oncogenicity of Ad mediated gene transfer. However, the virus does provoke an immune response that limits the transgene expression and can causes problems with vector-induced toxicity (See section 1.6.7).

1.6.1 Ad Serotypes

Currently 51 different human Ad serotypes and many more from chimps, pigs, mice and birds have been identified. The human Ad serotypes have been classified into groups A-F (Figure 1.5) based on their ability to agglutinate red blood cells, DNA sequence homology, genomic organisation, and their oncogenicity in rodents (Shenk, 1996). There is also some correlation between subgroup and tissue tropism (Table 1.3). Group B viruses were previously subdivided into 2 groups (B1 and B2) based on genetic differences. Group B1 (Ad3, Ad7, Ad16, Ad21 and Ad50) are mainly associated with acute respiratory infections and group B2 (Ad11p, Ad34 and Ad35) have mainly been associated with kidney and urinary tract infections. CD46 is the receptor for the majority of group B viruses (Gaggar *et al.*, 2003) with the exception of possibly Ad3 and Ad7 (Martilla *et al.*, 2005). A recent study has suggested a new classification system for the

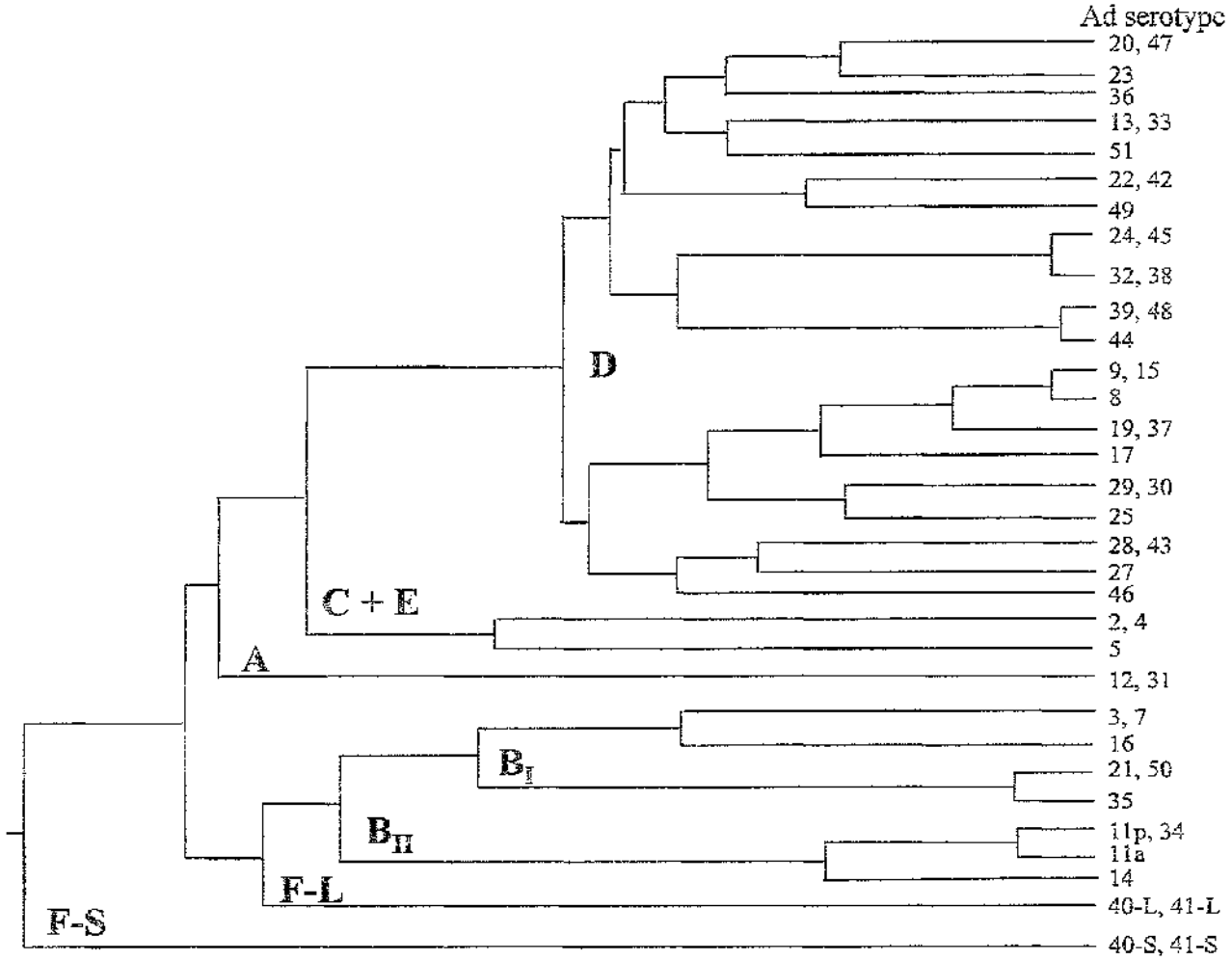


Figure 1.5 Phylogenetic tree of Ad serotypes. The tree is based on the amino acid sequence of the fiber. The subgroups are shown in bold at the branch point. Group F are divided into the short (S) and long (L) fibers. The diagram uses the original classification of group B viruses, however a new system that divides the group into 3 subroups has recently been proposed (Tuve et al., 2006) and is described in table 1.3. (Adapted from (Havenga et al., 2002)).

Group	Serotypes	Associated Infections (Zhang and Bergelson, 2005)	Main Receptor
A	Ad12, Ad18, Ad31	Respiratory	Bind CAR but not known if it is the primary receptor (Freimuth, 1999, Bewley *et al.*, 1999)
B I	Ad35, Ad50	Respiratory, kidney and urinary tract	CD46 (Gaggar *et al.*, 2003, Tuve *et al.*, 2006)
B II	Ad3, Ad7p, Ad14	Respiratory, kidney and urinary tract	Receptor X (an unidentified glycoprotein) (Tuve *et al.*, 2006)
B III	Ad11p	Respiratory, kidney and urinary tract	CD46 and receptor X (Tuve *et al.*, 2006)
C	Ad1, Ad2, Ad5, Ad6	Respiratory	CAR (Bergelson *et al.*, 1997, Tomko *et al.*, 1997).
D	Ad8, Ad9, Ad10, Ad13, Ad15, Ad17, Ad19, Ad20, Ad22, Ad23, Ad24, Ad25, Ad26, Ad27, Ad28, Ad29, Ad30, Ad32, Ad33, Ad36, Ad37, Ad38, Ad39	Epidermic keratoconjuctivitis	Some e.g. Ad37, 19a and 8 use sialic acid as their primary receptor (Arnberg *et al.*, 2000, Arnberg *et al.*, 2002, Burmeister *et al.*, 2004). CD46 might also be a primary receptor (Wu *et al.*, 2004, Trauger *et al.*, 2004, Zhang and Bergelson, 2005).
E	Ad4	Respiratory	Bind CAR (Roelvink *et al.*, 1998) but not known if it is the primary receptor
F	Ad40, Ad41	Gastroenteritis	Express 2 fibers. The long fiber binds CAR, the short fiber has no known receptor (Roelvink *et al.*, 1998, Nakamura *et al.*, 2003).

Table 1.3 Adenovirus subgroups. The most common primary cellular receptor for each group is shown, but this has not been determined for all Ad serotypes and there is some variation seen within subgroups.

group B viruses based on the identification of a novel receptor (called receptor X) for some of the serotypes (Table 1.3) (Tuve *et al.*, 2006).

1.6.2 Structure of adenoviruses

Adenoviruses are non-enveloped viruses with an icosahedral capsid consisting of 3 main structural proteins, hexon, fiber and penton base and several minor capsid proteins. Structural images of all 3 capsid components (Rux *et al.*, 2003, van Raaij *et al.*, 1999, Zubieta *et al.*, 2005) and the complete capsid (Stewart, 1993, Fabry, 2005) have been produced. The capsid consists of 240 trimeric hexon capsomeres (the main structural component of the capsid) and 12 pentameric penton bases (Figure 1.6A). The trimeric fiber protein protrudes from the penton base at each of the 12 vertices of the capsid (Figure 1.6A). Minor capsid proteins, protein IIIa (pIIIa), pVI, PVIII and pIX have roles in joining the hexon and penton subunits together (Fabry, 2005).

The fiber dictates haemagluttination and its main function is to mediate binding to the host cell receptor to tether the virus to the cells. All serotypes have the same basic fiber structure consisting of an N-terminal tail which binds to the penton base, a central shaft consisting of a repeated sequence and a C-terminal globular knob domain that provides a large surface area for interactions with the cellular receptor. The knob domain of the fiber is the most poorly conserved region between serotypes as it contains the receptor binding domains, typically on surface exposed loops. The N-terminal tail is highly conserved between serotypes, with normally more than 50% of residues identical to that of Ad5 (Nicklin *et al.*, 2005). The crystal structure of Ad2 in complex with the fiber demonstrated that 11 residues from the N-terminal tail make contact with the penton by inserting between 2 penton monomers. The fiber shaft contains a variable number of β repeats, ranging from 5.5 in Ad35 to 22.5 in Ad12 (Nicklin *et al.*, 2005). Each repeat is approximately 13Å long and consists of 15-20 amino acids that form 2 anti-parallel β-strands connected by a β-turn. These repeats join to form a very rigid and stable structure (van Raaij *et al.*, 1999). Although it might not be directly involved in receptor binding, the shaft can affect interactions with cellular receptors, as the longer fibers potentially cause less steric hindrance between the receptor and capsid. Furthermore the ability of

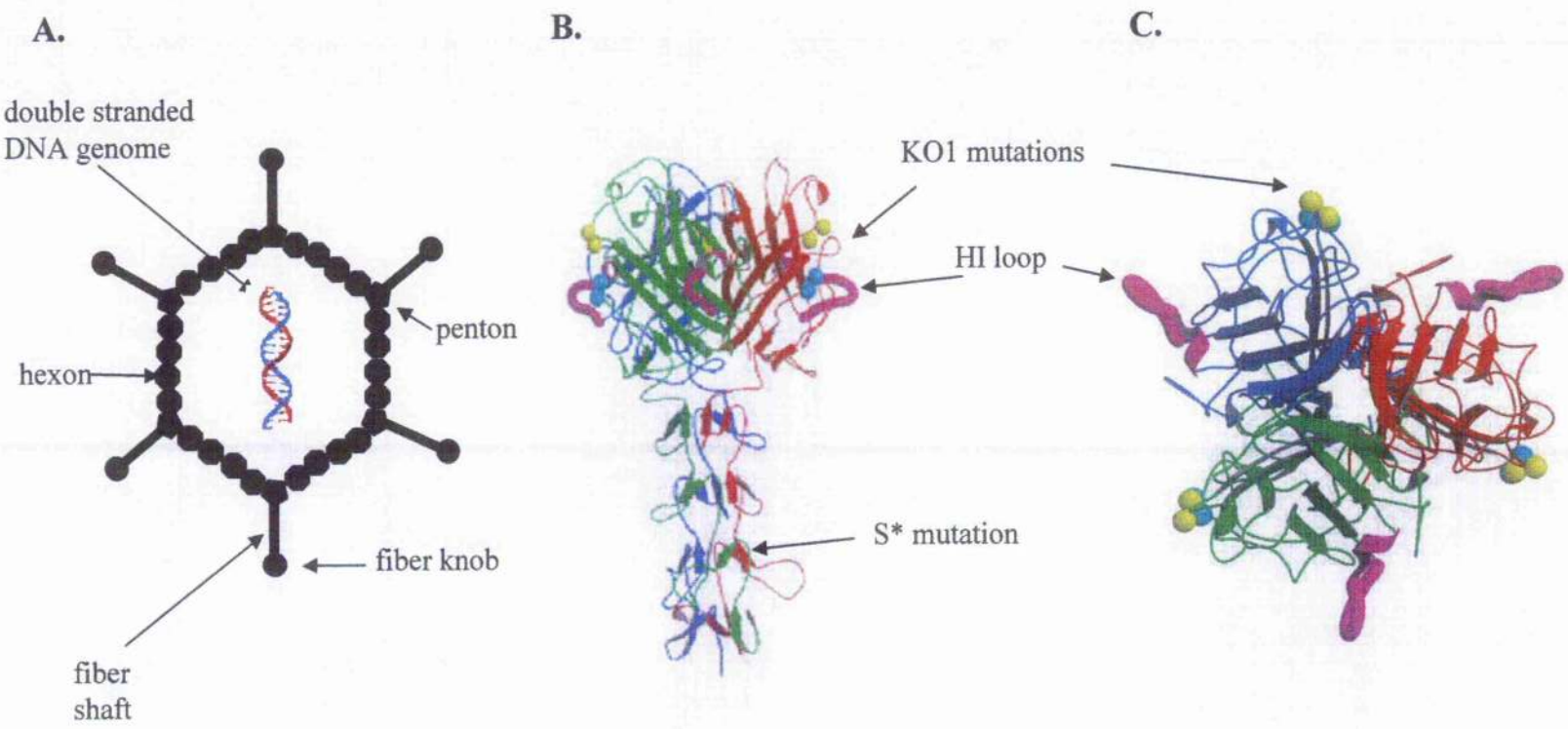


Figure 1.6 Structure of adenovirus . A. The position of the major capsid proteins hexon, penton, fiber are shown. **B.** Side view of the Ad5 fiber showing the sites of mutations used to detarget the virus and the HI loop where targeting peptides can be inserted. **C.** Top view of the fiber knob domain trimer. (Ribbon diagrams from V. Reddy, Scripps Institute, CA, USA)

the fiber to bend also seems to affect virus transduction. Electron microscopy has shown hinges in the fiber shaft of several group C viruses (Chroboczek *et al.*, 1995, Ruigrok *et al.*, 1990) that are thought to be caused by a 2-4 residue insertion in the β-turn in the 3[rd] β-repeat in several serotypes. Kinks in the fiber may reduce steric hindrance by allowing the fiber to bend, altering its orientation relative to the capsid (Wu *et al.*, 2003a).

Although there is a high degree of homology between fibers of different serotypes, there is some variation between the primary receptors they use. This is due to differences in primary amino acid sequence, the shaft length and rigidity and net charge of the fiber knob. It is only in the last 10 years that the cellular receptors have begun to be identified, so for many serotypes the cellular receptors are not yet accurately defined (Table 1.3).

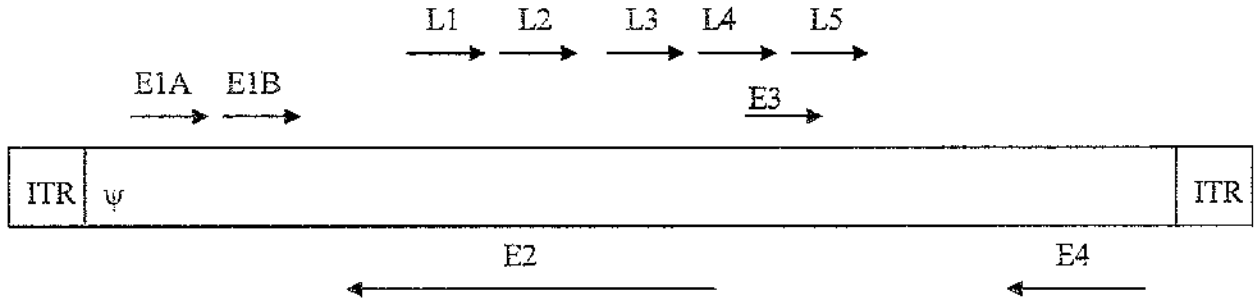
1.6.3 The adenovirus genome and vector development

The Ad genome is a linear double-stranded genome of approximately 36 kb. It encodes early genes that are involved in regulating replication (Figure 1.7) and the late genes that mainly encode structural proteins. The genes are encoded on both strands in a series of overlapping transcription units (Figure 1.7). At either end of the genome is an inverted terminal repeat (ITR) of 100-140bp.

There have been many advances in vector production that now enable Ads to be produced with the majority of the viral genome removed. The viruses are produced in cell lines that express the required viral genes or by co-infection of viruses that have the necessary genes for replication but lack the packaging signal, so the wild type genome cannot be packaged. Deletion of unrequired virus genes reduces the immune response caused by the virus and enables larger expression cassettes to be incorporated into the virus.

First generation Ads commonly have E1 and part or all of the E3 gene deleted. E1A is essential for virus replication so this produces replication defective viruses. It enables the

A.



B.

Gene	Function	Reference
E1A	Activates transcription of the other early genes and induces the host cell to enter S phase to provide the optimal environment for virus replication.	(Berk, 1986)
E1B 19K	Inhibits apoptosis to maximise virus yield	(Rao et al., 1992)
E1B 55K	Aids transport of viral mRNAs to the cytoplasm during late stage of infection	(Pilder, 1986)
E2	Needed for DNA replication, encodes DNA polymerase, preterminal protein, 72kDa ssDNA binding protein	(McConnell and Imperiale, 2004)
E3	Blocks host immune response	(McConnell and Imperiale, 2004)
E4	Involved in cell cycle control and transformation	(McConnell and Imperiale, 2004)

Figure 1.7 The Ad genome. A. Diagram of the basic structure of the Ad genome. ψ = packaging signal. **B.** Function of the early genes.

incorporation of expression cassettes of up to 8.2 kb (Bett *et al.*, 1993). The vectors still stimulate an immune response against transduced cells, possibly due to a low level of virus replication (Yang *et al.*, 1994, Yang *et al.*, 1995, Dai *et al.*, 1995). This results in transient gene expression that lasts about 2-3 weeks (Yang *et al.*, 1995). Despite this first generation vectors are still the most commonly used.

Second generation vectors also have E2 and E4 genes removed, which increases their packaging capacity. In some cell types this can increase the duration of transgene expression (Hu *et al.*, 1999, Engelhardt *et al.*, 1994). However second generation vectors do not provide longer-term transgene expression in vascular cells (Wen *et al.*, 2000). Second generation vectors are rarely used.

Helper-dependent or gutted viruses have all viral genes deleted leaving the 2 ITRs and the packaging signal required for DNA replication and packaging (Mitani *et al.*, 1995, Kochanek, 1999). This produces a virus with a packaging capacity of approximately 36 kb. *In vivo* studies suggest significantly longer transgene expression can be achieved with these vectors, although they still cause an innate immune response (Dellorusso *et al.*, 2002, Ehrhardt and Kay, 2002, Muruve *et al.*, 2004, Pastore *et al.*, 2004, Chen *et al.*, 1997, Oka *et al.*, 2001), probably because the vectors are still sequestered by Kupffer cells (macrophages found in the liver) (Schiedner *et al.*, 2003).

1.6.4 Mechanism of adenovirus infection

For the development of gene therapy vectors it is important that the natural mechanism of cell infection is understood as all stages of the infection process and not just the initial cell-binding event affect vector tropism.

Ad5 is the most extensively studied adenovirus and is the most commonly used as a gene therapy vector. Although there is some variation in the mechanism of Ad5 infection in different cell types *in vitro*, the general mechanism has been well characterised. For other serotypes a similar mechanism of infection is probably used but this has not been characterised. Ad5 binds to the host cell through an interaction between the fiber knob

and the cellular receptor, coxsackie and adenovirus receptor (CAR) (Bergelson *et al.*, 1997, Tomko *et al.*, 1997). This tethers the virus to the cell surface and brings the capsid close to integrins: no CAR mediated signal is actually required for internalisation (Shayakhmetov *et al.*, 2005a). The RGD motif in the penton base binds to α_v integrins to activate virus internalisation by causing rearrangements in the actin cytoskeleton (Wickham *et al.*, 1993, Wickham, 1994, Huang *et al.*, 1995, Li *et al.*, 2001, Huang *et al.*, 1996, Salone *et al.*, 2003). The need for Ad to bind both CAR and integrins at the same time means the fibers are required to be long (Shayakhmetov and Lieber, 2000) and flexible (Wu *et al.*, 2003a). An interaction between the fiber shaft and heparan sulphate proteoglycans (HSPGs) has also been implicated in mediating infection (Dechecchi *et al.*, 2000, Dechecchi *et al.*, 2001), but there is no direct evidence that binding occurs (Smith *et al.*, 2003a, Nicol *et al.*, 2004, Smith *et al.*, 2003b, Bayo-Puxan *et al.*, 2006)(A. Kritz, in press).

After receptor binding the virus is internalised into an endosome via a clathrin coated pit. (Varga *et al.*, 1991, Wang *et al.*, 1998). As the endosome becomes more acidic, virus disassembly occurs as the penton undergoes a conformational change. This exposes minor protein VI, which triggers a further pH drop and disruption of the membrane leading to virus escape from the endosome (Greber *et al.*, 1993, Wiethoff, 1998). Ad protease in the capsid also becomes exposed and this enhances virus release (Medina-Kauwe, 2003). Lysis of the endosome and release of the virion into the cytoplasm enables it to evade degradation by lysosomal enzymes. The virus then translocates to the nucleus via microtubules (Suomalainen *et al.*, 1999). At the nuclear membrane, complete virus disassembly occurs and the genome is released into the nucleus. Ad2 has been shown to do this by docking with the nuclear pore complex CAN/Nup214 and histone H1 (Trotman *et al.*, 2001). In the nucleus E1A is the first gene to be expressed. It transactivates the expression of the other early transcription units (E1B, E2, E3 and E4) and induces the cell to enter S phase, which provides the optimal environment for viral replication (Berk, 1986). This is followed by expression of the late genes. E1B is involved in the transport of late mRNAs into the cytoplasm where they are translated. The L4 protein associates with the hexon in the cytoplasm soon after translocation and

mediates hexon trimerisation. (Cepko, 1983). Hexon trimers are transported back to the nucleus (Woodrich, 2003), where they associate with the penton and minor capsid proteins to form the protein capsid. DNA encapsidation then occurs and Ad protease cleaves a subset of structural proteins into their mature form to produce fully infectious virions. Cell lysis and virus release then occurs, about 30 hours after the original infection (McConnell and Imperiale, 2004).

1.6.5 Ad5 tropism

CAR, a member of the immunoglobulin superfamily, is a cell membrane protein that is made up of a short leader sequence, an extracellular domain containing 2 immunoglobulin domains, a single membrane spanning domain and an intracellular domain (Bergelson *et al.*, 1997). The first immunoglobulin domain is sufficient for Ad binding (Bewley *et al.*, 1999, Freimuth, 1999). The exact expression profile of CAR has not been determined, but CAR mRNA has been detected in heart, brain, pancreas, intestine, lung, liver and kidney (Tomko *et al.*, 1997, Fechner *et al.*, 1999, Howitt, 2003). CAR is localised to the basolateral surface of polarised epithelial cells, specifically concentrated at the end of the lateral plasma membrane, a site associated with tight junctions and adherence junctions (Cohen *et al.*, 2001, Walters *et al.*, 2002). Its precise function is not known; however it has a role in homotypic cell-to-cell fusion (Honda, 2000). Cells that do not express CAR include many advanced tumours, skeletal muscle cells, SMC, haematopoietic stem cells and dendritic cells. Most tissues of transgenic animals that broadly over-express CAR, are more readily transduced by Ad (Tallone *et al.*, 2001), and in transgenic animals tissue-specific CAR expression has been shown to increase transduction of skeletal muscle (Nalbantoglu *et al.*, 2001) and lymphocytes (Schmidt *et al.*, 2000, Wan *et al.*, 2000) which are normally refractory to infection.

After systemic administration of Ad5 to rodents and non-human primates, about 95% of measurable transduction is found in liver hepatocytes (Huard *et al.*, 1995, Sullivan *et al.*, 1997, Alemany and Curiel, 2001, Mizuguchi *et al.*, 2002, Smith *et al.*, 2002, Leissner *et al.*, 2001). However, the majority of the virus injected does not successfully transduce any cells as it is rapidly cleared from the blood, with a half-life of less than 3 minutes in

mice (Alemany *et al.*, 2000, Koizumi *et al.*, 2003, Sakurai *et al.*, 2003). This is probably due to the virus being taken up very efficiently by Kupffer cells, where it is then inactivated and degraded (Lieber *et al.*, 1997, Wolff *et al.*, 1997, Worgall *et al.*, 1997a). Activated Kupffer cells stimulate an innate immune response by release of proinflammatory cytokines and chemokines (Liu and Muruve, 2003).

If Ad is delivered locally e.g. into a tumour, then vector dissemination to the blood and liver occurs (Mizuguchi *et al.*, 2002, Okada *et al.*, 2003, Hiltunen *et al.*, 2000).

The broad tropism of Ad and the high degree of transgene expression in the liver following systemic transduction are undesirable for many gene therapy applications, so studies have been carried out to produce Ad based vectors with altered tropism.

1.6.6 Ad-mediated cardiovascular transduction

Systemic delivery of Ad5 results in a very low level of transduction of vascular cells. However unmodified Ad can produce a high level of transgene expression in endothelial cells and some expression in SMC in vessels if the virus is applied locally and a high dose is used (Lemarchand *et al.*, 1993, Lee *et al.*, 1993, Merrick *et al.*, 1996). For example, in intact human vessels *ex vivo* transduction with an Ad vector can result in transduction of virtually all EC but transduction of intimal SMCs is low (1.3-3.8%) as the virus cannot cross the endothelium (Rekhter *et al.*, 1998b). If the endothelium is damaged then Ad can penetrate the elastic lamina and infect SMC (George *et al.*, 1998, Gruchala *et al.*, 2004). Ad infection causes an inflammatory response in the vessel wall that limits transgene expression to only 1-2 weeks (Newman *et al.*, 1995, Channon *et al.*, 1998).

It has been reported that Ad5 more efficiently transduces atherosclerotic vessels compared to healthy vessels (Ooboshi *et al.*, 1997, Rekhter *et al.*, 1998b), possibly because of an increase in VCAM-1 expression. VCAM-1 and CAR are both members of the immunoglobulin superfamily and have 43% homology in the region of CAR known to bind Ad5 (Chu *et al.*, 2001). This led to the proposal that VCAM-1 might act as a

receptor for Ad5 and it has been shown that Ad5 transduction was 5-10 fold higher in VCAM-1 expressing cells (Chu *et al.*, 2001).

1.6.7 Immune response to Ad vectors

One of the main disadvantages of Ad vectors is that they provoke a strong immune response. Within minutes of virus administration the innate immune response is triggered and this can result in inflammation, loss of transduced cells and systemic toxicity (McConnell and Imperiale, 2004). It causes 80-90% of first generation Ads to be removed from the liver within 24 hours of vector administration (Worgall *et al.*, 1997a). It is caused by the uptake of Ad by the reticuloendothelial system (RES), mainly by Kupffer cells. Although they lack CAR, uptake of virus is highly efficient and results in vector clearance from the blood and activates the inflammatory and adaptive immune response.

The adaptive cellular immune response is initiated by Kupffer cells and other antigen presenting cells, leading to presentation of virus and transgene proteins in the major histocompatability complex (MHC) class I. This activates cytotoxic T lymphocytes (CTLs) against both the virus and transgene. To activate T-cells viral gene expression is also required (Muruve *et al.*, 2004). Although first generation E1 deleted Ads cannot replicate, some viral gene expression remains and this is sufficient to activate the cellular immune response mediated by CTLs. (Yang *et al.*, 1994). This leads to the death of transduced cells and therefore the loss of transgene expression. Due to the removal of the majority of the virus genome, HdAds do not activate the cellular immune response so produce longer-term transgene expression than first generation Ads (Schiedner *et al.*, 1998).

The humoral response to Ad is activated by binding of the virus to immunoglobulins on the surface of B-cells. This results in the activation of B-cells and the production of anti-Ad antibodies, which prevent cellular entry and promotes uptake of the virus by macrophages, thereby limiting transgene expression. If the transgene is a foreign protein

then this can also provoke a humoral response that can reduce the action of the therapeutic protein.

Immunity that develops after receiving a dose of gene therapy can also prevent successful readministration of the same vector, but this depends on the dose and route of administration used. For example, intra-muscular administration of a low dose of Ad5 produced detectable transgene expression and did not affect a repeated intra-muscular injection, but intra-venous readministration failed to produce transgene expression (Chen *et al.*, 2000a). As the humoral and innate immune responses are mediated directly by the virus capsid they do not differ for first generation and HdAds (Alba *et al.*, 2005). The affect of anti-Ad antibodies can be reduced by using alternative serotypes (Kass-Eisler *et al.*, 1996, Mack *et al.*, 1997), coating the virus with polyethylene glycol (PEG) (O'Riordan *et al.*, 1999, Croyle *et al.*, 2001, Croyle *et al.*, 2005), or co-administering immunosuppressive drugs (Smith *et al.*, 1996, Fang *et al.*, 1995).

A further issue relating to the immune response to Ad is that about 80% of the population have pre-existing anti-Ad5 antibodies due to natural infection (Chirmule *et al.*, 1999, Vigne *et al.*, 2003). These can inhibit the effectiveness of gene therapy treatments (Varnavski *et al.*, 2005, Chirmule *et al.*, 1999, Chen *et al.*, 2000b, Varnavski *et al.*, 2002) and in mouse models pre-existing immunity has been shown to increase mortality rates (Varnavski *et al.*, 2005, Vlachaki *et al.*, 2002).

The host immune response to Ad vectors has also been implicated in causing serious adverse effects. In a pilot clinical trial for the monogenic disorder ornithine transcarbamylase (OTC) deficiency, a patient died because of the immune response to the treatment. Patients were given replication-defective Ad with the OTC transgene via the hepatic artery at increasing doses (Raper *et al.*, 2002). In one of two patients given the highest dose of the vector (6×10^{11} particles/kg), it caused a strong systemic inflammatory response that led to multiple organ failure and death 98 hours after injection of the vector (Raper *et al.*, 2003). The other patient given the same dose and patients given lower doses suffered from mild fevers, suggesting there is significant

patient variability in response to the same vector (Raper *et al.*, 2002). This is a critical issue in the translation of Ad based vectors to the clinic.

If targeted Ad vectors with a high level of cellular specificity can be produced, then this should in theory reduce virus dissemination and the dose required, and thereby minimise and localise the immune response against the treatment (Schoggins *et al.*, 2005).

1.7 Adeno-associated virus

AAV is classified as a *Dependovirus* in the *Parvoviridae* family as it usually requires co-infection with a helper virus (such as Ad or HSV) for it to undergo productive replication. AAV has many attributes that make it a promising vector for gene therapy. One of the main features of AAV based vectors is that they have been shown to produce stable transgene expression in a number of animal models (Snyder *et al.*, 1999, Herzog *et al.*, 1999, Flotte *et al.*, 1993, Xiao *et al.*, 1996, Woo *et al.*, 2005). AAV also have a relatively broad tropism, can infect both dividing and non-dividing cells (Monahan and Samulski, 2000b), are non-pathogenic in humans and are significantly less immunogenic than adenovirus vectors (Chirmule *et al.*, 1999, Chirmule *et al.*, 2000, Xiao *et al.*, 2000, Beck *et al.*, 1999). AAV2 is the most commonly used serotype for gene therapy vectors.

1.7.1 Structure of AAV

AAV is a small non-enveloped virus with an icosahedral capsid. It has a linear single-stranded DNA genome of approximately 4.7 kb, which encodes 2 genes rep (replication) and cap (capsid) flanked by ITRs. The ITRs are required for viral DNA replication. The rep gene encodes the non-structural proteins Rep78, Rep68, Rep52 and Rep40, which are involved in regulating gene expression. The cap gene encodes the 3 structural proteins that form the AAV capsid. It is transcribed to produce mRNA that is alternatively spliced to produce two transcripts. The largest transcript encodes virion protein 1 (VP1) and the shorter transcript encodes VP2 and VP3 by translation from 2 start codons. All 3 transcripts use the same stop codon, therefore VP2 (72 kDa) and VP3 (60 kDa) are truncated forms of VP1 (90 kDa), identical in the C-terminus. The capsid is formed from 60 subunits with a molar ratio of VP1, VP2 and VP3 of approximately 1:1:20

(Rabinowitz and Samulski, 2000). It has 12 five-fold axes of symmetry and at each one is a narrow pore, which is important for both viral infectivity and genome packaging (Bleker *et al.*, 2005). The most prominent feature of the AAV2 capsid surface is the three-fold-proximal peaks, which are formed by the interaction of two subunits (Xie *et al.*, 2002) (Figure 1.8A). The unique N-terminal region of VP1 contains a phospholipase A_2 domain (PLA_2) (Zadori *et al.*, 2001) that is thought to be required for virus translocation to the nucleus and initiation of viral gene expression (Girod *et al.*, 2002). The VP3 region found in all 3 capsid proteins contains the areas of the capsid that mediate cell receptor binding. The function of VP2 has not been elucidated and it has in fact been shown that viruses lacking VP2 can be produced (Warrington *et al.*, 2004, Grieger and Samulski, 2005). Each of the 60 capsid subunits has a β-barrel core structure which is commonly found in parvoviruses (Xie *et al.*, 2002). Long loops between the β-strands of the barrel are poorly conserved between parvoviruses and it is these loops which are displayed on the capsid surface and mediate interactions with cell surface receptors and antibodies (Xie *et al.*, 2002).

1.7.2 AAV serotypes

Over the last few years the number of AAV serotypes and genetic variants isolated has increased rapidly. The main reason for this is the development of a polymerase chain reaction (PCR) screening technique that has been used to detect AAV variants in non-human primates (Gao *et al.*, 2003). PCR screening of the hypervariable regions of the AAV capsid from primate tissues has demonstrated that AAV has undergone rapid molecular evolution in non-human primates (Gao *et al.*, 2003). Currently there are eleven different AAV serotypes (AAV1-11) and over 100 different variant capsid sequences have been identified (Gao *et al.*, 2004). By definition a serotype is a newly isolated virus that does not cross-react with neutralising sera for other serotypes. The serology of the variants has not yet been fully investigated therefore they are not yet classified as serotypes. Serotypes 1-6 were isolated as live viruses whereas AAV7-11 were identified by PCR screening of hypervariable region 3 of the AAV capsid in primate tissues (Gao *et al.*, 2003). As many of the serotypes have only recently been identified work is ongoing to fully characterise these viruses and determine their primary receptors

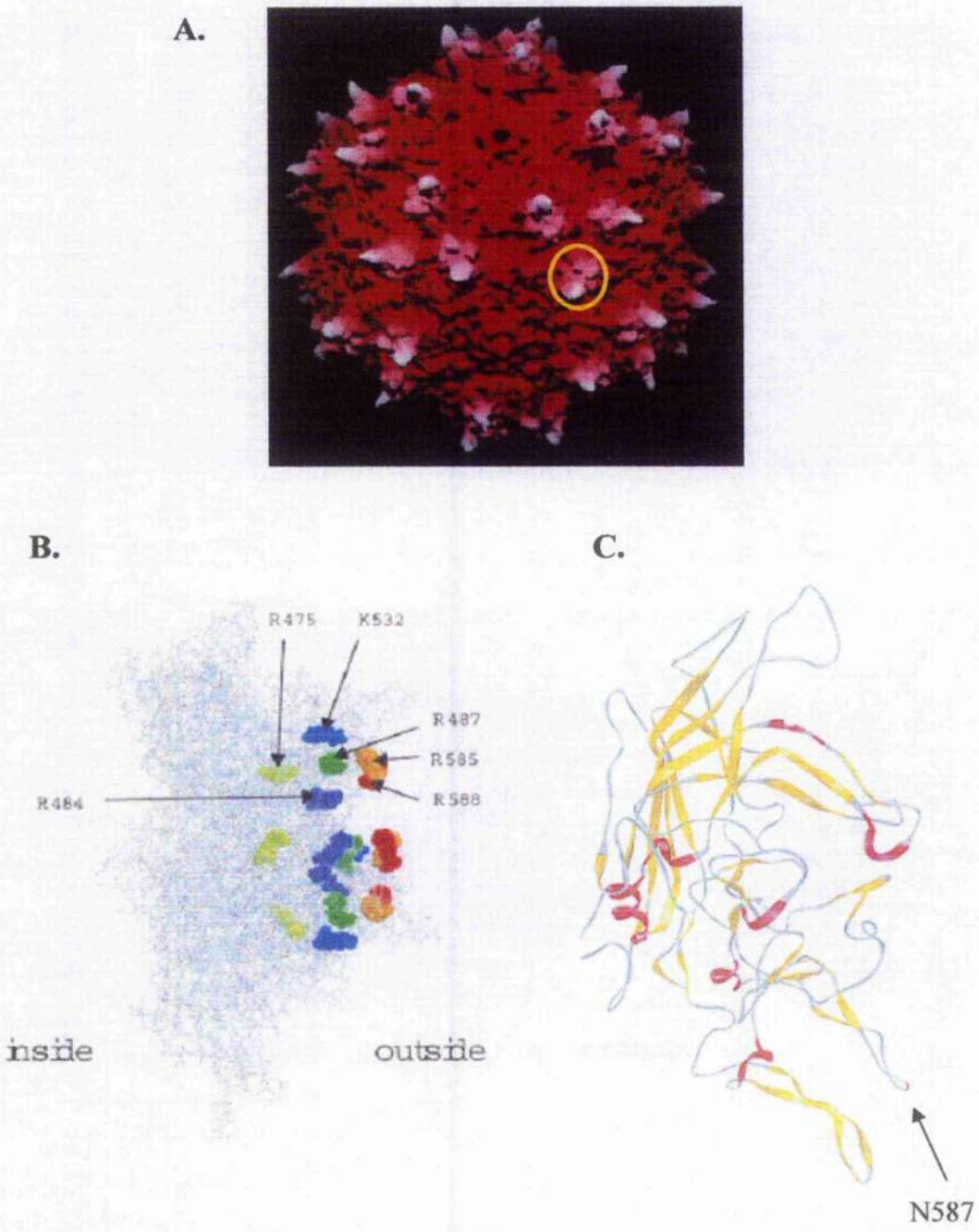


Figure 1.8 Structure of AAV2 **A.** Structure of the AAV2 capsid with a threefold peak region marked (Xie et al., 2002). **B.** Side view of a VP timer with the positions of amino acids involved in HSPG binding indicated (Kern et al., 2003). **C.** Structure of AAV2 VP1subunit (Image courtesy of Luca Perabo).

(Table 1.4). The novelty of some serotypes is under question as AAV6, 10 and 11 might not be true serotypes. AAV6 is almost serologically identical to AAV1 and it is thought to have formed due to a recombination event between AAV1 and 2 as the left ITR and p5 promoter region are almost identical to AAV2 and the rest of the genome is almost identical to AAV1 (Rutledge *et al.*, 1998, Xiao *et al.*, 1999). AAV10 and 11 are not yet fully characterised but do not cross react with anti-AAV2 antibodies (Zhijian *et al.*, 2006, Mori *et al.*, 2004).

1.7.3 AAV2 receptor binding and infection pathway

The most extensively studied serotype and the most commonly used as a gene therapy vector is AAV2. The primary receptor for AAV2 is HSPG (Summerford and Samulski, 1998), which is expressed on many different cell types, so the virus has a very broad tropism. It uses $\alpha_v\beta_5$ integrin (Summerford *et al.*, 1999), fibroblast growth factor receptor type 1 (FGFR1) (Qing *et al.*, 1999), hepatocyte growth factor receptor c-Met (Kashiwakura *et al.*, 2005) or laminin receptor (Akache *et al.*, 2006) as co-receptors to enable internalisation. The exact mechanism by which AAV2 enters the cell and traffics to the nucleus is still not fully understood.

Before the crystal structure of AAV2 was solved insertional mutagenesis studies identified residues that mediate binding of AAV2 to HSPG (Section 1.13.2 for more detail). Alignment of AAV2 capsid sequence with that of AAV1 and AAV3 that both bind HSPG and AAV4 and AAV5 that do not bind HSPG, identified 15 different residues that could be involved in HSPG binding (Wu *et al.*, 2000). The solving of the crystal structure in 2002 (Xie *et al.*, 2002) supported the results of mutational studies as it showed that the basic amino acids R484, R487, R585, R588 and H509 clustered around the 3-fold proximal peak and are critical for forming the HSPG binding site (Figure 1.8B) (Xie *et al.*, 2002). The primary amino acid sequence around these residues R_{484}QQRVSK_{490} and R_{585}GNR_{589} form motifs typical of sequences known to bind heparin (Xie *et al.*, 2002). Importantly, the 3D conformation of the amino acids in this region of the capsid resembles a heparin binding site, which is commonly a flat pocket with a positive charge (Hileman *et al.*, 1998).

Serotype	Primary Receptor	Species origin
AAV1	Sialic acid (Chen *et al.*, 2005)	Human or non-human primates
AAV2	HSPG (Summerford and Samulski, 1998)	Human
AAV3	HSPG (Rabinowitz *et al.*, 2001, Handa *et al.*, 2000)	Human
AAV 4	O-linked sialic acid (Kaludov *et al.*, 2001)	Non-human primates
AAV5	N-linked sialic acid (Kaludov *et al.*, 2001, Walters *et al.*, 2001) PDGF receptor (Di Pasquale *et al.*, 2003).	Human
AAV6	Sialic acid (Zhijian *et al.*, 2006)	Human
AAV7	Unknown	Rhesus monkey
AAV8	Laminin receptor (Akache *et al.*, 2006)	Rhesus monkey
AAV9	Laminin receptor (Akache *et al.*, 2006)	Rhesus monkey
AAV10	Unknown	Cynomolgus monkey (Mori *et al.*, 2004)
AAV11	Unknown	Cynomolgus monkey (Mori *et al.*, 2004)

Table 1.4 AAV serotypes.

Further mutational studies found that substitutions R585A and R588A caused the greatest reduction in HSPG binding, whereas making conservative substitutions to lysine caused no reduction in heparin binding or infectivity of the virus (Kern *et al.*, 2003, Opie *et al.*, 2003). Substitution of residues R484, R487 and R532 also caused reduction in heparin binding and infectivity, but to the extent seen with R585 and R588 substitutions (Opie *et al.*, 2003, Kern *et al.*, 2003). These experiments identified both the residues involved in HSPG binding and the importance of the positive charge of the residues.

The regions of the AAV2 capsid that bind to the co-receptors are yet to be identified. A recent mutational study has identified a region known as the 'dead zone' that lies adjacent to the heparin binding site (Lochrie *et al.*, 2006). This is not involved in heparin binding, but is required for transduction (Lochrie *et al.*, 2006). A model has been produced showing this 'dead zone' could potentially be the binding site for the co-receptor FGFR1 (Lochrie *et al.*, 2006). Although the areas of the capsid responsible for co-receptor binding are yet to be identified, it has still been possible to produce viruses with significantly altered tropisms.

Following receptor binding, the principal mechanism of entry into cells is via receptor-mediated endocytosis in clathrin-coated pits (Duan *et al.*, 1999, Bartlett *et al.*, 2000). Single virus tracing (SVT) has shown that a single AAV particle is transported in an endosome towards the nucleus by a mechanism likely to involve active transport via microtubules (Seisenberger *et al.*, 2001). AAV is released from the late endosome when it is at a low pH (Douar *et al.*, 2001, Bartlett *et al.*, 2000, Hansen *et al.*, 2001a). After release from the endosome, AAV2 trafficking is poorly understood and probably varies in different cell types and possibly even within the same cell. For example, in polarised human epithelial cells virus entry from the basolateral side results in efficient transduction, but from the apical side the ubiquitin-proteasome pathway inhibits it (Duan *et al.*, 2000b).

How the virus enters the nucleus is also yet to be determined, but it is thought to be accelerated by the presence of helper virus (Xiao *et al.*, 2002). It was originally thought that AAV2 enters the nucleus via nuclear pore complexes, but agents that block this do not effect transduction (Hansen *et al.*, 2001b). It is also not known whether the virus uncoats before or after entry into the nucleus. However, it is thought that a highly conserved basic region (amino acids 166-172) found within the VP2 region of all 11 AAV serotypes is required for the virus genome to enter the nucleus (Grieger *et al.*, 2006). Confocal microscopy has shown that mutation of this region causes the virus to accumulate at the surface of the nucleus but no viral genomes were detected inside the nucleus (Grieger *et al.*, 2006).

Once inside the nucleus the presence or absence of helper virus determines whether AAV2 enters either the lytic pathway or becomes latent. In the absence of a helper virus, AAV becomes latent and integrates into a specific region of human chromosome 19 known as AAVS1 (Kotin *et al.*, 1990, Kotin *et al.*, 1991). This region of the genome is the only area that contains the necessary features for AAV2 integration, a Rep binding site, a terminal resolution site and a spacer sequence (Meneses *et al.*, 2000). The mechanism by which this highly specific integration occurs is unknown, although it does require virus proteins Rep78 and Rep68 and the ITRs (Weitzman *et al.*, 1994, Huttner *et al.*, 2003). In the presence of helper virus or an agent such as UV irradiation or genotoxic compounds that cause DNA damage (Yakinoglu *et al.*, 1988), the single stranded genome is converted to double stranded DNA. This can occur by second strand synthesis or the annealing of a plus and minus strand from two separate virions that have infected the same cell (Figure 1.9A and 1.9B) (Ferrari *et al.*, 1996, Fisher *et al.*, 1996, Nakai *et al.*, 2000, Hauck *et al.*, 2004). DNA synthesis requires Rep78 and Rep68.

The first stage of virus assembly is the formation of empty capsids in the nucleus. A basic region in VP3 (amino acids 307-312) has been shown to be essential for capsid assembly to occur (Grieger *et al.*, 2006). The 4 Rep proteins then form a complex on the capsid surface. Sequence specific binding of the AAV genome to the large Rep proteins mediates translocation of the genome into the capsid (King *et al.*, 2001).

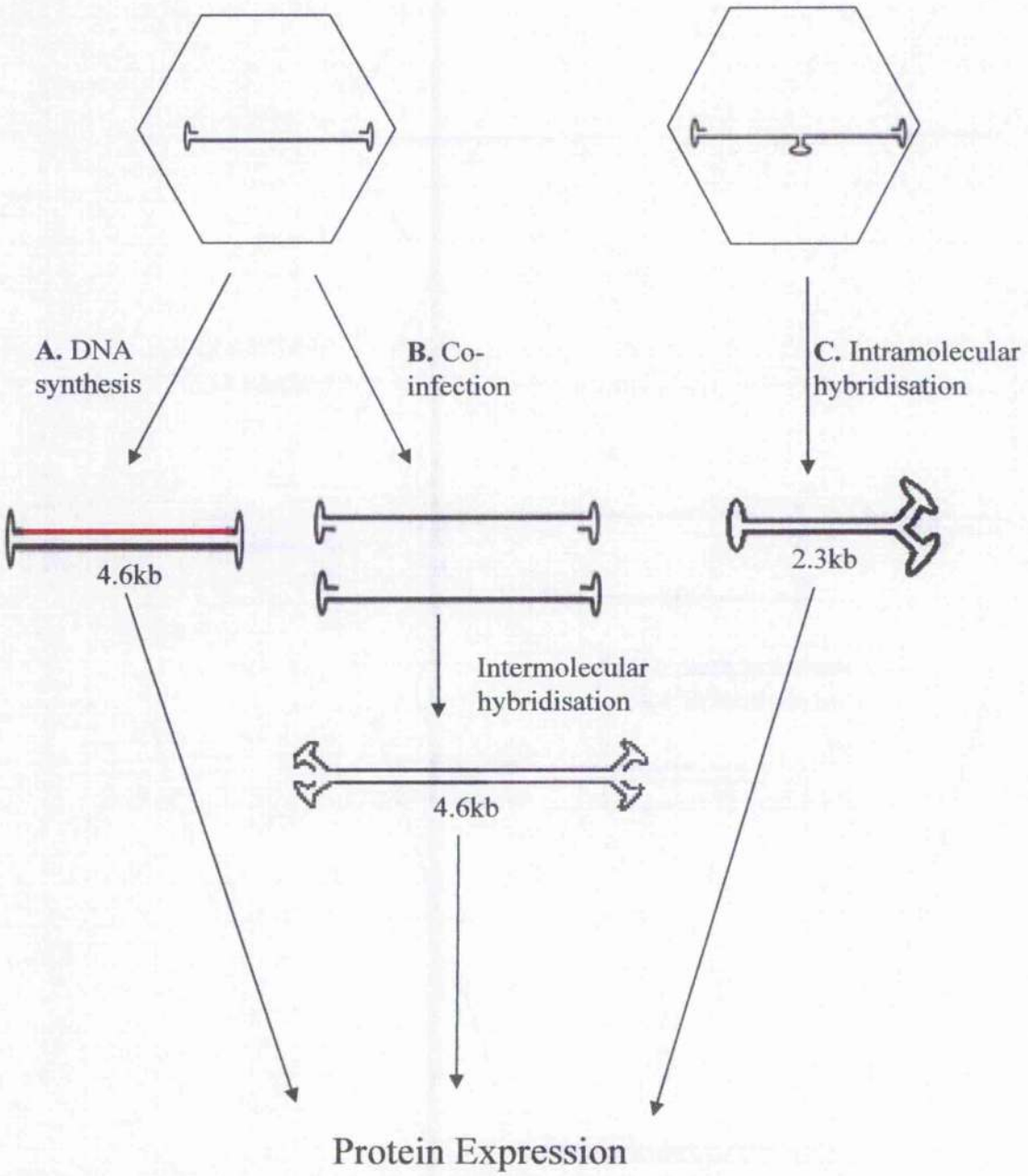


Figure 1.9 Mechanisms of transgene expression from AAV vectors. A. single stranded DNA genome is converted to double stranded DNA by DNA synthesis. **B.** Co-infection of 2 viruses enables positive and negative strand annealing to produce double stranded DNA. **C.** Intramolecular hybridisation of a self complementary genome produces double stranded DNA.

1.7.4 AAV2 tropism

AAV2 has a relatively broad tropism and can transduce a wide range of tissues *in vivo* including muscle, lung, the retina and the central nervous system (Acland *et al.*, 2001). However, following systemic administration the vast majority of virus is found in the liver (Nathwani *et al.*, 2001, Miao *et al.*, 2000).

1.7.5 AAV and vascular transduction

AAV2 poorly transduces vascular endothelial cells *in vitro* and *in vivo* (Gnatenko *et al.*, 1997, Maeda *et al.*, 1997, Richter *et al.*, 2000, Nicklin *et al.*, 2001a, Dishart *et al.*, 2003, Pajusola *et al.*, 2002, Vassalli *et al.*, 2003). This is despite ECs expressing HSPG and the virus co-receptors $\alpha_v\beta_5$ integrin and FGF receptor-1 (Pajusola *et al.*, 2002). This may be due to sequestration of the virus in the extracellular matrix by binding to HSPG (Pajusola *et al.*, 2002) and/or proteasomal degradation of the virus during the trafficking process (Duan *et al.*, 2000b, Nicklin *et al.*, 2001a, Denby *et al.*, 2005). The effect of the proteasome on AAV2 transduction seems to vary in different cell types as transduction enhancement varies when proteasome inhibitors are used (Yan *et al.*, 2002, Douar *et al.*, 2001). The mechanism by which the proteasome can block transduction is not fully understood as it may not be that the proteasome actually degrades the virion (Duan *et al.*, 2000b, Yan *et al.*, 2002), but somehow reduces nuclear uptake of the virus (Duan *et al.*, 2000b, Yan *et al.*, 2004).

When an intact blood vessel is exposed to AAV2 the majority of transduction is observed in smooth muscle cells and not the overlying endothelial cells (Richter *et al.*, 2000, Pajusola *et al.*, 2002), in complete contrast to Ad5 (Lemarchand *et al.*, 1993). Higher levels of transduction of SMC than EC have also been seen *in vitro* (Mohuczy *et al.*, 1999). This could be due to expression of the virus receptor or differences in efficiencies in the later steps of the transduction pathway in each cell type.

Comparing AAV2 and Ad5 transduction of mouse carotid arteries showed that both produced very low levels of transduction and transduction levels with AAV were 8-fold lower than Ad. However transgene expression from the AAV vector lasted up to 8 weeks

whereas with Ad a maximum of 2 weeks expression was seen (Vassalli *et al.*, 2003). Another study compared the transduction of rabbit arteries following intraluminal injection of AAV2 or Ad5 vectors (Gruchala *et al.*, 2004). AAV2 mainly transduced SMC and produced transgene expression for at least 100 days where as Ad transduced EC and transgene expression was undetectable after just 14 days (Gruchala *et al.*, 2004). Ad also caused a significant inflammatory response whereas that caused by AAV2 was not much greater than that seen with control infections (Gruchala *et al.*, 2004). These studies highlight major differences between the 2 vector types and suggest that they may be utilised for different conditions. For example AAV may be more suitable for treating chronic conditions such as atherosclerosis where sustained transgene expression will probably be required, whereas Ad may be more appropriate for inhibiting SMC proliferation after acute vascular injury where high levels of short term gene expression may be adequate (George *et al.*, 2000).

1.7.6 Advances in the development of AAV based vectors

One of the major limitations of using AAV as a gene therapy vector is the small packaging capacity of the virus, which has an optimum range of 4.1-4.9 kb (Dong *et al.*, 1996). Attempts to package larger expression cassettes have shown that up to 5.6 kb can be packaged, but the resulting viruses have decreased transduction efficiency (Grieger and Samulski, 2005, Hermonat *et al.*, 1997). Several methods are being developed to overcome this limitation.

A split-gene strategy where 2 vectors are used each expressing half of the gene with a splice donor and splice acceptor site incorporated, makes use of the fact that AAV genomes can naturally recombine. The 2 vectors are required to infect the same cell and form head to tail heterodimers from which the ITRs are then spliced out of the mRNA transcript to join the two halves of the transcript together (Duan *et al.*, 2003). Another method relies on homologous recombination to join the 2 halves of the transgene (Duan *et al.*, 2000a). However these processes produce much lower transgene expression than single vector constructs because of the added complexity involved and the requirement

for the 2 different vectors to infect the same cell (Reich *et al.*, 2003, Sun *et al.*, 2000, Duan *et al.*, 2001).

Another limitation of AAV vectors is the slow rate of conversion of the single stranded genome to double stranded DNA, which is required for transgene expression (Ferrari *et al.*, 1996, Fisher *et al.*, 1996). The development of self-complementary AAV (scAAV) vectors that contain a double stranded genome to overcome the rate-limiting step of second strand synthesis (McCarty *et al.*, 2001) has been shown to produce a higher level of gene expression more rapidly than vectors with a single stranded genome (McCarty *et al.*, 2003, Wang *et al.*, 2003, Ren *et al.*, 2005) (Figure 1.9). However this reduces the size of the expression cassette that can be used to only 2.3 kb.

1.7.7 Immune responses to AAV

There is virtually no innate immune response to AAV compared to that produced by Ad (Zaiss *et al.*, 2002). AAVs also induce a weaker cell-mediated immune response than Ads, probably because they less efficiently infect antigen presenting cells (Bessis *et al.*, 2004). However recent evidence suggests the activation of T-cells may be more of a problem than originally thought. Activation of T-cells has been implicated in liver toxicity seen in a clinical trial for haemophilia B (Manno *et al.*, 2006). It has also recently been shown that in both mice and non-human primates, intramuscular administration of AAV2 activated T-cells against the AAV2 capsid, where as AAV7 and AAV8 produced little T-cell activation (Vandenberghe *et al.*, 2006). Further work showed the T-cell activation was due to the heparin binding motif RGNR (residues 585-588), so it was proposed that HSPG-mediated uptake of AAV2 into dendritic cells (DC) leads to the activation of T-cells (Vandenberghe *et al.*, 2006). This was supported by evidence that showed vectors with a heparin binding motif bind DCs but those without the motif do not (Vandenberghe *et al.*, 2006). This suggests that vectors with peptides inserted after residue 587 can have both altered tropism and activate a reduced immune response compared to wild type AAV2, but this is peptide dependent.

The humoral response to AAV is also a problem. Between 50-96% of people have anti-AAV2 antibodies (Blacklow *et al.*, 1968), with 18-67.5% of these being neutralising (Chirmule *et al.*, 1999, Erles *et al.*, 1999, Moskalenko *et al.*, 2000). The effect of these on transgene expression from AAV vectors is under investigation. Repeated re-administration of AAV2 to rabbit lungs generated increasing levels of anti-AAV2 neutralising antibodies, which by the third dose reduced the level of transgene expression (Beck *et al.*, 1999). When a vector pseudotyped with AAV3 was used for the third dose transgene expression remained unchanged (Beck *et al.*, 1999). The same pattern of results was seen in a similar experiment in mice (Halbert *et al.*, 2000). It has been shown that if the animal is immunosuppressed at the time of the first delivery, then the same vector can be successfully readministered (Halbert *et al.*, 1998, Manning *et al.*, 1998).

Data from cystic fibrosis clinical trials has also shown that pre-existing anti-AAV2 antibody levels did not correlate with the amount of gene transfer produced (Aitken *et al.*, 2001, Wagner *et al.*, 2002). However, after receiving the AAV2-CFTR vector patients had higher levels of these antibodies and they prevented efficient transduction from subsequent administration of AAV2 (Peden *et al.*, 2004).

Capsid modifications that reduce the effect of neutralising antibodies have been identified. Peptide insertion into exposed loop regions of the capsid identified immunogenic domains around amino acids 534 and 573 and found that transduction of a virus with a peptide inserted at residue 587 was unaffected by the presence of human sera containing anti-AAV2 neutralising antibodies (Huttner, 2003). A recent study also showed that 2 mutants (R471A and N587A) were resistant to all human sera tested and identified other mutants that were resistant to some of the sera. From this 3 antigenic epitopes have been proposed, one at the heparin binding site including residue 587, another involving E548 and T550 (and possibly R471) at the side of the spike and a third involving residues N705 and V708 (Lochrie *et al.*, 2006). Two other antigenic regions involving amino acids 261 and 447 have previously been identified (Moskalenko *et al.*, 2000). As human sera contains a diverse range of antibodies against many different regions of the AAV2 capsid (Lochrie *et al.*, 2006), it is unlikely that just disrupting one

epitope will be sufficient to produce an antibody resistant virus suitable for use in the general population.

1.7.8 Safety concerns associated with AAV vectors

The potential for AAV vectors to mediate germ-line transmission has become a concern since the discovery of vector DNA in the semen of patients involved in a haemophilia clinical trial, (High *et al.*, 2004). However this was only detected transiently (High *et al.*, 2004) and in a previous study vector DNA was not detected in patient semen (Kay *et al.*, 2000). Animal studies in rats, rabbits and mice have detected AAV in the gonads following either myocardial, intramuscular or intravenous delivery of the vector (Pachori *et al.*, 2004b, Jakob *et al.*, 2005, Arruda *et al.*, 2001). However, no vector DNA was detected in sperm of their offspring (Arruda *et al.*, 2001, Jakob *et al.*, 2005) suggesting germ-line transmission was not occurring. Another study in mice has also shown that direct exposure of sperm to AAV does not result in germ-line transmission (Couto *et al.*, 2004). Therefore these studies suggest that the risk of germ-line transmission is low but will still require careful monitoring in clinical trials.

There are also concerns about the ability of AAV to integrate into the host cell genome. Although wild type AAV only integrates at a specific locus in the human genome, in some cases vectors have been found to integrate randomly due to the removal of Rep78 and Rep68 genes. However, more commonly the vector genome remains episomal (Nakai *et al.*, 2001, Schnepp *et al.*, 2003, Nakai *et al.*, 2003). Although this integration is a safety concern, studies have shown that it occurs only at a relatively low rate in the liver (Nakai *et al.*, 2001) and in skeletal muscle no integration could be detected (Schnepp *et al.*, 2003). Unlike retroviruses, integration of AAV vectors requires non-homologous end joining at existing chromosome break points (Miller *et al.*, 2004, Miller *et al.*, 2002) so it is less likely to occur. Recently a large-scale study looked for tumours in the livers of 695 mice that received AAV2 vectors and found only one tumor (Bell *et al.*, 2005) suggesting that AAV does not cause tumours, but vector integration will still require careful monitoring in clinical trials.

Currently over 20 phase I and II clinical trials have been performed using AAV vectors for treating diseases such as Parkinson's disease, Canavan's disease, α1 anti-trypsin deficiency, cystic fibrosis and hemophilia B (Carter, 2005). They have shown AAV to have a good safety profile as in more than 200 patients no serious side effects have yet been seen (Carter, 2005).

1.8 Targeting of viral vectors

One of the major challenges of gene therapy is the development of vectors that deliver the transgene to the target tissue type with a high degree of specificity. This is required to improve the efficiency of the vector when transduction of less permissive cell types is required, to prevent unwanted transgene in non-target tissues, to optimise the minimal dose required and therefore maximise the safety profile of the vector. Targeting can be achieved at the level of transduction, transcription or ideally both.

1.8.1 Transcriptional targeting

The most commonly used promoters in gene therapy vectors are based on the constitutive viral promoters from cytomegalovirus (CMV) and Rous sarcoma virus (RSV). They are active in a wide range of cell types so they are not ideal for producing tissue-specific transgene activity. Also, there are problems with transcriptional silencing of virus-based promoters, which limits the duration of transgene expression. For vascular-targeted gene therapy, several studies have shown that CMV and RSV promoters are relatively inefficient in vascular cells (Richter *et al.*, 2000, Gruchala *et al.*, 2004, Nitta *et al.*, 2005, Clesham *et al.*, 1996). It is also possible that transcriptional silencing of the CMV promoter may occur relatively quickly in the vasculature. A study comparing AAV transduction of the heart and the coronary artery in mice using a CMV promoter showed transgene expression was still relatively high in the heart 1 year after vector administration, but it was barely detectable in the artery after just 8 weeks, with gene expression having peaked after just 2 weeks compared to 8-26 weeks in cardiomyocytes (Vassalli *et al.*, 2003). Therefore other promoters may be more suitable for vascular-targeted gene therapy.

Several different promoters that are thought to possess endothelial specificity have been tested, including promoter sequences from fms-like tyrosine kinase-1 (Flt-1) (a receptor for VEGF), vascular endothelial growth factor receptor 1 (VEGFR-1), intracellular adhesion molecule (ICAM-2), von Willebrand factor (vWF), Tie 2, E-selectin and thrombomodulin (Melo *et al.*, 2004, Minami *et al.*, 2002, Aird *et al.*, 1995, Weiler-Guettler *et al.*, 1996, Cowan *et al.*, 1996, Cowan *et al.*, 1998). Nicklin *et al.* (Nicklin *et al.*, 2001b) compared the activity of Flt-1, ICAM-2 and vWF promoters in Ad vectors in ECs. Flt-1 and ICAM-2 produced expression levels equivalent to a CMV promoter but analysis in other cell types showed Flt-1has a higher degree of EC specificity than the ICAM-2 promoter (Nicklin *et al.*, 2001b). After systemic administration in mice the Flt-1 promoter produced a substantially lower level of transgene expression in the liver compared to the CMV promoter (Nicklin *et al.*, 2001b). A larger fragment of the Flt-1 promoter has also been shown to be upregulated by hypoxia, suggesting that under disease conditions its activity may be enhanced (Gerber *et al.*, 1997)

Gory *et al.* (Gory *et al.*, 1999) investigated using a 2.5 kb region of the promoter from VE-Cadherin and found it produced EC selective gene expression *in vivo*. However in some EC beds only a low level of expression is achieved. The efficiency of this promoter has been significantly improved by incorporating 4 kb of the 5' end of the first intron into the promoter system (Ilisatsune *et al.*, 2005).

As many eukaryotic promoters provide weaker transgene expression than viral promoters, another approach has been to use endothelial specific enhancer elements in combination with the murine leukemia virus promoter sequence. Enhancers from the promoters of preendothelin-1, E-selectin, ICAM-2, Flt-1 and vWF have produced enhanced specificity for EC in this system (Jaggar *et al.*, 1997, Mavria *et al.*, 2000, Richardson *et al.*, 2004).

There have also been some studies focusing on the development of vascular SMC specific promoters. The SM-22α promoter has been shown to be active in arterial but not venous SMC in mice The human vascular smooth muscle cell α-actin promoter is

active in vascular smooth muscle, cardiac and skeletal muscle when incorporated into a plasmid (Keogh *et al.*, 1999) or an Ad vector (Wills *et al.*, 2001). Another study combined the major immediate early murine cytomegalovirus enhancer/promoter (MIEmCMV), the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and fragments of the rabbit smooth muscle myosin heavy chain (SMMHC) promoter in an adenovirus vector (Appleby *et al.*, 2003). Compared to MIEmCMV alone the promoter increased transgene expression of SMC *in vitro* 90-fold and porcine coronary arteries *in vivo* 40-fold (Appleby *et al.*, 2003).

To produce disease-effective transcriptional control, vectors are being developed to produce gene expression regulated by conditions such as hypoxia, inflammation or increased shear-stress by combining a tissue specific promoter with regulated enhancer elements (Modlich *et al.*, 2000, Houston *et al.*, 1999). A retroviral vector with an expression cassette containing hypoxia response elements and endothelial cell specific enhancer elements from the kinase domain region (Flk-1) promoter increased transgene expression in endothelial cells in response to hypoxic conditions (Modlich *et al.*, 2000). A shear stress response element increased transgene expression *in vitro* and *in vivo* in response to shear-stress (Houston *et al.*, 1999). Studies focusing on treating ischemia have developed vectors which contain hypoxic response elements so transgene expression is more specifically localised to ischemic tissue (Su *et al.*, 2004, Pachori *et al.*, 2004a). These promoter systems may be utilised to target gene expression to only diseased areas of the vasculature to reduce potential side effects of unwanted gene expression in healthy tissue.

1.8.2 Transductional targeting

Three basic methods have been developed to target the transduction of viral vectors (Figure 1.10).

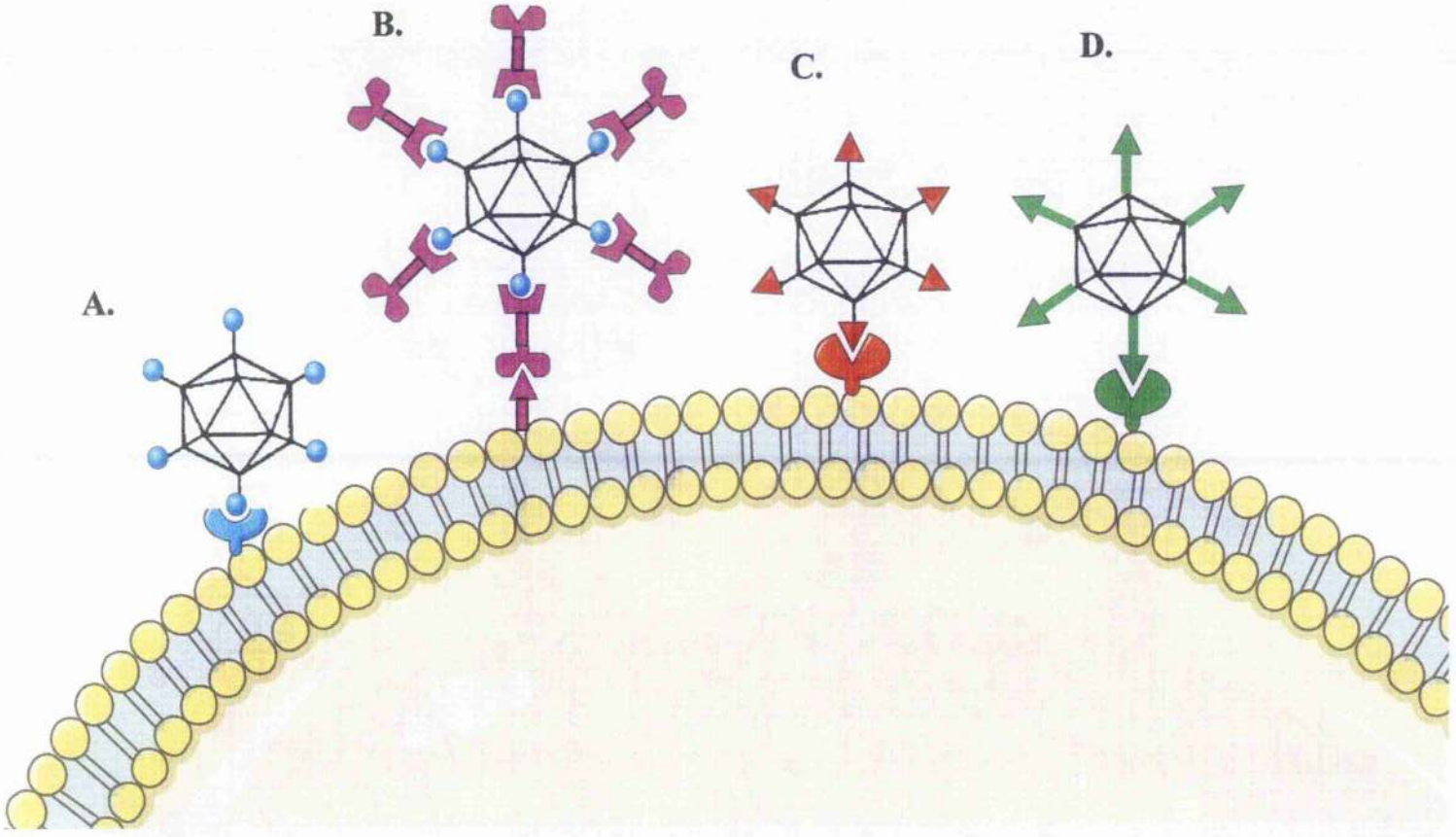


Figure 1.10 Techniques used to alter the tropism of Ad vectors. **A.** Natural method of cell attachment, with the fiber knob binding to the cellular receptor. **B.** Non-genetic targeting by coating the virus in a bispecific molecule that binds the virus capsid and the novel receptor. **C.** Ligand insertion into the fiber knob to enable the virus to bind an alternative receptor. **D.** Pseudotyping the virus by replacing part or all of the fiber protein with that of another serotype that utilises a different receptor.

1.8.2.1 Non-genetic targeting

Non-genetic modification of viruses provides the simplest way of altering vector tropism. It involves coating the virus in a bispecific molecule that binds to both the virus capsid and the novel receptor. Methods have been developed to utilise bispecific antibodies, antibody fragments and bispecific fusion proteins for re-targeting. One of the main advantages of using bispecific conjugates is that in most cases the natural tropism of the virus is eliminated due to steric hindrance.

Another modification strategy involves coating the virus with inert polymers such as polyethylene glycol (PEG) or poly[N-(2-hydroxy-propyl)methylacrylamide] (pHPMA). Ad vectors modified in this way have been found to have reduced toxicity, cause a reduced cell-mediated and humoral immune response (O'Riordan *et al.*, 1999, Croyle *et al.*, 2001, Croyle *et al.*, 2005) and have a longer plasma circulation time than unmodified Ad (Fisher *et al.*, 2001, Green *et al.*, 2004). However it also significantly reduces the efficiency of transduction due to steric hindrance, for example, PEGylation reduced Ad5 transduction of A549 cells (human lung carcinoma cells) 200-fold (Eto *et al.*, 2005). Conjugating a targeting ligand to the PEG can increase transduction and enables targeting of the vector (Romanczuk *et al.*, 1999, Fisher *et al.*, 2001). For example, a PEG and FGF2 coated Ad has been shown to infect cells via FGF2 receptor in a CAR independent manner, to increase transduction of tumour cells in a model of ovarian cancer and reduce transduction of non-target cells in other organs (Lanciotti *et al.*, 2003). Also, an E-selectin specific antibody combined with PEG has been used to target activated endothelial cells both *in vitro* and *in vivo* (Ogawara *et al.*, 2004).

One of the problems with non-genetic targeting is that it can be difficult to produce clinical grade uniform preparations of the complex without batch variation. Genetic retargeting may therefore provide a more reliable and attractive vector from a regulatory standpoint.

1.8.2.2 Pseudotyping

As different serotypes have slightly different caspids and therefore different cellular tropisms it is possible to exploit these differences to develop gene therapy vectors targeted to different cell types. Pseudotyping involves replacing part or all of the capsid of one serotype with that of another serotype to produce a vector with a novel tropism. For enveloped viruses pseudotyping is achieved by incorporating alternative envelope glycoproteins into the envelope.

1.8.2.3 Genetic targeting of viral vectors

Genetic modification of the virus capsid can be used to both detarget the virus from its native tropism and retarget it to a different cell type. Detargeting can be achieved by mutating residues in the capsid that are known to be involved in the virus binding to native receptors. Retargeting can be achieved by inserting peptides that bind to alternative cellular receptors into the capsid of viral vectors to alter their tropism. To be successful, the peptide needs to be incorporated into an exposed region of the capsid so that it is accessible for cellular binding and it needs to be in a locale that has no detrimental affect on capsid assembly or function. This requires both detailed knowledge of the capsid structure and the identification of efficient targeting peptides.

1.9 Phage display

Phage display is the most common method used to identify targeting peptides when there is no known suitable ligand. The technique was first used by Smith *et al.* (Smith, 1985) to identify peptides that bound to a specific monoclonal antibody, since then it has been developed to have a wide range of applications. For example, it can be used to identify peptides that bind to a specific target molecule, cell or tissue *in vitro* or *in vivo*. Phage display (also known as biopanning) involves screening large numbers of random peptides (known as libraries) expressed on the surface of bacteriophage to identify peptides that have a high binding affinity for a specific target. It is a very powerful technique as the libraries used can have a very high diversity (dependent on peptide length), with as many as 10^9 different peptides being displayed (Scott and Smith, 1990).

1.9.1 *In vitro* phage display

The basic method of *in vitro* biopanning involves incubating the phage library with the target (either a protein or a cell type), then the target is washed to remove any unbound phage. Phage that remain bound to the target are eluted and amplified before being reapplied to the target. This is repeated for several cycles to produce a pool of phage enriched in peptides that bind to the target with a high affinity. Sequencing of the phage genome is used to identify the targeting peptide motifs.

One of the most successful *in vitro* biopanning experiments identified the integrin binding peptide ACDCRGDCRCG (RGD4C) (Koivunen *et al.*, 1995). This peptide forms 2 disulphide bonds and was shown to have a 200-fold higher affinity for integrins than other linear RGD containing peptides that bind integrins (Koivunen *et al.*, 1995). It has subsequently been used in several *in vivo* studies. After intravenous administration into a mouse model of breast cancer the peptide was found to target the tumour cells more efficiently than the brain or the kidney (Pasqualini *et al.*, 1997). Coupling the peptide to the chemotherapeutic drug doxorubicin was found to increase the potency of the drug in a mouse model of breast cancer (Arap *et al.*, 1998). The peptide has also been used to target a number of viral and non-viral vectors to tumours (Seki *et al.*, 2002, Mitra *et al.*, 2005, Yamamoto *et al.*, 2006, Line *et al.*, 2005, Stachler and Bartlett, 2006) and SMC (Work *et al.*, 2004b).

Although *in vitro* phage display has been used to identify some useful molecular markers, it has several disadvantages. *In vitro* many tissue specific markers are no longer expressed so the cultured cell phenotype is not the same as *in vivo* (Borsum *et al.*, 1982). It has been shown that 40% of rat lung endothelial cell surface proteins cannot be detected when the cells are grown *in vitro* (Durr *et al.*, 2004). Also, some molecules that might not normally be accessible from the bloodstream may be bound during *in vitro* phage display.

1.9.2 *In vivo* phage display

In vivo phage display can be used to overcome some of the problems described for *in vitro* phage display. It ensures the identified binding region of the target receptor is accessible to the circulation and it combines a form of negative selection as phage with a higher specificity for other targets (e.g. ubiquitously expressed receptors) will bind preferentially to them. It also ensures that the phage selected is stable in the blood and not rapidly degraded. As well as identifying targeting peptides, phage display has also been used to better characterise protein expression on the endothelium.

The basic *in vivo* phage display method was developed by Pasqualini and Ruoslahti (Pasqualini and Ruoslahti, 1996) (Figure 1.11). The library was injected intravenously into the animal and the phage left to circulate for approximately 5 minutes before animals were perfused. Perfusion is an important step as it removes phage that are bound non-specifically or with only a weak affinity for the target and removes phage that remain in the blood (Rajotte *et al.*, 1998). Tissues of interest are removed and phage are extracted for analysis. With longer circulation times, phage that internalise into cells can be specifically identified (Barry *et al.*, 1996). Repeated rounds of biopanning produce a restricted library with a high degree of selectivity for the target tissue.

This basic method can be modified to include additional steps, such as an initial *ex vivo* step involving biopanning on cells from the target tissue to produce an enriched library for *in vivo* screening (Hoffman *et al.*, 2004). This pre-selection can reduce the number of *in vivo* rounds required and also seems to identify peptides which are more efficiently internalised into the target cells (Ruoslahti, 2004). Also, the delivery of non-infectious phage before delivery of the library can be used to saturate binding sites in the reticulo-endothelial system to prevent non-specific uptake of phage (Hoffman *et al.*, 2004).

The first described example of *in vivo* phage display was carried out in mice (Pasqualini and Ruoslahti, 1996). After 3 rounds of biopanning peptides that were selective for the brain and the kidney endothelium were isolated (Pasqualini and Ruoslahti, 1996). This suggested that the endothelium of different vascular beds expressed unique molecular

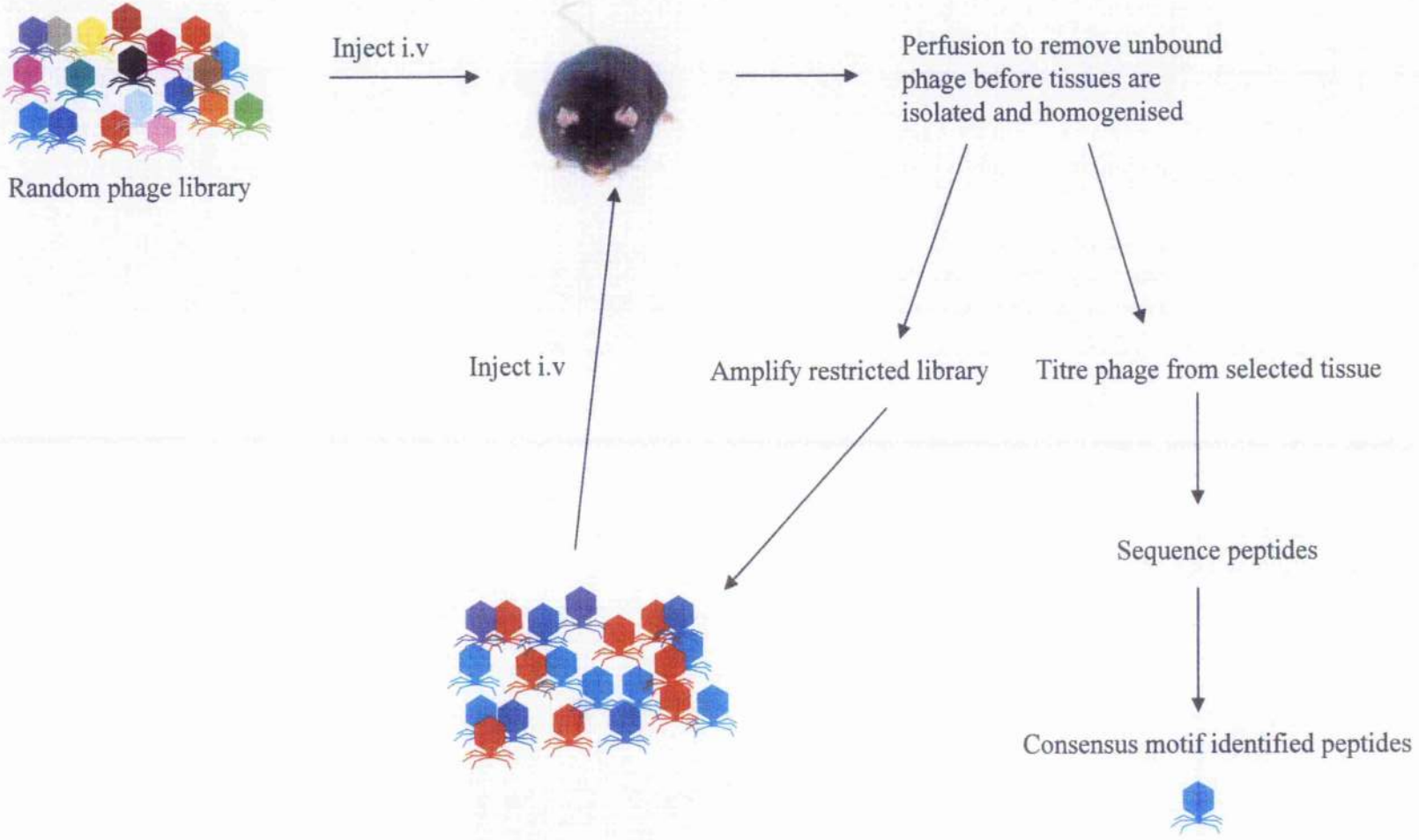


Figure 1.11 Diagram illustrating *in vivo* biopanning. A random phage library is injected intravenously into the animal and left to circulate for about 5 minutes before the target tissues are harvested and bound phage isolated. Isolated phage are re-amplified and the process is repeated until titering and sequencing show a consensus motif has been identified.

markers and that these could be exploited for tissue-specific targeting. Further *in vivo* biopanning in mice has identified unique molecular markers on the endothelium of the lung, pancreas, intestine, uterus, adrenal gland, retina (Rajotte *et al.*, 1998) and muscle (Samoylova and Smith, 1999). These tissue specific molecular markers have become known as the 'vascular address system' (or ZIP code) as they can be exploited to target vectors to specific tissues with a high level of selectivity. EC gene expression varies between different vascular beds as in different tissues the EC are exposed to different environments e.g. varying oxygen concentrations in the blood and are required to carry out different functions depending on their location (Minami and Aird, 2005, Lacorre *et al.*, 2004, Aird, 2004).

The vasculature of diseased tissues also expresses unique molecular markers. Biopanning has now been performed to identify peptides that bind to several types of cancers including nasopharyngeal cancer (Lee *et al.*, 2004b), prostate cancer (Arap *et al.*, 2002a) and breast cancer (Essler and Ruoslahti, 2002). It has also been used to identify peptides that target pancreatic and skin tumour cells at different stages of the tumour development (Hoffman *et al.*, 2003), demonstrating that malignant and pre-malignant tissues express different molecular markers. This highlights the high level of selectivity that can be achieved using biopanning.

However, there are also disadvantages to *in vivo* phage display. Using animal models can result in the identification of peptides that bind to a receptor that is not well conserved between species so might not bind to the human form of the protein. Or, if the disease model used is not accurate the receptor may not actually be expressed in the human disease state. For example, prostate-specific membrane antigen is expressed in human prostate cancer and human tumour vasculature but in the mouse it is normally expressed in the brain and kidney (Arap *et al.*, 2002b). So it is essential that an accurate animal model is used and the peptides are tested across a range of species wherever possible. However species-specific peptides do not seem to be a common occurrence (Ruoslahti, 2002).

In vivo phage display has been carried out in humans (Arap *et al.*, 2002a). The first example of this was in a patient with a B cell tumour that had been pronounced brainstem dead. 4716 peptide motifs were isolated from different organs and analysed to identify tri-peptide motifs that were commonly found in each organ (Arap *et al.*, 2002a). However, due to the diversity of the human population, one round of biopanning in one person is not sufficient to draw many conclusions and there are obvious ethical problems in expanding this kind of study. Recently a phage display study in 8 patients with breast, melanoma and pancreatic cancers was carried out to isolate tumour-targeting phage (Krag *et al.*, 2006). In 3 of the patients 2-3 rounds of biopanning were possible (Krag *et al.*, 2006). Repeated peptides and some consensus motifs within peptides were identified, some of which were found to have homology to known human proteins that have been associated with cancer (Krag *et al.*, 2006). Some of the phage identified were tested to see if they bound to corresponding cancer cell lines, but in most cases no binding or reduced binding was seen compared to cells from the original tumour (Krag *et al.*, 2006). Therefore it is important that these peptides are tested further to check that they are not patient-specific.

To try and reduce the numbers of both animals and humans required for *in vivo* biopanning Kolonin *et al.* (Kolonin *et al.*, 2006) have developed a technique where by one animal can be used to identify phage that target several tissues. The method involved statistical analysis of large numbers of peptides to identify tripeptide motifs (Kolonin *et al.*, 2006). To overcome ethical problems with performing phage display in humans, biopanning has also been carried out on human tissues transplanted into mice. For example, this has been successfully performed with synovial tissue from arthritis patients (Lee *et al.*, 2002).

1.10 Targeting of lentivirus vectors

Lentivirus vectors used for gene therapy are commonly based on human immunodeficiency virus (HIV), which has a natural tropism for CD4+ expressing T-cells. To broaden the range of cells that can be infected by the vector, pseudotyped viruses with

the vesicular stomatis virus glycoprotein (VSV-G) incorporated into the virus envelope have been produced (Cronin *et al.*, 2005).

Several studies have compared the level of transduction of vascular cells achieved by lentiviral and other viral vectors. Dishart *et al.* (Dishart *et al.*, 2003) showed an HIV-1 vector pseudotyped with VSV-G produced better transduction of human saphenous vein SMC and EC than AAV serotypes 2, 3, 4, 5 and 6. Comparing this vector to Ad5 vector showed that both human coronary artery ECs and human coronary artery SMCs were also more efficiently transduced by the lentivirus (Cefai *et al.*, 2005). Although these results seem promising for producing vascular-targeted vectors, due to the broad tropism of VSV-G modified vectors (Follenzi *et al.*, 2002), following systemic delivery *in vivo* they are unlikely to produce selective transduction of the vasculature, but may be utilised for local delivery.

To increase the selectivity of these vectors for ECs the use of different promoters has been investigated. Comparing promoter sequences from Tie1, Tie2, Flk-1, VE-Cadherin and ICAM-2 genes in a VSV-G modified vectors showed that the Tie2 promoter and enhancer sequences provided the best specificity for endothelial cells *in vitro* and tumour endothelial cells *in vivo* in a mouse model (De Palma *et al.*, 2003). This demonstrated that transcriptional targeting could be used to increase the selectivity of VSV-G modified lentiviruses administered systemically.

Another study has investigated the transduction of endothelial cells with a lentivirus vector containing a Hantavirus glycoprotein (HTNV-pseudotyped lentivirus)(Qian *et al.*, 2006). *In vivo* studies in balloon injured carotid arteries of rabbits showed that the HNTV-pseudotyped lentivirus produced significantly higher levels of reporter transgene expression than either VSV-G pseudotyped lentivirus or adenoviral vectors following local administration (Qian *et al.*, 2006). When HTNV-pseudotyped lentivirus expressing human extracellular superoxide dismutase (EC-SOD) was administered to rabbits with balloon injured carotid arteries a significant reduction in the intimal/media ratio was seen 6 weeks after administration, indicating a reduction in stenosis (Qian *et al.*, 2006). These

studies suggest that target lentiviruses may be developed into vectors that can be used for cardiovascular gene therapy.

1.11 Targeting Ad vectors

1.11.1 Non- genetic retargeting of Ad

There are many studies that have used non-genetic targeting techniques to successfully modify the tropism of Ad5 *in vitro*. The first proof of concept used an anti-knob neutralising antibody fragment (Fab) conjugated to folate to retarget the vector to a folate receptor over-expressed on cancer cells (Douglas *et al.*, 1996). Similar methods have been used to target other molecules including FGF receptor on Kaposi's sarcoma cells (Goldman *et al.*, 1997), epidermal growth factor (EGF) receptor (Miller *et al.*, 1998) and CD40 on dendritic cells (Tillman *et al.*, 1999). Another system has used soluble CAR protein fused to an anti-CD40 antibody or EGF to retarget the virus to CD40 (Pereboev *et al.*, 2002) and EGF receptor respectively (Dmitriev *et al.*, 2000).

There are several examples of non-genetic targeting of Ad to vascular cells. A single chain variable fragment (scFv, a fusion of the variable regions of the heavy and light chains of an antibody) has been used to target Ad5 to endothelial cells by creating a fusion protein of an EC targeting peptide SIGYPLP (identified by phage display) with an anti-Ad scFv (Nicklin *et al.*, 2000). This increased transduction of ECs by about 15-fold compared to unmodified Ad (Nicklin *et al.*, 2000). Wickham *et al.* (Wickham *et al.*, 1996) used a bi-specific antibody that binds to the Ad penton base and α_v integrins to increase Ad5 transduction of both vascular EC and SMC and Harari *et al.* (Harari *et al.*, 1999) used the a bi-specific antibody to target Ad5 to E-selectin to generate a vector which targeted activated ECs. Although these studies have demonstrated the production of vectors with altered tropism *in vitro*, *in vivo* testing is required to fully validate these systems.

One of the first examples of using this technology *in vivo* used the 9B9 antibody that binds to the Ad5 knob and angiotensin converting enzyme (ACE) to target Ad vectors to the pulmonary endothelium *in vivo* following i.v. delivery (Reynolds *et al.*, 2000). This

produced 20-fold higher transgene expression in the lung compared to untargeted vector but levels of virus in the liver remained relatively high (Reynolds *et al.*, 2000). To increase the specificity of the vector the viral promoter was replaced with the Flt-1 promoter. This resulted in a 15-fold increase in transgene expression in the lung and a 67% reduction in liver expression, and demonstrated that transcriptional and transductional targeting can be combined to have a synergistic effect on transgene expression *in vivo* (Reynolds *et al.*, 2001). The efficiency of the vector carrying endothelial nitric oxide synthase (eNOS) transgene was tested in stroke-prone spontaneously hypertensive rats (SHRSP) to determine if the level of targeting achieved was sufficient to have a therapeutic effect. Following systemic administration of the vector, gene expression was predominantly observed in the lung vasculature, and this had a significant hypotensive effect whereas an untargeted Ad5 produced no change in blood pressure (Miller *et al.*, 2005). This was the first study to demonstrate a therapeutic effect achieved using a targeted viral vector *in vivo*.

1.11.2 Genetic retargeting of Ad

The HI loop is an exposed region on the surface of the fiber knob of Ad5 (Figure 1.6B and 1.6C). It consists mainly of hydrophilic amino acids that connect β-strands H and I (Xia *et al.*, 1994). This loop region is not involved in any intramolecular interactions within the knob so insertion of additional amino acids into this region was thought to be feasible without affecting fiber trimerisation. Insertion into the HI loop results in the incorporation of 36 copies of the peptide into each virion.

One of the first examples of genetically retargeting Ad was performed by Wickham *et al* (Wickham *et al.*, 1997). Several targeting peptides (including an RGD peptide for integrin binding and KKKKKKK (K_7) for heparin binding) were inserted at the C terminus of the knob to expand the tropism of the virus (Wickham *et al.*, 1997). The RGD modified virus produced higher levels of transduction of EC and SMC and the K_7 modified virus produced between 5-550 fold increase in transduction of several cell lines including EC, SMC, macrophages and fibroblasts (Wickham *et al.*, 1997). Dmitriev *et al.* (Dmitriev *et al.*, 1998) inserted an RGD peptide into the HI loop of Ad5 and showed this

increased transduction of human umbilical vein ECs and human ovarian cancer cells independently of CAR. Since these first studies were published many different targeting peptides have been inserted into the HI loop (commonly after residue T542) to expand the tropism of Ad vectors and enable enhanced transduction of a range of cells. It has since been shown that insertions of up to 100 amino acids can be tolerated in this position (Belousova *et al.*, 2002).

This technology has also been used *in vivo*. For example, local administration into the tumours of a B16 mouse melanoma model of a vector with RGD inserted into the HI loop produced a 40-fold increase in transgene expression in the tumour and an 8-fold decrease in expression in the liver (Mizuguchi and Hayakawa, 2002). However, due to the broad expression of integrins this vector would be unsuitable for systemic delivery to produce highly selective transgene expression, but it may have applications for *ex vivo* gene therapy. For example Hay *et al.* (Hay *et al.*, 2001) showed that insertion of an RGD peptide into the HI loop of Ad5 increased transduction of EC in rabbit jugular veins *ex vivo* to produce a 4-fold increase in gene expression.

Although the majority of studies use the HI loop for peptide insertion other sites have been investigated. Hexon is the most abundant capsid protein, so peptide insertion into it results in display of the highest number of copies of peptide (720); therefore it could enable highly efficient retargeting. Hexon is highly conserved between Ad serotypes but there are hypervariable loop regions that extend from the surface of the virus. Insertion of an 11-mer RGD containing peptide into hypervariable region 5 of the Ad5 hexon resulted in a 30% increase in transduction of vascular SMC using 10-fold less virus (Vigne *et al.*, 1999). This demonstrated that hexon can tolerate peptide insertion and that it can be used to alter the virus tropism.

Eighty trimers of the minor capsid protein pIX are incorporated into the capsid to stabilise hexon-hexon interactions (Vellinga *et al.*, 2005). It is possible to insert relatively large peptides into the surface exposed C-terminus of pIX (Dmitriev *et al.*, 2002, Meulenbroek *et al.*, 2004). To extend the peptide away from the virus surface so

that it is more exposed, an α helical spacer is incorporated with the peptide (Vellinga *et al.*, 2004). However, as this position is less well exposed than the HI loop it is unlikely to provide such efficient targeting.

Although these studies have demonstrated that it is possible to genetically alter the tropism of Ad vectors *in vitro*, for many gene therapy vectors a highly specific gene therapy vector is required. Therefore, as well as expanding the tropism of the virus to target the vector to a novel cell type, ablation of the natural vector tropism is also required. Targeting of Ad vectors could enable tissue specific transgene expression following systemic administration of the vector, could reduce vector dissemination and toxicity and enable the use of lower doses of vector. In addition to this, fiber modified viruses do not provoke as strong as immune response as unmodified vectors (Schoggins *et al.*, 2005).

1.11.3 Detargeting Ad vectors

Early attempts at ablating CAR binding to reduce the natural tropism of Ad5 involved the replacement of the fiber knob with a trimerisation domain to enable fiber formation and targeting ligands such as a the myc epitope, a 6-His tag or RGD peptide (van Beusechem *et al.*, 2000, Magnusson *et al.*, 2001). Kransyth *et al.* (Krasnykh *et al.*, 2001) deleted the entire fiber knob and shaft except the N-terminal 2 amino acids and replaced it with a truncated form of bacteriophage T4 fibritin protein linked to a 6-His tag. This vector no longer interacted with CAR and produced a 100-fold increase in transduction of cells expressing a 6-His receptor (Krasnykh *et al.*, 2001).

The residues in the knob domain that mediate binding to CAR were identified by alignment of sequences from 14 Ad serotypes that bind CAR to find conserved residues (Roelvink *et al.*, 1999). Site directed mutagenesis and transduction studies in A549 cells (that express CAR) were used to identify the critical CAR binding residues (Roelvink *et al.*, 1999). It was concluded that the main CAR binding site consists of residues in the AB loop (S408, P409, K417), the B β sheet (K420) and the DE loop (Y447), and that residues in the FG loop (mainly Y491) are indirectly involved (Roelvink *et al.*, 1999).

The results of this, other mutational studies (Kirby *et al.*, 1999, Kirby *et al.*, 2000) and the solving of the crystal structure of Ad12 bound to CAR (Bewley *et al.*, 1999) confirmed these residues were important residues for CAR binding. This led to the development of CAR-binding ablated forms of Ad5, such as AdKO1 that has the point mutations S408E and P409A. These have been shown to reduce viral infectivity by about 99% *in vitro* (Jakubczak *et al.*, 2001).

By combining genetic retargeting strategies with the CAR ablating mutations it is possible to generate vectors with novel, highly specific tropisms. Nicklin *et al.*(Nicklin *et al.*, 2001c) generated a modified virus that was both CAR binding ablated and retargeted by combining the KO1 mutations with insertion of the EC targeting peptide into the HI loop (Figure 1.6). This virus transduced human umbilical vein EC and human saphenous vein EC but not vascular SMC more efficiently than AdKO1, showing that the virus had some selectivity for ECs (Nicklin *et al.*, 2001c).

In a similar study 2 12-mer peptides identified by phage display performed on human umbilical vein ECs were inserted into the HI loop of Ad5 in combination with the KO1 mutations. The vectors were found to be selective for human umbilical vein ECs but not human aortic ECs, human saphenous vein SMCs or human fibroblasts showing that the cellular selectivity of the peptides was maintained after the peptides were inserted into the HI loop (Nicklin *et al.*, 2004). But following systemic administration, both viruses produced high levels of transgene expression in the liver even though the *in vitro* tropism had been shown to be independent of CAR binding (Nicklin *et al.*, 2004). Despite the high level of liver transduction the viruses were still found to be selective for venous ECs compared arterial ECs (Nicklin *et al.*, 2004), so they may be useful for local gene delivery e.g. to vein grafts.

1.11.4 *In vivo* modification of Ad tropism

As demonstrated by the previous example, attempts to modify the tropism of Ad vectors have shown that although binding to CAR can have an important role in Ad transduction *in vitro*, *in vivo* its role is less certain. Several studies that have tried to ablate the natural

tropism of Ad by mutating CAR binding residues have shown that *in vivo* this does not have a great affect on reducing hepatotropism (Table 1.5). Therefore to produce a truly retargeted virus with a highly selective tropism *in vivo*, additional mutations are required.

Mutation of the RGD motif in the penton base that is involved in integrin binding shows it is not vital for infection *in vitro*, although it does cause virus internalisation to occur at a much slower rate (Bai *et al.*, 1993), and it reduces the efficiency of endosomal escape (Shayakhmetov *et al.*, 2005a). It is not known whether the mutated virus uses an integrin independent method of internalisation or if a different part of the capsid is able to interact with the integrin. It has also been shown that blocking virus interaction with HSPGs can significantly reduce transduction *in vitro* (Dechecchi *et al.*, 2000, Dechecchi *et al.*, 2001). As a result of these studies, combinations of mutations that block interactions with CAR, integrins and HSPGs have been tested in a number of *in vivo* studies.

Einfield *et al.* (Einfeld *et al.*, 2001) found that combining CAR binding mutations and mutation of the RGD motif caused a 700-fold decrease in liver tropism in mice, whereas mutating CAR or RGD alone caused only 10- and 20-fold decreases in liver transduction, respectively. In rats mutation of RGD was found to cause a 30% decrease in transduction and in combination with the CAR mutation caused a 99% decrease in transduction (Nicol *et al.*, 2004). However, in primates mutation of RGD alone had no affect on liver transduction, although transduction of all other tissues tested was significantly decreased (Smith *et al.*, 2003a). These studies have shown that combining mutations to block binding to both CAR and integrins synergistically reduced transduction, but the extent of this varies in different species.

Investigating the effect of blocking HSPG interaction *in vivo* showed that mutation of the putative binding domain KKTK (residues 91-94 in the 3rd repeat of the fiber shaft) to GGAG (known as the S* mutation) (Figure 1.6) caused a 15-fold decrease in liver transduction following systemic administration in mice (Smith *et al.*, 2003b). Combining the heparin binding mutation with the CAR binding mutation (KO1) reduced liver transduction 1000-fold (Smith *et al.*, 2003b). A similar study compared the

Mutation	Tropism compared to unmodified Ad5 following intravenous injection	Reference
Y477A mutation in DE loop	There was no difference in vector biodistribution in mice.	(Alemany and Curiel, 2001)
S408E mutation in AB loop	There was no difference in vector biodistribution in mice.	(Leissner *et al.*, 2001)
Mutation of TAYT in the FG loop	There was no difference in vector biodistribution in mice.	(Mizuguchi *et al.*, 2002)
S408E and P409A in AB loop	No decrease in liver transduction in non-human primates.	(Smith *et al.*, 2003a)
S408E and P409A in AB loop	In rats there was no decrease in liver transduction.	(Nicol *et al.*, 2004)
R412S, A425G, E416G and K417G in AB loop	10-fold decrease in liver transduction in mice.	(Einfeld *et al.*, 2001)
K420A in B β sheet of the fiber knob	9-fold decrease in liver transduction in mice.	(Yun *et al.*, 2005)
P409E and K417A in AB loop, in combination with mutation of RGD in the penton base	There was no difference in vector biodistribution in mice.	(Martin *et al.*, 2003)
S408E and P409A in AB loop	In mice there was no decrease in liver transduction.	(Smith *et al.*, 2002, Smith *et al.*, 2003b)

Table 1.5 The affect of CAR ablating mutations on *in vivo* tropism of Ad5.

biodistribution profiles of AdKO1, AdS* and AdKO1S* after systemic administration to cynomolgus monkey (Smith *et al.*, 2003a). Only the AdS* and AdKO1S* vectors showed reduced accumulation in the liver compared to unmodified Ad5, and they also showed reduced amount of virus in all other tissues tested including the spleen, lung, kidney and heart. A similar study in rats supported this work as it showed that the KO1S* mutations caused a 95-fold decrease in liver transduction although the S* mutation alone caused just a 2-fold decrease in liver transduction (Nicol *et al.*, 2004).

These studies confirmed that *in vivo* CAR binding is not a significant factor in mediating Ad transduction, but that by combining CAR ablating mutations with mutation of the KKTK or RGD motif does significantly reduce Ad infectivity.

Koizumi *et al.* (Koizumi *et al.*, 2003) used a slightly different approach to produce a detargeted virus by combining a CAR ablated Ad5 knob with, the shorter fiber shaft of Ad35 (that has no KKTK motif) and a mutant Ad5 penton base without an RGD motif. The double mutant virus was found to produce a 270-fold decrease in liver transduction following systemic administration in mice, however in combination with the Ad35 fiber this caused a 300,000-fold decrease in liver transduction (Koizumi *et al.*, 2003). However, both vectors were cleared from the blood very rapidly (Koizumi *et al.*, 2003), so the lack of transgene expression was likely to be due to degradation of the vectors. No retargeting of this vector has been demonstrated.

Due to discrepancies in CAR expression levels and virus biodistribution profiles (Fechner *et al.*, 1999), and the lack of effect seen on *in vivo* tropism by mutating CAR, the mechanism of Ad transduction *in vivo* is currently under debate and the role of CAR binding is being questioned. Recent studies have proposed that an interaction between Ad and blood coagulation factors might have a more significant role in determining Ad tropism. Shaykhmetov *et al.* (Shayakhmetov *et al.*, 2005b) showed that coagulation factor IX (FIX) and complement component C4-binding protein (C4BP) bind to the virus and to cellular HSPG and LDL receptor related protein (LRP) to act as a bridge between the virus and cell, mediating virus uptake into hepatocytes and Kupffer cells. A virus

unable to bind CAR, C4BP and FIX (with knob mutations Y447A, deletion of TAYT in the FG loop and insertion of a 12 amino acid peptide in the HI loop at position 547) was found at a 50-fold lower level in the liver of intravenously injected mice compared to unmodified Ad5 (Shayakhmetov *et al.*, 2005b). Another recent study showed that Ad5 hepatocyte transduction both *in vitro* and *in vivo* is enhanced by virus binding to the vitamin K-dependent coagulation factors FX, protein C, FVII and FIX (Parker *et al.*, 2006). *In vivo* warfarin was used to down-regulate the coagulation factors and this resulted in significantly reduced liver transduction that could be restored by injection of FX (Parker *et al.*, 2006). Direct binding of FX to both unmodified Ad5 and AdKO1 was also demonstrated (Parker *et al.*, 2006). These studies have provided important information about alternative mechanisms of Ad transduction *in vivo* that can be used to further develop more selective Ad vectors. By blocking coagulation factor binding and therefore uptake into Kupffer cells, the immune response to Ad vectors should be reduced and the selectivity of targeted vectors could be improved.

Although there are many examples of vector modifications that increase transgene expression in the target tissue, as yet there have no been no examples of peptide targeted Ads that have also been successfully detargeted from the liver following systemic administration. To improve the degree of detargeting it is likely that additional detargeting mutations will be required e.g. to block binding to coagulation factors. It may also be improved by incorporating targeting peptides with a stronger affinity for their receptor and peptides that also enhance virus internalisation and/or trafficking.

1.11.5 Pseudotyping Ad

Pseudotyping of Ad most commonly involves replacing the entire fiber or part of the fiber of one Ad serotype with that of another. Normally Ad5 is used as the basis of the vector as it is the best characterised and can be produced efficiently. The technique was first described by Kransyth *et al.* (Krasnykh *et al.*, 1996) who produced an Ad5 vector with the knob of Ad3.

Several studies have analysed vascular cell transduction mediated by pseudotyped vectors (Table 1.6). Although many of these studies have identified pseudotyped viruses that more efficiently infect vascular cells than Ad5, *in vivo* many of the viruses have a broad tropism therefore would require further modifications to be developed into highly selective vectors.

Generally, subgroup B viruses transduce primate vascular cells more efficiently than Ad5, but there seem to be species-specific factors that suggest they are less efficient in rat and pig cells (Havenga *et al.*, 2001). CD46, the receptor for most group B viruses (Gaggar *et al.*, 2003) is not expressed in mice except in the testes so they are unlikely to provide a good platform for developing vascular targeted gene delivery vectors in animal models. Also CD46 is a membrane bound protein involved in complement regulation and is expressed on all nucleated human cells, so is therefore unlikely to provide the required level of selectivity. For example, although Ad5/35 and Ad5/11 have been shown to transduce ECs more efficiently than Ad5 (Havenga *et al.*, 2001), following intravenous injection into baboons the viruses were detectable in all major organs including liver, spleen, heart, brain and bone marrow (Ni *et al.*, 2005).

As yet, no pseudotyped viruses that selectively transduce vascular cells have been produced, but one of the more promising vectors is Ad5 pseudotyped with Ad19p, which has been shown to have a reduced tropism for rat liver *in vivo* and mouse, rat and human hepatocytes *in vitro* (Denby *et al.*, 2004). It also transduces human endothelial cells and smooth muscle cells at least as efficiently as Ad5 (Denby *et al.*, 2004). With additional genetic modifications to improve its selectivity it may be possible to develop it into a highly efficient vascular-targeted vector.

One of the main problems with pseudotyping is that it can reduce the titre of vector achieved (Kreppel *et al.*, 2005). However it has the advantage of producing vectors to which the population has a lower level of pre-existing antibodies. Although administration of Ad5/35 and other pseudotyped vectors have been shown to induce anti-

Vector	Result	Reference
Ad5 with the Ad3 fiber (Ad5/3)	Increased transduction of human vascular SMC .	(Su *et al.*, 2001)
Ad5, 5/12, 5/16, 5/28, 5/40L (groups C, A, B, D, F respectively)	Ad5/16 produced the highest level of transduction (10-100 fold higher than Ad5) of SMC from human umbilical vein, iliac artery, left interior mammary artery, aorta and saphenous vein. However in rat and pig SMC Ad5 transduction was 100 and 5-10 fold higher than Ad5/16, showing species specificity. Ad5/16 produced higher levels of transduction than Ad5 in human vessels *ex vivo*.	(Havenga *et al.*, 2001)
Ad5 pseudotyped with group B viruses 11, 35 and 41	Pseudotypes transduced human ECs more efficiently than Ad5.	(Havenga *et al.*, 2001)
Ad2 pseudotyped with Ad17 fiber	More efficient transduction of human umbilical vein ECs than Ad2.	(Chillon *et al.*, 1999)
Ad11 (group B), 4 (group E), 41 (group F)	Infect human microvascular ECs more efficiently than Ad5	(Zhang *et al.*, 2003)
Ad5 pseudotyped with Ad37 or Ad19p (group D)	Do not transduce human, mouse or rat hepatocytes as efficiently as Ad5 but transduce human EC and SMC at least as efficiently as Ad5.	(Denby *et al.*, 2004)

Table 1.6 Transduction of pseudotyped Ads in vascular cells.

Ad5 antibodies (Ophorst *et al.*, 2004, Gall *et al.*, 1996) as both hexon and fiber are targets for neutralising antibodies (Sumida *et al.*, 2005, Vigne *et al.*, 2003).

1.12 Targeting AAV

1.12.1 Targeting AAV using bi-specific molecules

Bi-specific antibodies, which bind to both the AAV2 capsid and an alternative receptor on the target cell surface, have been used to retarget AAV2 vectors. Bartlett *et al.* (Bartlett *et al.*, 1999) carried out the first proof of concept using a bi-specific antibody that targeted the virus to integrins. *In vitro*, this was shown to expand the tropism of AAV2 as it did not inhibit the natural tropism of the virus and it enabled infection of normally non-permissive human megakaryocytic cells (Bartlett *et al.*, 1999).

A more advanced strategy involved insertion of an IgG binding domain into the capsid of AAV2 at residue 587 to create a vector that could be used to test the targeting capacity of different ligands (Ried *et al.*, 2002). Antibodies against α1 integrin, c-kit stem cell receptor and CXCR4 were used to target the virus to M-07e, Jurkat and Mec1 cells with a high degree of selectivity, although the level of transduction remained lower than that seen with AAV2 (Ried *et al.*, 2002). The low efficiency of the vectors may have been due to the insertion of a relatively large peptide (34 amino acids) into the capsid.

An alternative non-genetic targeting strategy involved producing biotinylated AAV2 which was then conjugated to streptavidin linked EGF. The vector produced enhanced transduction of ovarian cancer cells expressing EGF receptor but also retained the native AAV2 tropism (Ponnazhagan *et al.*, 2002). These studies provide proof-of-concept of non-genetic targeting of AAV2 *in vitro* but their stability *in vivo* and large-scale production needs further examination and the specificity of the retargeting may be compromised if the native tropism of the vector is retained.

1.12.2 Genetic targeting of AAV

The first example of genetic targeting of AAV2 was performed by Girod *et al.* (Girod *et al.*, 1999) before the crystal structure of AAV2 was solved. By comparing the AAV2

amino acid sequence with the crystal structure of canine parvovirus, 6 possible positions were identified that were predicted to be in a surface exposed position that could tolerate peptide insertion (Girod *et al.*, 1999). A 14 amino acid RGD containing peptide (L14) was inserted at these sites. The most affective insertion was after residue 587. This produced a virus that transduced an integrin expressing cell line, B16F10 that is normally non-permissive to AAV2. Transduction could be inhibited by synthetic RGD peptide and in cell lines normally permissive to AAV2, soluble heparin reduced but did not ablate transduction (Girod *et al.*, 1999). This suggests that transduction was mediated by the RGD peptide binding to integrins, but that the virus was not completely detargeted from HSPG. Subsequent publication of the AAV2 capsid structure showed that amino acid 587 is located in the three-fold symmetry axis of the capsid, protruding from the external surface of the virus and facing the inside of the crown like structure that forms the heparin binding site (Xie *et al.*, 2002) (Figure 1.8).

Several other groups have also carried out insertional mutagenesis studies to identify other regions of the capsid that can tolerate peptide insertion. Variable success has been achieved as peptide insertion has been shown to produce a number of different outcomes. It can either prevent virus assembly, reduce the titre of the resulting virus, reduce virus infectivity by either preventing cell binding or inhibition of a later stage of the transduction process or it can produce a virus with expanded or altered tropism (Figure 1.12). Due to the organisation of the AAV2 genome it is difficult to modify any of the VP proteins individually other than the unique 137 residues at the N-terminal of VP1. The N-terminal region of VP1 is highly conserved among the different AAV serotypes (Gao *et al.*, 2003) and contains the phospholipase domain, so there are few sites suitable for peptide insertion. Residue 34 has been used as an insertion site for a serpin receptor targeting peptide to produce a vector that infects the normally unpermissive cell line HB3, but heparin binding was still required for infectivity (Wu *et al.*, 2000). The N-terminus of VP2 contains a hypervariable region that has been shown to tolerate some peptide insertions. Insertion of peptides after residue 138 is well tolerated and the peptide is displayed on the capsid surface (Wu *et al.*, 2000). Several different peptides have been successfully incorporated at this position to expand the tropism of the virus by enabling it

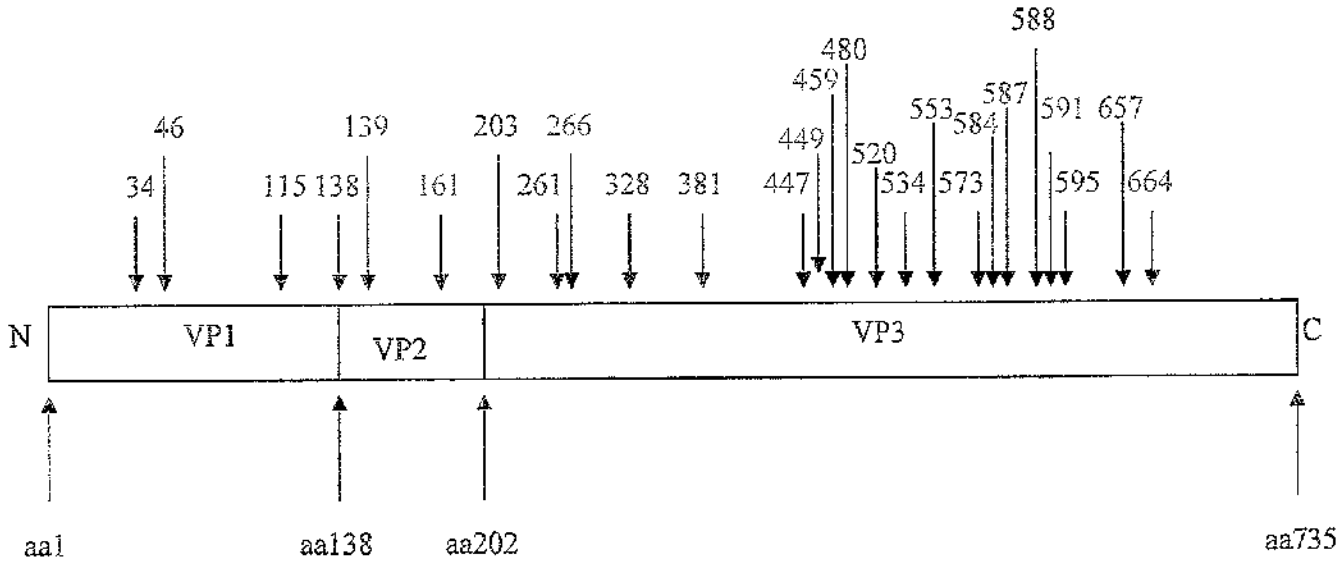


Figure 1.12 Affect of peptide insertions into the AAV2 capsid on virus production and infectivity. The amino acid (aa) position and affect of peptide insertions into the capsid protein are shown (Wu et al., 2000, Shi et al., 2001) (Shi and Bartlett, 2003, Girod et al., 1999, Grifman et al., 2001). It is important to note that the length and sequence of the peptide inserted also affect the production and efficiency of the modified virus, so some variability has been seen in these results.

to use an additional receptor for cell binding, but the vectors are still able to bind heparin (Wu *et al.*, 2000, Shi *et al.*, 2001, Shi and Bartlett, 2003, Loiler *et al.*, 2003). For example, insertion of a lutenizing hormone receptor targeting peptide at residue 138 was shown to enhance transduction of OVCAR-3 cells even in the presence of soluble heparin, even though the vector could still bind heparin (Shi *et al.*, 2001). Eleven of the twelve hypervariable regions of the capsid lie within the VP3 region of the proteins and encode surface exposed loops between the critical structural domains of the capsid. The majority of successful peptide insertions are in the VP3 region which results in the incorporation of 60 copies of the peptide into the capsid. Recently a double mutant vector has been produced with the targeting peptide RGD4C inserted after both residues 520 and 584 (Shi *et al.*, 2006). Previously insertion of peptides at residue 520 has been shown to produce vectors which had very low titres due to instability and an inability to efficiently package the genome (Shi *et al.*, 2001) and insertion of RGD4C peptide after residue 584 alone retains some heparin binding ability (Shi *et al.*, 2006). But these deficiencies are overcome in the double mutant which is unable to bind heparin and was shown to infect several cells by integrin binding mediated by the inserted peptide (Shi *et al.*, 2006).

Despite the identification of some other possible peptide insertion sites amino acid 587 remains the favoured position with which most success in producing viruses with modified tropisms have been achieved. There have been several examples of vascular targeting peptides incorporated in this position (Table 1.7). Despite the insertion of targeting peptides into the heparin binding site of the capsid, in some cases the vector retains the ability to bind HSPG.

Peptide inserted	Target	Results	Reference
NGRAHA	Sarcoma cells. Peptide binds CD13, which is expressed by angiogenic vasculature and many tumour cell lines.	Transduced tumour vasculature but still bound heparin to some degree.	(Grifman *et al.*, 2001)
SIGYPLP	Endothelial cells	Selective for ECs. Low transduction of SMCs and hepatocytes. Heparin independent transduction.	(Nicklin *et al.*, 2001a)
RGD-4C peptide	α_v integrin	Vector bound α_v integrin and enhanced transduction of cells expressing α_v integrin up to 40-fold, independently of HSPG binding. Also targeted tumour cells in a murine model of ovarian cancer, providing the first evidence of genetic retargeting of AAV2 *in vivo*.	(Shi and Bartlett, 2003)
EYHHYNK	SMC	Up to 70-fold increase in human SMC trasnsduction.	(Work *et al.*, 2004a)
MTPFPTSNEANL	Venous EC	Higher transduction of venous EC but not arterial EC *in vitro* and *in vivo*. Heparin independent transduction.	(White *et al.*, 2004)
QPEHSST	Brain microvasculature	Higher transgene expression seen in rat brain compared to rAAV. No difference in liver transduction. Heparin independent transduction.	(Work *et al.*, 2006)
VNTANST	Pulmonary endothelium	Higher transgene expression seen in rat lungs compared to unmodified AAV. No difference in liver transduction. Heparin independent transduction.	(Work *et al.*, 2006)

Table 1.7 Examples of peptide-modified AAV2 vectors targeted to the vasculature. Peptides were inserted into the capsid after residue 587.

1.12.3 Alternative AAV serotypes and pseudotyping

Pseudotyping is being used to exploit the natural variation of the AAV serotypes to produce vectors targeted to different tissues. The similar structural organisation of the genome and capsid of the different AAVs makes production of pseudotyped vectors relatively easy. The majority of pseudotyped AAVs utilise an AAV2 transgene expression cassette in combination with the capsid of another serotype. Many studies in animals have been carried out to determine the tropism of vectors pseudotyped with the alternative capsids (Table 1.8). Due to the different tropisms of these viruses, different pseudotypes have the potential to be developed for different forms of gene therapy. For example AAV5 efficiently transduces airway epithelial cells (Sumner-Jones *et al.*, 2006) so may be useful for cystic fibrosis gene therapy. Whereas AAV6 efficiently transduces skeletal muscle (Blankinship *et al.*, 2004) so could potentially be used to treat muscular dystrophies. AAV8 has a strong hepatic tropism so is being developed for treating haemophilia by mediating hepatic expression of FIX (Davidoff *et al.*, 2005, Nathwani *et al.*, 2005).

Several AAV pseudotypes are thought to be particularly efficient for cardiac targeted gene therapy. AAV1, AAV6, AAV8 and AAV9 have been shown to efficiently transduce skeletal and cardiac muscle (Blankinship *et al.*, 2004) (Wang *et al.*, 2005, Gregorevic *et al.*, 2004, Inagaki *et al.*, 2006, Pacak *et al.*, 2006, Du *et al.*, 2004, Su *et al.*, 2006, Kawamoto *et al.*, 2005). These vectors may be utilised for treating myocardial ischemia following myocardial infarction. For example, an AAV1 vector with a hypoxia induced promoter (comprising of hypoxia-response elements and the promoter of myosin light chain2v) expressing VEGF was injected intramyocardially into mice following ligation of the coronary artery (Su *et al.*, 2006). It caused a significant increase in neovascularisation and improved cardiac function (Su *et al.*, 2006).

Although the serotypes do have different tropisms, they generally have a broad tropism following systemic administration, and produce higher levels of transgene expression than AAV2 in most tissues (Müller *et al.*, 2006, Grimm *et al.*, 2003). Therefore they are

Serotype	Tropism	Ref
AAV2	Wide tissue tropism including liver, spleen, muscle, CNS and lung.	(Nathwani *et al.*, 2001)
AAV1	Higher transduction of skeletal muscle than AAV2, and lower transduction of the liver. Higher transduction of the CNS than AAV2.	(Xiao *et al.*, 1999, Burger *et al.*, 2004)
AAV4	Strong tropism for ependymal cells in CNS.	(Davidson *et al.*, 2000)
AAV5	Higher transduction of skeletal muscle, retinal and airway epithelia and CNS compared to AAV2.	(Hildinger *et al.*, 2001, Zabner *et al.*, 2000, Davidson *et al.*, 2000, Burger *et al.*, 2004, Rabinowitz *et al.*, 2001)
AAV6	Higher transduction of airway epithelia than AAV2. Efficient cardiac gene transfer. Efficient skeletal muscle transduction. Transduces liver more efficiently than AAV1 and AAV2.	(Halbert *et al.*, 2001, Gregorevic *et al.*, 2004, Blankinship *et al.*, 2004, Grimm *et al.*, 2003)
AAV7	Transduction of skeletal muscle equivalent to AAV1.	(Gao *et al.*, 2002)
AAV8	10-100 fold higher transduction of mouse liver compared to any other AAV serotype. Efficient transduction of the heart and skeletal muscle following systemic administration.	(Gao *et al.*, 2002, Wang *et al.*, 2005, Nakai *et al.*, 2005)
AAV9	Broad tropism, produces higher transduction than most serotypes in most tissues. Transduces myocardium more efficiently than AAV 2 and AAV2/1 following intravenous administration. 5-10 fold higher transduction of the myocardium than AAV8.	(Muzyczka and Warrington, 2005, Inagaki *et al.*, 2006, Pacak *et al.*, 2006)
AAV10	Broad tropism - 6 weeks after systemic administration in mice transduction of liver, heart, muscle, lung, kidney and uterus was detectable.	(Mori *et al.*, 2004)
AAV11	Broad tropism - 6 weeks after systemic administration in mice transduction of muscle, kidney, spleen, lung, heart and stomach was detectable. Unlike most serotypes, liver transduction is poor.	(Mori *et al.*, 2004)

Table 1.8 Tropism of pseudotyped AAV vectors. Pseudotypes contained the AAV2 ITRs and the capsid of the serotype indicated.

unlikely to provide the high specificity of infection required for some gene therapy treatments, but they might provide a better platform vector to which further modifications could be made to enable the creation of a vector with the desired tissue selectivity. As yet, there are few examples of genetic modifications being made to the capsid of any serotype other than AAV2. One example is the insertion of the RGD4C peptide at residue 590 of AAV1 (Stachler and Bartlett, 2006). Unmodified AAV1 transduces vascular endothelial cells more efficiently than AAV2 (Chen *et al.*, 2005) and AAV1-RGD4C transduces human umbilical vein EC and human saphenous vein EC better than AAV1 *in vitro* (Stachler and Bartlett, 2006). However due to the wide range of cells that express the target integrin receptor, this virus would have low selectivity *in vivo*.

Recently AAV serotypes 2-5 and AAV2 pseudotyped with AAV1-5 capsids were tested to compare their transduction of human and rat aortic ECs (Chen *et al.*, 2005). At 7 days post infection AAV1 gave the highest level of transduction in both cell lines, however at days 14 and 21 AAV5 was producing higher levels of transgene expression despite the expression from AAV1 remaining stable (Chen *et al.*, 2005). *In vivo* AAV1 and AAV5 produced more transgene expression than AAV2 in rat aortic endothelial and smooth muscle cells (Chen *et al.*, 2005). A previous study carried out by Dishart *et al.* (Dishart *et al.*, 2003) comparing AAV2-6 transduction of human umbilical vein EC and human saphenous vein EC showed they were all inefficient vectors but that AAV2 produced the highest transduction at day 5, but later time points were not investigated (Dishart *et al.*, 2003). Denby *et al.* (Denby *et al.*, 2005) compared the transduction of AAV2, AAV7 and AAV8, in EC and SMC, but found that neither were more effective than AAV2 and all three serotypes were affected by proteasomal degradation.

Differences in tropism may not just be due to binding of different cellular receptors. Despite AAV2 and 8 having 83% amino acid similarity, AAV8 produces a 20-fold higher level of transduction of mouse liver than AAV2 (Nakai *et al.*, 2005). It is thought that this increase may be due to more efficient virus uncoating rather than cellular binding (Thomas *et al.*, 2004). Although AAV3 has been shown to bind HSPG, it has a different tropism from AAV2, possibly due to the use of different co-receptors. For example,

AAV3 can transduce haematopoietic cells that cannot be transduced by AAV2 (Handa *et al.*, 2000).

In addition to producing a virus with a novel tropism AAV pseudotyped vectors may be more efficient than AAV2 as studies have shown that a smaller percentage of the population have pre-existing antibodies to the alternative serotypes. Although people have been found to have existing neutralising antibodies against serotypes 1, 3, 4, 5, 6 and 8, they occur at a lower rate than for AAV2 (Lochrie *et al.*, 2006, Erles *et al.*, 1999, Hildinger *et al.*, 2001, Xiao *et al.*, 1999, Halbert *et al.*, 2006). Also, many of the novel serotypes and variants are from non-human sources so the existing immunity to these may be relatively low. Existing anti-AAV2 antibodies have no detrimental effect on the transduction of AAV5 (Hildinger *et al.*, 2001, Peden *et al.*, 2004), AAV6 (Halbert *et al.*, 2000), AAV7 (Gao *et al.*, 2002), AAV8 (Gao *et al.*, 2002) or AAV4 (Kok *et al.*, 2005).

1.12.4 Hybrid vectors

The production of hybrid vectors that combine the high efficiency of infection of Ad with the longevity of expression of AAV2 are under investigation. The AAV ITR flanked expression cassette is packaged into the Ad capsid to combine the most desirable properties of the 2 vectors. This has been tested in a mouse model of glycogen storage disease type II, using an AAV2 expression cassette in an Ad5 capsid to express human acid α-glucosidase in acid α-glucosidase knock out mice (Sun *et al.*, 2003). Transgene expression was measured for 6 months and was shown to have a therapeutic effect (Sun *et al.*, 2003). Hybrid vectors have also been produced with serotyped Ad fibers. The Ad35 fiber was pseudotyped onto Ad5 capsid and combined with an AAV2 expression cassette. It provided stable transgene expression in human haematopoietic cells (Shayakhmetov *et al.*, 2002). This study shows the versatility of the different components of viral vectors and that by combining different virus components a more appropriate vector with the desired features can be produced.

1.13 Gene therapy for atherosclerosis

As atherosclerosis is a multifactorial disease there are many different ways in which gene therapy might be used to treat the condition. It could be used to prevent plaque development, cause plaque regression or plaque stabilisation to prevent plaque rupture. Plaque regression is likely to be harder to achieve than inhibiting plaque development and is also more difficult to prove experimentally, so there are fewer published examples of gene therapy to treat existing atherosclerotic plaques. Most groups have focused on reducing plasma cholesterol levels to try and increase plaque stability, but delivery of genes to control dislipidemia, the inflammatory response, SMC regulation and extracellular matrix production/degradation may all be advantageous.

1.13.1 Endothelial dysfunction

Therapeutics that reduce oxidative stress and increase NO bioavailability will improve endothelial function and reduce inflammation, so overexpression of anti-oxidant enzymes such as superoxide dismutase (SOD) and NOS in the endothelium should reduce ROS and have a vasoprotective effect. For example, local delivery of an Ad vector expressing eNOS to the carotid artery of hyperlipidemic rabbits resulted in a reduction in inflammatory cell infiltration and lipid accumulation after just 3 days (Qian and Neplioueva, 1999). Another study using local delivery of a first generation Ad5 vector to the carotid artery of ApoE-/- mice resulted in the development of smaller plaques containing fewer macrophages 5 weeks after treatment (Mujynya-Ludunge *et al.*, 2005). Delivery of an Ad vector expressing manganese SOD into the carotid artery of hypercholesterolemic rabbits has been shown to improve endothelial dysfunction 4 days post delivery (Zanetti *et al.*, 2001). Longer-term studies are required to determine if any of these treatments could have a significant therapeutic effect.

Intra-ventricular administration of an Ad5 vector expressing heme-oxygenase 1 (HO-1) into ApoE-/- mice was found to significantly reduce lesion formation in the aorta 6 weeks after vector administration (Juan *et al.*, 2001). When the same vector was delivered by tail vein injection it had no affect on the size of plaques that developed (Juan *et al.*,

2001), showing that localised transgene expression was required in this case. If systemic administration is necessary then a vascular targeted vector will need to be utilised. The mechanism by which HO-1 is atheroprotective is not fully understood, although it is known to prevent oxidative injury and is anti-proliferative and anti-inflammatory (Juan *et al.*, 2001, Morita, 2005).

These treatments all require a highly invasive procedure, so the development of EC specific vectors that could be delivered systemically would reduce the need for surgery.

1.13.2 Inflammation

Systemic approaches to reducing the inflammatory component of plaque development have been shown to have some success in animal models. Interleukin 10 (IL10) is an anti-inflammatory cytokine that is thought to have anti-atherogenic effects as it reduces the production of pro-inflammatory cytokines, chemokines and endothelial adhesion molecules (Pinderski Oslund *et al.*, 1999). Intramuscular administration of an AAV5 expressing IL10 into 4 week old ApoE-/- caused mice to develop smaller lesions with reduced MCP-1 expression and the mice had lower total serum cholesterol levels (Yoshioka *et al.*, 2004). The reduction in cholesterol was thought to be caused by inhibition of cholesterol synthesis (Yoshioka *et al.*, 2004). Another study in LDLR-/- mice using intravenous injection of AAV2 expressing IL10 demonstrated the protein was detectable in plasma up to 10 weeks after administration and showed the animals developed less atherosclerosis than controls or animals that received a vector expressing the pro-inflammatory cytokine granulocyte macrophage-colony stimulating factor (GMCSF) (Liu *et al.*, 2005).

TGFβ is a cytokine that is found in healthy vessels and a decrease in its expression results in an accumulation of inflammatory molecules within the vessel wall (Gamble *et al.*, 1993). $TGF\beta_1^{ACT}$, an active mutant form of the protein was expressed from an AAV2 vector systemically administered to LDLR-/- mice fed a high fat diet (Li *et al.*, 2006). Transgene expression could be detected 18 weeks after administration and treated

animals had lower levels of markers of oxidative stress and less lipid accumulation in their aortas, suggesting TGFβ may have an atheroprotective role (Li *et al.*, 2006).

Although these studies have shown therapeutic effects by over expressing genes involved in controlling inflammation, if these therapies were to progress to humans it is likely that specific plaque localised gene expression will be required as systemic changes to the immune system may be detrimental to the patients ability to respond to infection.

1.13.3 Lipid Balance

It is thought that gene delivery of ApoAI-Milano is likely to improve dyslipidemia (See section 1.2.4). Intravenous delivery of an AAV1, 2 or 5 vector with the ApoAI-Milano transgene into ApoA-I deficient mice resulted in sustained transgene expression for at least 24 weeks, with the highest levels achieved by the AAV1 vector (Sharifi *et al.*, 2005). Whether the levels achieved are high enough to have a therapeutic effect needs further investigation. In another study a second generation Ad vector expressing ApoA-I was found to cause a 70% reduction of existing fatty streaks in LDLR-/- mice just 4 weeks after administration (Tangirala *et al.*, 1999). Another group used a HdAd vector to deliver human ApoAI to LDLR-/- mice with advanced lesions (Belalcazar *et al.*, 2003). 24 weeks after treatment a 50% reduction in lesion progression was measured and the plaques had a more stable phenotype (Belalcazar *et al.*, 2003). Long-term expression of ApoAI-Milano seems to provide a promising treatment for preventing plaque progression.

There have also been several studies investigating the effect of ApoE delivery. One study using HdAds to deliver ApoE to ApoE-/- mice showed a single administration caused a lifetime correction (2.5 years) of hypercholesterolemia and prevented plaque development (Kim *et al.*, 2001). This study demonstrated the sustained transgene expression that can be achieved using HdAds (Kim *et al.*, 2001). It also showed that readministration of the same vector is ineffective due to neutralising antibodies against the capsid, but if a different serotype is used readministration can be successful (Kim *et al.*, 2001). Several other groups have also used Ad vectors to deliver ApoE to ApoE

deficient mice to cause regression of fatty streaks (Tsukamoto *et al.*, 1999, Desurmont *et al.*, 2000) and advanced lesions (Harris *et al.*, 2002, Tangirala *et al.*, 2001). In a long-term study ApoE-/-mice were fed a high cholesterol diet for 30 weeks before administration of an HdAd expressing ApoE3. 36 weeks later no plaque regression had occurred, but plaque progression was significantly reduced compared to untreated animals (Oka and Chan, 2005).

Other studies have focused on lowering LDL cholesterol levels by delivery of LDL receptors (Ishibashi *et al.*, 1993, Kozarsky *et al.*, 1994) or very low density lipoprotein receptor (VLDLR) (Oka *et al.*, 2001). Use of first generation Ads to deliver LDLR to LDLR deficient mice (Ishibashi *et al.*, 1993) and Watanabe heritable hyperlipidemic rabbits (Kozarsky *et al.*, 1994) was unsuccessful as it resulted in only transient lowering of plasma cholesterol probably due to an immune response against the LDLR and vector used (Kozarsky *et al.*, 1994). In a similar study where LDLR-/- mice received a HdAd expressing LDLR, mice that received a lower dose (5×10^{12} vp/kg) maintained low cholesterol levels for at least 108 weeks and developed significantly less atherosclerosis than control animals (Nomura *et al.*, 2004). No immune response against the vector were detected in these mice, but 2/10 mice that received a higher dose (1.5×10^{13} vp/kg) developed anti-LDLR antibodies (Nomura *et al.*, 2004).

The lipid lowering proteins described above do not just act by reducing lipoprotein concentration. ApoA-I elevates HDL cholesterol, which has anti-inflammatory and anti-oxidative properties as well as promoting reverse cholesterol transport. ApoE has an anti-atherogenic affect via many mechanisms including, an anti-oxidant effect, inhibition of T cell proliferation, inhibition of SMC proliferation and migration, inhibition of reverse cholesterol transport and inhibition of platelet aggregation {Davignon, 2005 #58. Gene therapy vectors that alter the lipoprotein balance are produced some of the most successful studies in animal models and could be developed into successful therapeutics.

Another approach investigated was the delivery of a soluble form of the macrophage scavenger receptor (sMSR) to reduce oxLDL uptake into macrophages and so reduce

plaque development. An AAV2 vector expressing sMSR was delivered to LDLR-/- mice by tail vein injection at the same time as fat feeding was begun {Jalkanen, 2003 #5633}. sMSR could be detected in serum up to 6 months after administration and this resulted in a 21% reduction in the size of lesions that developed in the aorta (Jalkanen *et al.*, 2003).

1.13.4 Matrix remodeling

TIMPs have an important role in the regulation of MMP activity and extracellular matrix remodeling which occurs during plaque progression. Tail vein injection of an HdAd expressing TIMP1 or TIMP2 into ApoE-/- mice fed a high fat diet showed that TIMP2 expression in plasma reduced development of lesions whereas TIMP1 expression had no effect (Johnson *et al.*, 2006). The plaques that did develop in mice that received TIMP2 had a more stable phenotype, containing more SMC and less macrophages (Johnson *et al.*, 2006). This was proposed to be due to TIMP2 inhibiting the migration of macrophages and inhibiting smooth muscle cell apoptosis (Johnson *et al.*, 2006). TIMP2 may therefore be a good candidate gene for a treatment aiming to stabilise vulnerable plaques.

Another study used tail vein injection of ApoE-/- mice of an Ad5 vector for systemic expression of decorin, a matrix proteoglycan that regulates cell proliferation, migration and growth factor activity (Zen *et al.*, 2005). Decorin could be detected in plasma for up to 4 weeks after vector administration and resulted in a significant reduction in atherosclerosis (Zen *et al.*, 2005).

1.13.5 Thrombus formation

There are also several studies that have investigated the use of gene therapy to prevent thrombus formation. For example Waugh *et al.* (Waugh *et al.*, 1999a) have produced an Ad vector that expresses tissue plasminogen activator (tPA), which catalyses the rate limiting step in fibrinolysis. The vector was administered into rabbit arteries 3 days before thrombus formation was induced (Waugh *et al.*, 1999a). It inhibited thrombus formation without having any affect on systemic coagulation factors (Waugh *et al.*,

1999a). Similar results were achieved using an Ad vector expressing the anticoagulant protein thrombomodulin in the same model (Waugh *et al.*, 1999b).

Tissue factor pathway inhibitor (TFPI) inhibits the coagulation cascade and therefore blocks thrombus formation. Local gene delivery of an Ad vector expressing TFPI to rabbit carotid arteries inhibited subsequent induced thrombus formation (Nishida *et al.*, 1999). Another study has also shown a protective role of TFPI in preventing thrombus formation (Zoldhelyi *et al.*, 2000). Gene delivery of other genes including hirudin (Rade *et al.*, 1996) and cyclo-oxygenase (Zoldhelyi *et al.*, 1996) have also shown some inhibition of thrombus formation in animal models. It is important that anti-coagulants such as TFPI are only active at the site of unstable plaques, so they are good candidates for therapeutic genes delivered using vectors targeted specifically to unstable plaques.

All the examples described above have used either local delivery to the vessels or systemic expression of proteins to try and reduce atherosclerosis. There are no published examples of the use of vectors targeted specifically to established atherosclerotic plaques. Ideally this is what is required, so a non-invasive procedure can be used to deliver a highly selective vector. The majority of studies have also focused on preventing plaque development rather than the targeting of existing plaques, the likely clinical scenario. Also, the majority of the animal studies described only short-term transgene expression, but validation of both short and long term gene expression is required. So, there is a need for the development of vectors that can be used to specifically target gene expression to areas of atherosclerosis to either stabilise or cause regression of existing plaques.

Chapter 2:

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 Tissue culture

All tissue culture work was performed under containment level 2, in sterile conditions using a vertical laminar flow hood. Table 2.1 describes the cell types used in this study.

2.2.1 Maintenance of cell lines

Cell lines were maintained in the appropriate cell culture media (Table 2.1) and incubated at 37°C in a 5% CO_2 atmosphere. Confluent cultures of cells were passaged using trypsin-ethylendiamine tetra-acetic acid (trypsin-EDTA). Briefly, cells were washed twice in phosphate buffered saline (PBS) (Biowhittakers, Berkshire, UK) and incubated in a minimal volume of trypsin-EDTA (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 8 ml media. Cells were harvested by centrifugation at 480 x 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 of cell lines

Cells were harvested as described in section 2.2.1 and re-suspended at a density of approximately 1-2 x 10^6 cells/ml in complete cell culture media supplemented with 10% dimethyl sulphoxide (DMSO). Cell suspensions were aliquoted into sterile 2 ml cryo-preservation vials and cooled at a constant -1°C/minute to -80°C using isopropanol. Vials were then transferred to liquid nitrogen and stored indefinitely.

For recovery of cryo-preserved cells, vials were removed from liquid nitrogen and warmed in a 37°C water bath until the cells were completely thawed. Cells were then

Cell Type	Description	Supplier	Culture Media
293	Transformed human embryonic kidney cell line	Microbix (Toronto, Canada)	Minimal essential media (MEM) (Biowhittakers, Berkshire, UK) supplemented with 10% (v/v) fetal calf serum (FCS), 100 IU/ml penicillin (Gibco, Invitrogen, Paisley, UK), 100 µg/ml streptomycin (Gibco, Invitrogen, Paisley, UK) and 2 mM L-glutamine (Gibco, Invitrogen, Paisley, UK).
633	Derived from A549 cells. Inducibly express Ad5 E1A and constitutively express the Ad5 fiber protein	Scripps Institute (La Jolla, CA, USA)	Dulbecco's Minimal Essential media (DMEM) with Glutamax, 4500 mg/l glucose (Gibco, Invitrogen, Paisley, UK), supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 200 µg/ml hygromycin B (Gibco, Invitrogen, Paisley, UK), 300 µg/ml zeocin, (Gibco, Invitrogen, Paisley, UK), 200 µg/ml neomycin sulphate
HSVEC	Primary human saphenous vein endothelial cells	Prepared from vein sections (section 2.2.3)	Large vessel endothelial cell basal medium supplemented with 50x large vessel endothelial cell growth supplement (TCS Cellworks, Buckinghamshire, UK) 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
HCAEC	Primary human coronary artery endothelial cells	PromoCell (Heidelberg, Germany)	Endothelial cell growth medium (PromoCell, Heidelberg, Germany)
RGE	Rat glomerular endothelial cell line	DSMZ-Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (Braunschweig, Germany)	MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
SVEC 4-10	Mouse endothelial cell line from the vascular epithelium of the axillary lymph node	American Type Culture Collection (LGC Promochem, Middlesex, UK)	MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
IP-1B	Mouse endothelial cell line from the vascular epithelium of the axillary lymph node	American Type Culture Collection (LGC Promochem, Middlesex, UK)	MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
HT1080	Human fibrosarcoma cells	Gift from G. Murphy (University of Cambridge)	DMEM with Glutamax, 4500 mg/l glucose, supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, 50x HT supplement (Gibco, Invitrogen, Paisley, UK), 1.5 mM xanthine, 0.016 mM mycophenolic acid
HT1080-MT1-MMP	Human fibrosarcoma cells stably transfected with human MT1-MMP	Gift from G. Murphy (University of Cambridge)	DMEM with Glutamax, 4500 mg/l glucose, supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, 50x HT supplement (Gibco, Invitrogen, Paisley, UK), 1.5 mM xanthine, 0.016 mM mycophenolic acid
CRL-2254	Mouse hepatocytes	American Type Culture Collection (LGC Promochem, Middlesex, UK)	DMEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin , 2 mM L-glutamine,
HepG2	Human hepatocellular carcinoma cell line	European Collection of Animal Cell Cultures (Sailsbury, UK)	MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine
HeLa	Human cervical carcinoma cell line	European Collection of Animal Cell Cultures (Sailsbury, UK)	MEM supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine

Table 2.1 Cell types and culture media used.

transferred to a T-25 flask containing 5 ml of pre-warmed cell culture media and incubated overnight at 37°C. The following day they were placed in fresh media.

2.2.3 Isolation of human saphenous vein EC (HSVEC)

Saphenous veins were obtained from patients undergoing coronary artery bypass graft surgery. Ethical permission was obtained; reference number 04/161 (1) and 03CA022VRW.

HSVEC were isolated by collagenase digestion. Veins were washed in RPMI (Roswell Park Memorial Institute) media supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin. One end of the vein was clamped with a surgical clip to seal it and enable the vein to be filled with collagenase solution (2 mg/ml in PBS). The vein was then sealed and incubated at 37°C for 15 minutes. The collagenase solution was collected from the vein, then the process was repeated with fresh collagenase solution. The vein was washed with RPMI and the solution collected and pooled with the collagenase fractions. Cells were harvested by centrifugation at 200 x g for 10 minutes. Cells were re-suspended in cell culture media and placed in a T-25 flask. Cells were maintained for up to 7 passages.

2.3 Animal models

8 week old C57Bl6/129SvJ ApoE-/- mice were maintained on a diet of 21% beef lard supplemented with 0.15% cholesterol (Special Diets Services, Witham, Essex, UK) for 10 weeks (maintained by Bristol University Biological Services Staff). C57Bl6 ApoE-/- mice were obtained from Charles River Laboratories (Manston, Kent, UK) to set up a breeding colony maintained by University of Glasgow Biological Services Staff. For *in vivo* studies these mice were maintained on a diet of 21% beef lard supplemented with 0.15% cholesterol for 12 weeks from the age of 6 weeks. C57/Bl6 mice obtained from (Harlan, Oxfordshire, UK) were maintained on a standard rodent chow diet.

All animals were housed under controlled environmental conditions. Temperature was maintained at ambient temperature with 12 hour light/dark cycles. Food and water were

provided *ad libitum*. All work was performed in accordance with the Animals and Scientific Procedures Act 1986 under project licenses 60/3011 held by Professor A. Baker and 30/2151 held by Dr C. Jackson.

2.4 Plaque histology

Mice were anesthetised by intra-peritoneal injection of Euthatal (Pentobarbital Sodium 200 mg/ml) (Merial Animal Health, Essex, UK) before perfusion with PBS. The BCAs were removed and fixed overnight in 10% formalin then transferred to PBS. Histological analysis was performed by Dr R. McDonald (University of Bristol). BCA sections were stained with haematoxylin and eosin stain (H and E) to show the plaque structure and Elastic von Gieson (EVG) stain to determine the elastin content of the plaque.

2.5 Production of viruses

2.5.1 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% confluency 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 x 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 stripping of the Ad fiber from the capsid). The mixing was repeated. The suspension was centrifuged at 850 x 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.

2.5.2 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) fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. 24 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 x g for 10 minutes at room temperature. The pellet was resuspended in 10 ml of PBS. Cell lysis was caused by freezing the suspension in liquid nitrogen and thawing it in a 37°C waterbath, three times. The suspension was centrifuged at 850 x g for 15 minutes at room temperature. The supernatant was stored at -80°C until it was purified on a CsCl gradient.

2.5.3 Production of fiber modified Ad viruses

Peptide modified viruses were produced using the previously developed transfection/infection protocol where the modified fiber gene is supplied by a plasmid (Von Seggern *et al.*, 1998, Jakubczak *et al.*, 2001, Nicklin *et al.*, 2001c). Table 2.2 describes the plasmids used to produce each of the Ad platform vectors. All the plasmids express the Ad fiber gene from a CMV promoter.

2.5.3.1 Cloning of peptides into the Ad fiber gene

Overlapping oligonucleotides encoding the peptides flanked by the *Bsp*EI restriction site (Table 2.3) (not required for Ad19p fiber as blunt ligation was used) were obtained from MWG-Biotech (Edersberg, Germany). Oligos were annealed by mixing 1 µM of each oligo (in 100 µl deionised water), heating to 98°C for 10 minutes and then leaving them to cool to room temperature. Oligos were electrophoresed on a 16% polyacrylamide gel to confirm annealing had occurred.

Virus	Plasmid	Fiber Modification
Control (AdCTL)	pDV111 (Nicklin *et al.*, 2001c)	Ad5 fiber with unique *Bsp*EI site inserted after amino acid T542 in the HI loop.
AdKO1	pDV137 (Nicklin *et al.*, 2001c)	Ad5 fiber with mutations S408E and P409A and a *Bsp*EI site inserted after amino acid T542 in the HI loop.
AdKO1S*	pcDNA3.1-KO1S* (A. Kritz, in press)	Ad5 fiber with the KO1 mutations S408E and P409A, the S* mutation $_{91}$KKTK$_{94}$ to GAGA in the third repeat of the Ad5 fiber shaft and a *Bsp*EI site in the HI loop.
Ad5/19p	pDV145 mod (L. Denby, submitted for publication)	Ad19p fiber with the HI loop modified to contain an *Eco*47III site.

Table 2.2 Plasmids used to produce Ad vectors with modified fiber proteins.

	***Bsp*EI**	**C**	**A**	**P**	**G**	**P**	**S**	**K**	**S**	**C**	***Bsp*EI**
Ad CAP F. 5'	CCGGA	TGT	GCT	CCT	GGA	CCT	TCT	AAG	TCA	TGC	G
Ad CAP R. 3'	T	ACA	CGA	GGA	CCT	GGA	AGA	TTC	AGT	ACT	CGGCC
		C	**N**	**H**	**R**	**Y**	**M**	**Q**	**M**	**C**	
Ad CNH F. 5'	CCGGA	TGC	AAC	CAC	AGA	TAC	ATG	CAG	ATG	TGC	G
Ad CNH R.	T	ACG	TTG	GTG	TCT	ATG	TAC	GTC	TAC	ACG	CGGCC
		C	**Q**	**E**	**P**	**T**	**R**	**L**	**K**	**C**	
Ad CQE F. 5'	CCGGA	TGC	CAG	GAG	CCT	ACC	CGG	CTG	AAG	TGC	G
Ad CQE R.	T	ACG	GTC	CTC	GGA	TGG	GCC	GAC	TTC	ACG	CGGCC

Table 2.3 Sequence of oligos encoding the atherosclerotic plaque targeting peptides. The encoded amino acids are shown above. Oligos were used to produce the peptide modified Ad vectors.

Plasmids were digested overnight at 37°C with *Bsp*EI (New England BioLabs, Hitchin, UK) or *Eco47*III (Promega, Southampton, UK) in the reactions shown below:

Plasmid (50 µg)	50 µl
*Bsp*EI 10 U/µl	10 µl
Buffer 3	10 µl
Water	30 µl

Plasmid (50 µg)	50 µl
*Eco*47III 5 U/µl	10 µl
Buffer D	10 µl
Water	30 µl

1 µg of digested plasmid was dephosphorylated using 5 U Shrimp Alkaline Phosphatase (SAP) (Promega, Southampton, UK) by incubation at 37°C for 15 minutes, followed by incubation at 65°C for 15 minutes to deactivate the phosphatase.

Dephosphorylated plasmid and oligo duplexes were ligated using Quick T4 ligase (New England BioLabs, Hitchin, UK). 50 ng vector, 5 pmoles 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 waterbath for 30 seconds and then placed back on ice for 2 minutes. 450 µl of warmed SOC media (20 g/l. bactotryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose) was added to the tube, which was then placed in an orbital shaker for 1 hour at 37°C and 180 rpm shaking. 100 µl was plated onto Luria Broth (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 of ampicillin. Plates were incubated overnight at 37°C.

Colonies were amplified and the plasmid was isolated by performing small-scale plasmid DNA preparations 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 (primer for AdKO1 plasmids 5'-CACTTGAGTTGTGTCTCCTCCACC-3', primer for AdKO1S* plasmids 5'-AAGTGTAACAGGATGTGG-3' and primer for Ad5/19p plasmids 5'-TCTTTGATTGTGGTCGCAGG-3'), 0.5 μl v3.1 Ready Reaction mix (Applied Biosystems, MA, USA) and 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 preparations of correctly sequenced plasmids were performed 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 6000 x g for 15 minutes at 4°C. The cells were lysed by alkaline lysis and centrifuged at 20000 x 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.Cl, 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 these were stored at -80°C.

2.5.3.2 Production of fiber-modified and pseudotyped Ads

Ad vectors with genetically modified fibers were produced in 293-T cells that express the adenovirus E1 gene, which is essential for virus replication. Twenty 10 cm 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, 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 1 M $CaCl_2$, 21 µg plasmid DNA per plate was made up. This was added dropwise while the plate was gently rocked. As a positive control one plate was transfected with an eGFP expressing plasmid. 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 eGFP 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 deleted first generation virus). When the cytopathic effect had caused the majority of cells to detach, the cells were harvested by centrifugation at 850 x g for 10 minutes at room temperature. The pellet was resuspended in 10 ml of PBS. Cells were lysed by freeze/thawing three times in liquid nitrogen and at 37°C. The suspension was centrifuged at 850 x g for 15 minutes at room temperature. The supernatant was stored at -80°C until purified on a CsCl gradient.

2.5.4 Adenovirus purification using a CsCl gradient

To purify and concentrate the crude Ad stocks, centrifugation on CsCl density gradients was used. Fourteen ml cellulose-nitrate ultra-clear centrifuge tubes (Beckman Coulter (UK) 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, 3 ml of CsCl with a density of 1.32 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 (RPS40T-859) and centrifuged at 90,000 x g for 1.5 hours at 4°C with maximum acceleration and free deceleration. Following centrifugation a band containing complete virus can be seen (Figure 2.1). 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°C.

2.5.5 Titration of adenovirus by end-point dilution

Unmodified viruses were titred using the end-point dilution method (Nicklin and Baker, 1999). Briefly, 293 cells were passaged into 8 rows of 10 wells in a 96 well plate, so that they would be 50-60% confluent the following day. Serial dilutions (from 10^{-4}-10^{-11}) of the viral stock were made in media. The media in each row was replaced with 100 µl of the correct adenoviral dilution (10 wells/dilution). Cells were incubated for 18 hours at 37°C and then the virus containing media was replaced with 200 µl fresh media. This media was then replaced every 2-3 days until a cytopathic effect was seen. After 8 days, the number of wells containing plaques was counted and the titre was calculated in pfu/ml using the equation:

The proportionate distance =

$$\frac{\text{\% positive above 50\% -50\%}}{\text{\% positive above 50\%-50\% positive below 50\%}}$$

and log ID_{50} (infectivity dose) =

log dilution above 50% + (proportionate distance x dilution factor)

For example: titration gives:

@ 10^{-4} all wells positive [10/10]
@ 10^{-5} [10/10]
@ 10^{-7} [10/10]
@ 10^{-8} [9/10]
@ 10^{-9} [3/10]
@ 10^{-10} [0/10]
@ 10^{-11} [0/10]

The proportionate distance = $\frac{90-50}{90-30} = 0.67$

log ID_{50} = -8 + (0.67 x -1) = -8.67

$ID_{50} = 10^{-8.67}$

$TCID_{50}$ (tissue culture infectivity dose 50) = $\frac{1}{10^{-8.67}}$

$TCID_{50}$ /100 µl = $10^{8.67}$

X dilution factor (=10)

$TCID_{50}$ /ml = $10^{9.67}$

= 4.67 x 10^9 $TCID_{50}$/ml

1 $TCID_{50} \cong 0.7$ pfu

Final titre = 3.27 x 10^9 pfu/ml

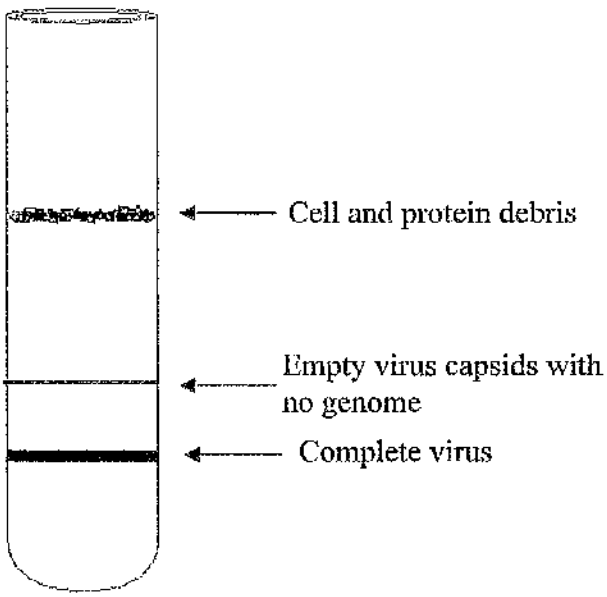


Figure 2.1 CsCl purification of Ad virus. Viruses were layered on a CsCl gradient and centrifuged at 90,000 x g for 1.5 hours at 4°C to produce separation of complete virus particles from empty capsids and cell debris.

2.5.6 Determining Ad virus particle titres

As fiber modified Ads do not infect 293 cells with the same efficiency, the end-point dilution method cannot be used to determine the virus titre. Instead the particle titre is calculated based on the protein content of the virus stock using the Micro BCA (bicinchoininc 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. 150 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. 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 x 10^9 viral particles (Von Seggern *et al.*, 1998)

2.5.7 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 blots 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) polacrylamide (30%), 3.75 mM Tris pH 6.8, 0.1% SDS 300 μl APS and 30 μl TEMED) was used with each gel.

$2x10^{10}$ 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 2 hours.

Proteins were transferred onto Hybond-P membrane (Amersham Bioscience UK Limited, Buckinghamshire, 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 1:1000 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 1:2000 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.

2.5.8 Production of AAV vectors

All AAV vectors were produced, purified and titred by Dr. H. Buening (University of Cologne, Germany) using a previously described method (Girod *et al.*, 1999, Nicklin *et al.*, 2001a). Briefly, oligonucleotides encoding the peptides CAPGPSKSC (CAP) and CNHRYMQMC (CNH) were annealed and ligated into the *Mlu*I-*Asc*I site of the plasmid pRC'99 to produce plasmids pRCcap and pRCcnh. pRC'99 encodes the AAV wild type capsid, so oligonucleotide insertion results in the incorporation of the peptides after amino acid 587 of the capsid protein (Figure 1.8).

Viruses were produced using a transfection protocol. Fifteen plate of 293 cells at 80% confluence were cotransfected with 37.5 μg of the plasmid expressing the virus capsid (pRC), pZnL and pXX6 at a 1:1:1 ratio. pXX6 provides the adenovirus genes required for helper function. pZnL encodes the AAV ITRs flanking the *LacZ* reporter gene and a CMV promoter (Girod *et al.*, 1999). 48 hours after transfection cells were harvested by centrifugation and the cell pellet was re-suspended in 150 mM NaCl, 50 mM Tris-HCl pH 8.5. This was freeze-thawed several times to lyse cells and treated with Benzonase (50 U/ml) for 30 minutes at 37°C to remove cellular DNA and RNA. Cell debris was removed by centrifugation and the supernatant was then purified on an iodixanol gradient. Genomic titres of the vector were determined by real-time PCR.

Viruses encoding the self-complementary eGFP gene were produced using the same method but the plasmid pscAGFPFG2 (which contains the eGFP flanked by AAV2 ITRs) was used instead of pZnL.

2.6 AAV heparin column binding

Heparin column binding was performed by Dr. H. Buening (University of Cologne, Germany). Virus was loaded onto HiTrap Heparin HP (Amersham Bioscience, Buckinghamshire, UK), and the initial flow through was collected. The column was washed twice with PBS containing 1 mM $MgCl_2$, 2.5 mM KCl and then bound virus was eluted by applying two 5 ml aliquots of PBS containing 1 mM $MgCl_2$, 2.5 mM KCl, 1 M NaCl. The virus content of each of the fractions was analysed by real time PCR.

2.7 *In vitro* infections

2.7.1 *In vitro* infection with adenovirus

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 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.

2.7.2 *In vitro* infection with adenovirus in the presence of anti-CAR antibody

Cells were seeded in 96-well plates and incubated overnight at 37°C to produce 70-80% confluence. Cells were pre-incubated on ice for 30 minutes in serum free media, media containing control rabbit serum or anti-CAR antibody diluted 1 in 200 in serum free media (a gift from J. Bergelson, University of Pennsylvania School of Medicine, Philadelphia, USA). Cells were infected with an MOI of 10000 for 3 hours, washed and incubated in fresh media for 48 hours.

2.7.3 *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.

2.7.4 *In vitro* infections with AAV in the presence of heparin

Cells were seeded in 96-well plates and incubated overnight at 37°C to produce 70-80% confluence. Cells were infected with an MOI of 1000 for 24 hours in the presence or absence of 1 U of heparin per 1 x 10^6 gp. Cells were washed in PBS then incubated in fresh media for 48 hours.

2.7.5 *In vitro* infections with AAV in the presence of proteasome inhibitors

Cells were seeded in 96-well plates and incubated overnight at 37°C to produce 70-80% confluence. Cells were infected with an MOI of 1000 for 24 hours in the presence or absence of proteasome inhibitors 40 μM LnLL (Calbiochem , Merck Biosciences Ltd., Nottingham, UK) or 4 μM MG132 (Calbiochem, Merck Biosciences Ltd., Nottingham, UK). Cells were washed in PBS then incubated in fresh media for 48 hours.

2.7.6 *In vitro* peptide competition assay

Cells were seeded in 96-well plates and incubated overnight at 37°C to produce 70-80% confluence. Cells were washed in PBS then placed in media containing the required

concentration of the peptide (Jerini Peptide Technologies GmbH, Berlin, Germany). Virus was then added to each well. AAV (MOI of 1000) was incubated with the cells for 24 hours before they were washed and placed in fresh media for a further 48 hours. Cell lysate was used in the β-gal assay.

2.8 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 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.

2.9 Quantification of β-gal expression in cell lysates

To quantify β-gal expression the Tropix Galacto-Light Plus system (Applied Biosystems, MA, USA) was used. Briefly, infected cells were washed in PBS then lysed in 80 µl of Lysis Solution (0.2% (v/v) Triton X-100 in PBS). 20 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 $MgCl_2$). 70 µl was then added to each well and incubated for 1 hour at room temperature. 100 µl of light emission accelerator was added to each well. Luminescence was measured using a Wallac Victor^2 plate reader. β-galactosidase activity was then normalised to protein content of samples to give relative light units per mg protein (RLU/mg protein).

2.10 Determination of protein concentration in cell 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.

2.11 *In vivo* virus biodistribution profiles

Mice received the required dose of virus via tail vein injection. After the required duration mice were anesthetised by intra-peritoneal injection of Euthatal (Merial Animal Health, Essex, UK), blood samples were taken by cardiac puncture and then the mice were perfused with PBS. For real-time PCR tissues were removed and snap frozen in liquid nitrogen. For immunohistochemistry (IHC) tissues were dissected and fixed overnight in 10% formalin then transferred to PBS.

2.11.1 Real time PCR

Real-time PCR (Taqman, UK) was used to quantify the number of virus genome particles in tissue extracts.

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 (up to 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. 200 µl ethanol was added to the samples, mixed and then they were 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 2 buffers 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). All samples were then diluted to 20 ng/µl, and 100 ng DNA was used in each reaction. A standard curve was produced from serial dilutions of the virus. All samples and standards were analysed in duplicate.

For detection of viruses with the eGFP transgene the SYBR green PCR kit (Applied Biosystems, MA, USA) was used with 300 nM forward (5'-CTCGATGTTGTGGCGGAT-3') and reverse primers (5'-GCGCCGAGGTGAAGTT-3'). The cycle conditions used were, step 1 denaturation, 95°C for 10 minutes, step 2 amplification and annealing, 95°C for 15 seconds, 60°C for 1 minute repeated for 50 cycles, step 3, melting point analysis 95°C for 15 seconds, 60 °C for 15 seconds and 95 °C for 15 seconds. Results were analysed using Taqman data analysis software (Applied Biosystems, MA, USA).

For detection of viruses with the *Lac Z* transgene or AAV library particles with the rep gene, Taqman Universal PCR Master Mix, No AmpErase UNG was used (Applied Biosystems, MA, USA) with gene specific primer and probe sets. Probes were labeled 5' with a FAM fluorophore (6-carboxyfluorescein) and 3' with a TAMRA fluorescence quencher (6-carboxytetramethylrhodamine). TAMRA quenches the fluorescence of FAM until the probe hybridises to the target sequence and is cleaved by the 5' exonuclease activity of the polymerase. For the rep gene, 300 nM of the forward primer (5'-CCGCAGATGTCAACACACAAG-3') and reverse primer (5'-CCCCTGAAGGTACACATCTCTGT-3') were used in combination with 200 nM of the probe (5'-CGTTCTTCCAGGCATGGTCTGGCA-3'). Cycle conditions were Step 1, 50°C for 2 minutes, 95 °C for 10 minutes, Step 2, 95 °C for 15 seconds, 60 °C for 1 minute, repeated for 50 cycles.

For the *lac Z* gene reactions contained 200 nM sense (5'-TACTGTCGTCGTCCCCTCAAA-3') and antisense (5'-TAACAACCCGTCGGATTCTCC-3') *LacZ* primers and 300 nM probe (5'-TATCCCATTACGGTCAATCCGCCG-3') (Senoo *et al.*, 2000). Cycle conditions were, Step 1, 50°C for 2 minutes, 95°C for 10 minutes, Step 2, 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, repeated for 50 cycles.

2.11.2 Immunohistochemistry

Formalin fixed tissues were embedded in paraffin, then 6 μm sections were cut and placed on silane coated slides. Slides were baked for 3 hours at 65°C for 3 hours, then 40°C overnight. Paraffin was removed from the sections by 2 x 7 minute washes in Histoclear (Fisher Scientific, Leicestershire, UK). Sections were rehydrated by passing them through an alcohol gradient of 100%, 95%, 70% ethanol for 7 minutes each. Slides were then washed in deionised water for 7 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 10 minutes. IHC was performed using Vectastain ABC rabbit IgG kit (Vector Laboratories, Peterborough, UK). Briefly, sections were placed in blocking solution (goat serum) and incubated for 1 hour at room temperature in a humidified chamber to prevent sections from drying out. The primary antibody, rabbit anti-β-gal (MP Biomedicals, Ohio, USA) and the negative control antibody rabbit IgG (DakoCytomation, Glostrup, Denmark) were diluted to 6.3 μg/ml in blocking solution. 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 (Vector Laboratories, Peterborough, UK) was diluted 1:200 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 brown/black.

2.12 Phage methods

2.12.1 Phage libraries

All phage experiments were carried out using the T7Select 10-3b phage display system (Novagen, EMD Biosciences, Darmstadt, Germany). In this system phage express 5-15 copies of the inserted peptide in the capsid protein 10B. A constrained and a linear 8-mer library and non-recombinant control phage (with no peptide insertion) were obtained as packaged libraries that required amplification before use (gift from E. Ruoslahti, Burnham Institute, USA). All phage amplification and titering was performed using BLT5615 *E.coli* (Novagen, EMD Biosciences, Darmstadt, Germany), which carry an ampicillin resistance gene. Expression of the capsid protein 10A requires addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) as the lacUV5 promoter drives its expression.

2.12.2 Amplification of phage libraries

Phage were amplified using the liquid lysate amplification protocol as described in the Novagen T7 select system manual. Briefly, a single BLT5615 colony was picked from a freshly streaked plate and amplified overnight in 50 ml LB, supplemented with 50 μg/ml ampicillin, in an orbital shaker at 37°C and 180 rpm. 5 ml of overnight culture was added to 500 ml LB supplemented with 50 μg/ml ampicillin in a 2 L flask, and incubated for approximately 2.5 hours at 37°C in an orbital shaker at 180 rpm. The absorbance at 600 nm was read at intervals. When it reached approximately 0.5, 5 ml 100 mM IPTG was added to the flask and the incubation was continued for a further 30 minutes. The number of cells in the culture was calculated using the formula:

$$\text{Number of cells} = OD_{600} \times \frac{(2 \times 10^8 \text{ cells/ml})}{0.5\ OD_{600}} \times \text{culture volume}$$

Stock phage was added at an MOI of 0.005. The culture 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 x g for 10 minutes to remove cell debris. The supernatant was transferred to a sterile bottle for PEG precipitation.

2.12.3 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 x g for 30 minutes at 4°C. The pellet was resuspended in 5 ml precipitation buffer (1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), transferred to microcentrifuge tubes and vortexed vigorously. This was centrifuged for 10 minutes at 12600 x g. The supernatant was removed to a fresh microcentrifuge tube and the pellet was washed in another 2 ml precipitation buffer. The supernatants were pooled and titred. 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.

2.12.4 Titering of phage

A single BLT5615 colony was picked from a freshly streaked plate and amplified overnight in 25 ml LB, supplemented with 50 µg/ml ampicillin, 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. The culture was diluted to an OD_{600} of approximately 1.

LB plates supplemented with 50 µg/ml ampicillin were pre-warmed in a 37°C incubator. Serial dilutions of phage were made in LB. Agar tops (10 g/L bacto-tryptone, 5 g/L yeast extract, 1 g/L Mg $Cl.6H_20$, 7 g/L agarose) was melted and 3 ml aliquots were made and placed in a 50°C waterbath. 120 µl 100 mM IPTG was added to each aliquot. 200 µl of BLT5615 culture were 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 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 x dilution factor x 10

2.12.5 Sequencing of phage peptides

Individual plaques were picked from the agar using a glass pipette. Each plaque was placed in 250 µl BLT5615 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. 5 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 produces 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 of this was 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.

2.12.6 UV irradiation of phage

Phage were titred and then diluted to the required concentration in precipitation buffer. Phage were irradiated using the Stratalinker UV Crosslinker (Stratagene, CA, USA). UV irradiation was performed for 9 hours at 4°C using 120 mJ UV irradiation. Following irradiation the phage were re-plated to confirm their inactivation through a lack of plaque formation. Non-irradiated phage was used as a positive control.

2.12.7.1 *In vivo* phage display

In vivo phage display was performed in 18 week old C57Bl6/129SvJ ApoE-/- mice that had been maintained on a diet of 21% beef lard supplemented with 0.15% cholesterol for 10 weeks. Mice were anesthetised by i.p injection of 0.125 ml/10 g of 5% (v/v) Rompon (PANTEX, De Hoeve, Holland) 10% (v/v) Ketaset (Fort Dodge Laboratories Inc., Iowa, USA) diluted in PBS. Where a predose was required it was injected via the tail vein and left to circulate for 15 minutes, before injection of phage libraries. All animals received phage library via tail vein injection and it was left to circulate for 15 minutes. Mice were further anesthetised by intraperitoneal injection of approximately 0.1 ml Euthatal (Merial Animal Health, Essex, UK). Blood samples were taken by cardiac puncture before mice were perfused with PBS until all organs appeared clear of blood (approximately 5 minutes). Tissue samples were taken and snap frozen in liquid nitrogen then stored at -80°C.

2.12.7.2 Extraction of phage from tissues

Phage were extracted from the entire BCA, carotid artery and aorta or approximately 100 mg of the other tissues. 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 sec 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-idet P40 (NP40) was added and incubated on ice for 5 minutes. This was followed by the addition of 30 ml 1% sodium azide. Samples were stored at 4°C for up to 5 days before titering was performed.

2.12.7.3 Bulk amplification of phage from tissue homogenates

The same protocol was used as for the initial amplification of the libraries (section 2.12.2) but instead of calculating the amount of phage to add to the liquid culture, all the remaining tissue homogenate from the animals in the group was added to the culture.

2.12.7.4 Amplification of individual phage

The standard amplification protocol was followed (section 2.12.2) but 5 ml cultures were used and 100 µl of the small-scale phage amplification used for PCR was added to the culture. Once lysed the cultures were pooled for purification.

2.13 AAV Library Methods

2.13.1 Production of AAV library

The AAV libraries were produced by Dr L. Perabo, University of Cologne, Germany (Perabo *et al.*, 2003). Briefly, a library of AAV plasmids (p587Lib7) was synthesised by inserting oligonucleotides encoding 7 amino acids into *Asc*I and *Not*I restriction sites in the cap gene, so that the peptides would be expressed after residue 587 (Figure 2.2). The plasmid library was transformed into DH5α *E.Coli* and amplified. Viruses were produced by co-transfection of the library plasmids and pXX6, using a protocol similar to that used to make peptide-modified AAVs (Section 2.5.8). Virions were purified on an iodixanol gradient and titred by dot blot analysis.

2.13.2.1 *In vitro* biopanning with the AAV library

Cells were seeded in T25 flasks and incubated overnight at 37°C to produce 70-80% confluence. RGE cells were co-infected with wild type Ad at MOI 500 and AAV library at MOI 20000 for 24 hours before the cells were washed in PBS and placed in fresh media. HeLa cells were co-infected with wild type Ad at MOI 10 and AAV library at MOI 20000 for 2 hours and then washed and placed in fresh media. 5 days after infection, cells were washed in PBS and resuspended in 500 µl AAV lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5). Cells were lysed by freeze/thawing three times in liquid nitrogen and a 37°C waterbath. The supernatant was removed and the cell pellet was re-suspended in 200 µl PBS. 400 µl of supernatant was used to infect cells for the

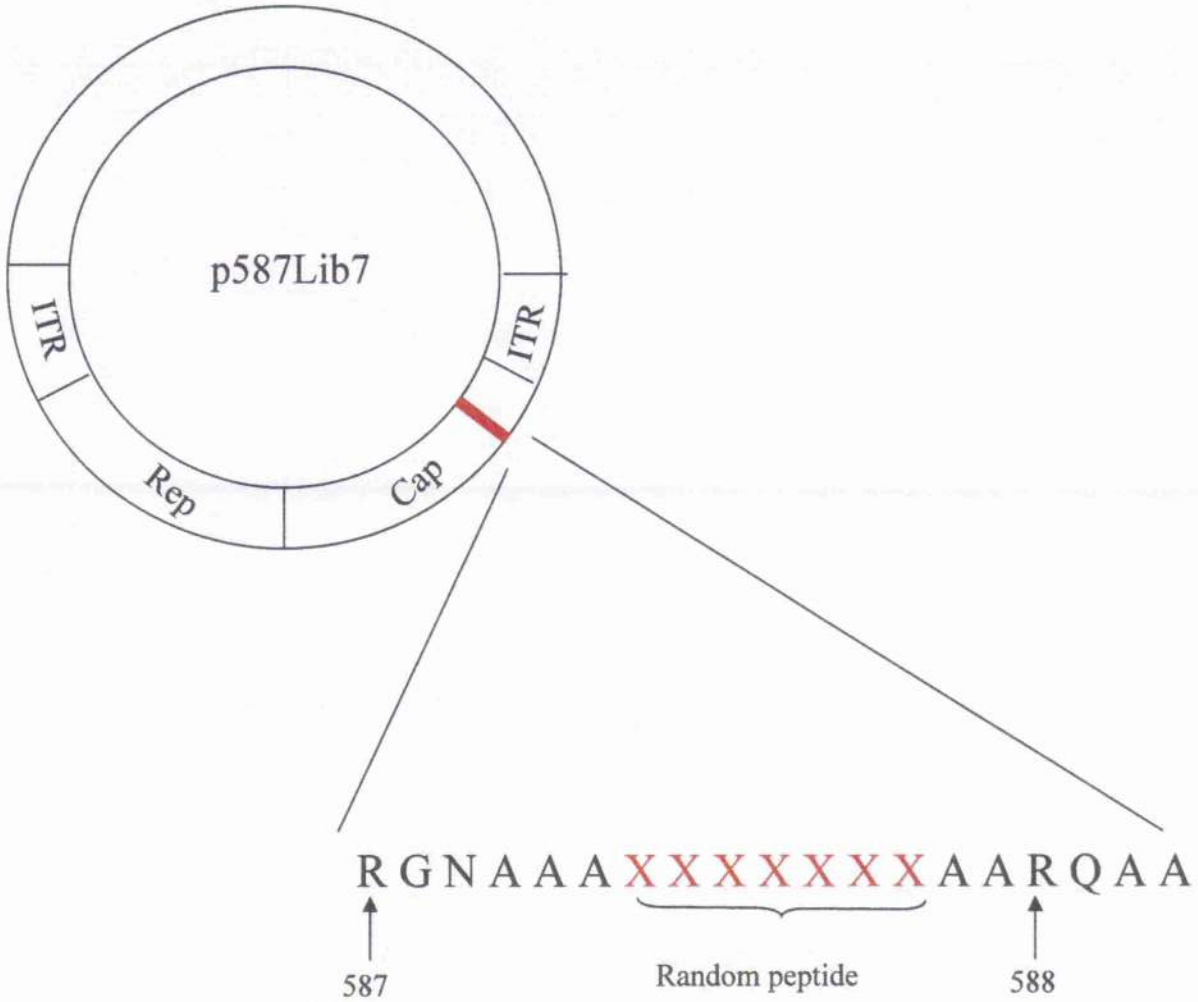


Figure 2.2 Schematic diagram of p587Lib, the plasmid used to produce the AAV library. The amino acid sequence at the peptide insertion site in the cap gene is shown. PCR mutagenesis was used to insert 5 alanine residues into the plasmid after residue 587 of the cap protein, to incorporate the *Asc*I and *Not*I restriction sites, which were required for cloning of the random peptides.

next round of biopanning and the remaining 100 µl was used for real time PCR and sequencing.

2.13.2.2 PCR and sequencing analysis of cell fractions from AAV library biopanning

DNA was extracted from biopanning cell media, pellet and supernatant samples using the QIAamp DNA Mini Kit (Qiagen, CA, USA) as per manufacturer's instructions. PCR was performed using primers (AAVF 5'-AAGCCACAAGGACGATGAAGA-3' and AAVR 5'-ATGTCCGTCCGTGTGTGGAAT-3') to amplify a 330 bp region of the cap gene that contains the peptide insertion. Reactions contained 5 µl of template DNA, 200 µM each dNTP (Promega, Southampton, UK.), 2.5 U Taq DNA polymerase (Promega, Southampton, UK.) and 0.25 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 95°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1 minute. PCR products were analysed by agarose gel electrophoresis. The PCR products were gel extracted using QIAquick Gel extraction kit (Qiagen, CA, USA) as per manufacturer's instructions. Briefly, the band was cut from the gel and placed in buffer at 50°C for 10 minutes to dissolve the gel. The solution was applied to a spin column and centrifuged to adsorb the DNA onto the membrane. The column was washed and then DNA was eluted in deionised water.

To sequence peptides from individual virions, the PCR product was TA cloned using Invitrogen TA cloning kit (Paisley, UK) as per manufacturer's instructions. Briefly, the purified PCR product was ligated to the plasmid pCR2.1using T4 DNA ligase. The plasmid was then transformed into TOP10 competent *E.coli* by heat shock. Blue/white screening was used to identify positive colonies, as colonies containing a ligated PCR product appeared white on X-gal plates. Each white colony was picked into 400 µl LB supplemented with 100 µg/ml ampicillin and grown in an orbital shaker at 37°C with shaking at 180 rpm for 4 hours. 1 µl of each culture was used as a template for PCR using M13 forward and reverse primers (5'-GTAAAACGACGGCCAG-3' and 5'-CAGGAAACAGCTATGAC-3' which anneal to pCR2.1 either side of the cloning site. Reactions contained 1 µl of template, 200 µM each dNTP (Promega, Southampton, UK.),

1.25 U Taq DNA polymerase (Promega, Southampton, UK.) and 0.25 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 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute. PCR products were analysed by agarose gel electrophoresis and cleaned using AmpPure (Agencourt Bioscience Corporation, MA, USA) as per manufacturer's instructions. Purified PCR products were re-suspended in 40 μl water and 10 μl of this was used in the sequencing reaction. Sequencing reactions contained 3.2 pmoles of AAVF 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 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 using SeqScape v2.0.

2.13.2.3 Analysis of AAV library peptide composition

Peptide composition was analysed using receptor ligands contact (RELIC) programs (http://relic.bio.anl.gov/relicPeptides.aspx).

2.13.3 Production of heparin binding and non-binding AAV libraries

The AAV library was split into heparin binding and non-binding pools based on affinity for a heparin column as described in section 2.6. This was performed by Dr L. Perabo, University of Cologne, Germany (Perabo *et al.*, 2006b).

2.13.4 *In vitro* biopanning with the heparin non-binding AAV library

Cells were seeded in 10 cm^2 tissue culture dishes (Nunc Wiesbaden, Germany) and incubated overnight at 37°C to produce 70-80% confluence. Cells were co-infected with wild type Ad at MOI 10 and AAV library at MOI 20000 for either 3 or 24 hours before the cells were washed in PBS and placed in fresh media. Seventy-two hours after infection, cells were washed in PBS and resuspended in 1.5 ml AAV lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5). Cells were lysed by freeze thawing three times in

liquid nitrogen and a 37°C waterbath. The supernatant was removed and the cell pellet was resuspended in 500 µl PBS. 1 ml of supernatant was used to infect cells for the next round of biopanning and the remaining supernatant was used for real time PCR and sequencing.

2.13.5 *In vivo* biodistribution studies with the AAV library

18 week old C57Bl6 ApoE-/- mice maintained on a diet of 21% beef lard supplemented with 0.15% cholesterol. for 12 weeks received 4 x 10^{10} gp AAV library or AAV control via tail vein injection. Twenty-four hours after injection, blood samples were taken by cardiac puncture, then mice were perfused with PBS. Tissues were removed and snap frozen in liquid nitrogen. DNA was extracted from tissues using the QIAamp DNA mini kit (Qiagen, CA, USA). This was used as a template for PCR and sequencing or real-time PCR for biodistribution analysis.

To produce a restricted library for the second round, the peptide containing region of virions extracted from the target tissue had to be amplified by PCR, cloned into p587Lib7 and used to synthesise a new pool of the library. PCR was carried out using primers 5'-GTAGCCATGGAAACTAGATAAGAAAGAATACG-3' and 5'-TACCAGCTCCCGTACGTCCTCGGC-3' which amplifies a 1.3 kb region of the genome which can be ligated into the plasmid using restriction sites *SnaB*I and *BsiW*I. PCRs contained 5 µl of tissue extracted DNA as the template, 200 µM each dNTP (Promega, Southampton, UK.), 1.25 U Taq DNA polymerase (Promega, Southampton, UK.) and 0.25 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 95°C for 40 seconds, annealing at 59°C for 40 seconds and extension at 68°C for 2 minutes. PCR products were analysed by agarose gel electrophoresis and cleaned using AmpPure (Agencourt Bioscience Corporation, MA, USA) as per manufacturer's instructions.

Cloning of the PCR products was carried out by Dr. H. Buening (University of Cologne, Germany) using the original protocol for producing the library (Section 2.13.1).

For the second round of biopanning the same method was used, but each mouse received 2×10^{10} gp as the titre of the virus pools were lower than that of the original library.

2.13.6 *In vivo* biodistribution of heparin non-binding and binding AAV libraries

C57/Bl6 mice received 4×10^{9} gp of A3, non-binding library, AAV-RC or binding library via tail vein injection. 24 hours after injection, blood samples were taken by cardiac puncture, then mice were perfused with PBS. Tissues were removed and snap frozen in liquid nitrogen. DNA was extracted from tissues using the QIAamp DNA mini kit (Qiagen, CA, USA) and used as a template for real-time PCR to analyse the biodistribution profiles of the viruses.

2.14 Surface plasmon resonance analysis

Surface plasmon resonance experiments were carried out by Dr J. McVey (Imperial Collage London) using a Biacore X instrument (Biacore, Stevenage, UK). Human MT1-MMP was immobilised onto a CM5 biosensor chip according to the manufacturer's instructions. Virus was passed over the chip at a flow rate of 20 µl/min.

2.15 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 was used to analyse the results, which were considered significant when $p<0.05$.

Chapter 3:
Production and Characterisation of Atherosclerotic Plaque Targeted Viral Vectors

3.1 Introduction

Genetic interventions aiming to prevent rupture of unstable atherosclerotic plaques are likely to be most effective using a vector that can provide transgene expression specifically in the plaque following intravenous injection. This is due to the need for an adequate dose of therapeutic agent to be provided locally to act on the appropriate target cells. Development of a vector with target tissue selectivity is required to prevent unwanted transgene expression in other tissues, to reduce the chance of possible side effects and enable the use of a lower dose to reduce potential vector toxicity. Therefore the ideal vector needs to be amenable to engineering to install targeting, stable in blood, non-toxic, efficient at cell internalisation and producing transgene expression and producible at high titres. Viral vectors may provide the best tool for achieving this as they provide a highly efficient and adaptable gene delivery system. However, as yet no vectors that selectively target atherosclerotic plaques have been identified, although methods of targeting vectors are now well established and may be applied to overcome this problem.

Healthy vasculature and atherosclerotic plaques are clearly very different at the cellular and molecular level (Ross, 1993). Molecules that are selectively upregulated on the surface of plaques could potentially function as receptors for gene therapy vectors. If peptides that target these molecules can be identified, it should be possible to exploit these differences to target viral vectors to plaques with a high degree of specificity. Liu *et al.* (Liu *et al.*, 2003) performed biopanning with an M13 phage library in an ApoE-/- mouse model of atherosclerosis to investigate molecular differences between atherosclerotic and healthy vasculature. The biopanning identified 103 plaque-targeting peptides, 3 of which have been used in this study.

3.1.1 CAPGPSKSC (CAP) peptide

The most commonly occurring peptide motif ($^{P}/_{Q}{}^{G}/_{Y}PS^{K}/_{R}SC$) was found at a frequency of 9.7% and is contained within the CAPGPSKSC (CAP) peptide. Further work showed that this peptide was unable to bind the aortic endothelium of healthy mice, but did bind to human arterial atherosclerotic lesions *ex vivo*, suggesting that the peptide has a plaque-specific targeting capacity (Liu *et al.*, 2003). It also suggests that the disease model maybe a reliable reflection of atherosclerosis in humans in this

context, as it demonstrated that the peptide was not species or model specific. BLAST (Basic local alignment search tool) searching showed the CAP peptide has homology to Chymase I, a serine protease that is involved in angiotensin II generation. Affinity chromatography followed by mass spectrometry identified glucose-regulated protein 78 (Grp78) (also known as BiP) as the putative receptor for this peptide (Liu *et al.*, 2003). Grp78, a member of the hsp70 family, was originally identified as an endoplasmic reticulum (ER) chaperone protein where it has a role in the unfolded protein response defense mechanism (Kaufman, 1999, Lee, 2001, Reddy *et al.*, 2003). It is constitutively expressed in the ER of almost all cell types (Kim *et al.*, 2006) and has been shown to be expressed on the surface of cells under stress (Delpino and Castelli, 2002). Its expression is upregulated in many cells under pathological conditions including cancer cells (Shin *et al.*, 2003, Asplin *et al.*, 2000), rheumatoid fibroblasts (Misra *et al.*, 1997), cardiomyocytes following myocardial infarction (Thurerauf *et al.*, 2006) and ischemia (Szegezdi *et al.*, 2006) and in atherosclerotic plaques (Bhattacharjee *et al.*, 2005). This upregulation is thought to be caused by conditions such as ischemia, hypoxia and glucose deprivation, which often occur in these disease states. Higher cellular levels of Grp78 expression result in its increased localisation to the cell membrane (Delpino *et al.*, 1988).

In atherosclerosis Grp78 is expressed on the endothelium of plaques and on the surface of monocytes and foam cells within plaques (Bhattacharjee *et al.*, 2005) (Outinen *et al.*, 1999). At the cell surface it can associate with the low-density lipoprotein receptor-related protein (Misra *et al.*, 2002), which is also upregulated during plaque progression (Llorente-Cortes *et al.*, 2004). There are several mechanisms that may cause an increase in Grp78 expression in plaques. The ER stress/unfolded protein response pathway that increases expression of Grp78 is active at all stages of development of atherosclerosis (Zhou *et al.*, 2005). Peroxynitrate, an oxidant (generated by NO reacting with superoxide) that causes ER stress and is associated with progression of atherosclerosis can upregulate Grp78 in human ECs (Dickhout *et al.*, 2005). Hyperhomocysteinemia a known risk factor for atherosclerosis (Wald *et al.*, 2002) is also thought to cause an increase in Grp78 expression, as it has been shown that stimulating human umbilical vein ECs with homocysteine causes an upregulation in Grp78 expression (Kokame *et al.*, 1998). Inducing hyperhomocysteinemia in ApoE-/- mice has also been shown to increase

expression of Grp78 in advanced plaques, and it was found to be mainly localised to the plaque cap (Zhou *et al.*, 2004).

The role of Grp78 in atherosclerotic plaques is still unclear. It may have a protective role in inhibiting atherosclerosis by decreasing the procoagulant activity of tissue factor, through direct binding of tissue factor in the cell membrane (Watson *et al.*, 2003, Bhattacharjee *et al.*, 2005). It may also be involved in inhibiting homocysteine-induced gene expression (Werstuck *et al.*, 2001). It is speculated that homocysteine causes cellular stress as it reduces NO bioavailability (Outinen *et al.*, 1999). This alters the intracellular redox potential and produces reactive oxygen species, which results in the accumulation of unfolded proteins in the ER. Grp78 may have a protective role in overcoming this (Kokame *et al.*, 1998, Eikelboom and Lonn, 1999). Homocysteine also promotes SMC growth, which may contribute to the development of atherosclerosis (Tsai *et al.*, 1994).

Interestingly Grp78 has been proposed to act as a native receptor for some viruses. It can associate with class 1 major histocompatability protein (MHC class I) on the cell surface and this complex has been shown to act as a co-receptor for coxsackie virus A9 (CAV9) in combination with the receptor $\alpha_v\beta_3$ integrin (Triantafilou *et al.*, 2002). It has also been proposed to act as a receptor for dengue virus serotype 2 (Jindadamrongwech *et al.*, 2004). As other viruses use Grp78 as a receptor, this shows that it must be accessible for virus binding and can enable vector internalisation, which is an important requirement for successful gene delivery.

Other phage display studies have identified Grp78 as a potential receptor for peptides. Biopanning on a human metastatic melanoma cell line identified the peptide CTVALPGGYVRVC that uses Grp78 as a receptor (Kim *et al.*, 2006). Phage display for antibody fingerprinting (phage display on immobilised patient immunoglobulins) identified Grp78 as a potential receptor for targeting tumours (Mintz *et al.*, 2003). In another phage display study immobilised Grp78 was used as the target and this identified the targeting peptides WIFWIQL and WDLAWMFRLPVG (Blond-Elguindi *et al.*, 1993), which target tumour cells in breast and prostate cancer models and human prostate cancers *ex vivo* (Arap *et al.*, 2004). These studies demonstrate

that Grp78 expression may be upregulated in several cancers and verified that Grp78 can act as a molecular target. None of the Grp78 targeting peptides share any common motifs, implying that they bind to different regions of the protein.

3.1.2 CQEPTRLKC (CQE) peptide

The second most commonly occurring motif from the biopanning was ($E^{P}/_{G}{}^{W}/_{T}R^{L}/_{A}{}^{K}/_{T}C$), which occurred in 4.9% of peptides and is found in the peptide CQEPTRLKC (CQE) (Liu *et al.*, 2003). BLAST studies identified homology with a novel protein described as an interferon-induced protein (Liu *et al.*, 2003). No further experiments were carried out to provide any information about the possible receptor for the peptide.

3.1.3 CNHRYMQMC (CNH) peptide

Another motif ($CN^{Q}/_{T}R^{H}/_{Y}$MQMSC) that occurred at a relatively high frequency (1.8%) was contained in the peptide CNHRYMQMC (CNH). This peptide is of particular interest as BLAST scanning showed it has homology to tissue inhibitor of matrix metalloproteinases 2 (TIMP2) (Liu *et al.*, 2003). TIMPs are naturally occurring inhibitors of matrix metalloproteinases (MMPs), a group of 24 structurally related zinc proteases that degrade extracellular matrix proteins. Many MMPs are known to be expressed in atherosclerotic plaques where they have a role in vascular remodeling, SMC migration, neointima formation, angiogenesis and plaque disruption (Rajavashisth *et al.*, 1999, Brown *et al.*, 1995, Galis *et al.*, 1994, Li *et al.*, 1996, Henney *et al.*, 1991, Nikkari *et al.*, 1995, Southgate *et al.*, 1996, Johnson *et al.*, 2005a). There are four members of the TIMP family, TIMPs -1, -2, -3 and -4, which vary in their tissue distribution, transcriptional regulation and metalloproteinase specificity, although there is some overlap (Lafleur *et al.*, 2003). For example, TIMPs 2-4 inhibit membrane type 1 matrix metalloproteinase (MT1-MMP) whereas TIMP1 has no effect (Nagase *et al.*, 2006).

TIMP2 has 2 functions, it is required for the activation of MMP2 and it acts as an inhibitor of proteases activated by MT1-MMP. Activation of pro-MMP2 occurs when TIMP2, pro-MMP2 and 2 MT1-MMP molecules form a complex in the cell membrane (Strongin *et al.*, 1995) (Butler *et al.*, 1998). MT1-MMP then cleaves pro-MMP2 to produce the active form of the protein. This process is dependent on the

TIMP2 concentration. An excess of TIMP2 prevents the second MT1-MMP molecule from binding so inhibits MMP2 activation (Hernandez-Barrantes *et al.*, 2001). High levels of TIMPs -3 and –4 also inhibit pro-MMP2 activation (Hernandez-Barrantes *et al.*, 2001). However, in the absence of TIMP2, MT1-MMP undergoes autolytic proteolysis to an inactive form, so TIMP-2 has both a positive and negative affect on MT1-MMP activity (Hernandez-Barrantes *et al.*, 2000).

As TIMP2 is known to interact with MT1-MMP at the cell surface, it is possible that the TIMP2 homologous peptide CNH may also bind to MT1-MMP. TIMP2 competitively inhibited the binding of the CNH peptide to human umbilical vein endothelial cells (HUVEC), suggesting that the peptide does bind to a TIMP2 receptor (Liu *et al.*, 2003). However, the region of TIMP2 homologous to the peptide differs from the regions of TIMP2 known to be involved in contact with MT1-MMP (Fernandez-Catalan *et al.*, 1998) so it has been proposed that it may be binding to an alternative previously unrecognised TIMP2 receptor (Liu *et al.*, 2003, Chesler *et al.*, 1995, Corcoran and Stetler-Stevenson, 1995).

Relatively high levels of TIMP2 expression have been detected in atherosclerotic plaques (Rajavashisth *et al.*, 1999), where its metalloproteinase activity might limit plaque progression (George, 2000). MT1-MMP is expressed in both normal and atherosclerotic human arteries and has been localised to macrophages and SMC within plaques (Rajavashisth *et al.*, 1999, Galis *et al.*, 1994). In the presence of inflammatory molecules such as oxLDL, IL-1α and TNFα, smooth muscle cell expression of MT1-MMP is upregulated (Rajavashisth *et al.*, 1999). Therefore expression levels are higher in atherosclerotic plaques than healthy vasculature. This suggests MT1-MMP may be a promising target for a gene delivery system selective for atherosclerotic plaques. MT1-MMP is a 64 kDa protein, and is one of six MMPs that have a single transmembrane domain. The catalytic domain is displayed on the exterior of the cell (Sato *et al.*, 1994). It is involved in activation of MMP2 and degradation of many extracellular matrix proteins including gelatin, fibronectin, proteoglycans, aggrecan and type I, II and III collagens (Pei and Weiss, 1996, Ohuchi *et al.*, 1997, d'Ortho *et al.*, 1997). Recently MT1-MMP has also been shown to be

involved in monocyte migration and transmigration through activated endothelial cells (Matias-Roman *et al.*, 2005).

The three peptides CAP, CNH and CQE are therefore promising candidate peptides for targeting vectors to atherosclerotic plaques, so it is necessary to test their targeting capacity in a range of vector platforms.

3.1.4 Viral vector platforms

The most commonly used viral vectors for cardiovascular disease gene therapy are based on Ad5. However, following systemic administration, Ad5 does not transduce the vasculature, as it is highly efficient at infecting other organs, particularly the liver and spleen. Strategies used for producing genetically targeted Ads with altered tropism are now well established (See section 1.11.2). Insertion of targeting peptides into the HI loop of the virus fiber has proven to be an efficient way of expanding viral tropism by enabling the virus to bind a novel receptor (Krasnykh *et al.*, 1998) (Dmitriev *et al.*, 1998, Nicklin *et al.*, 2001c). To produce a virus with a high level of cellular specificity it is also necessary to detarget the virus from its native receptors. Mutations that ablate Ad5 binding to its receptors CAR (Roelvink *et al.*, 1999, Jakubczak *et al.*, 2001), integrins (Wickham *et al.*, 1993, Wickham, 1994, Huang *et al.*, 1995) and HSPG (Smith *et al.*, 2003a, Smith *et al.*, 2003b, Nicol *et al.*, 2004) have been identified and shown to reduce the natural tropism of the virus. By combining the detargeting mutations with the plaque targeting peptides it should be possible to produce a highly selective vector.

Ad5 can also be pseudotyped to produce vectors with altered tropism. As yet, no pseudotypes with a high degree of selectivity for vascular cells have been identified but there are some promising pseudotypes that might provide a useful platform for the development of a plaque-targeted vector. Ad5 pseudotyped with the fiber of Ad19p, a subgroup D virus, has been shown to have a reduced tropism for rat liver *in vivo* and mouse, rat and human hepatocytes *in vitro* (Denby *et al.*, 2004). It also transduces human endothelial cells and smooth muscle cells at least as efficiently as Ad5 (Denby *et al.*, 2004). Ad19p also has a favourable safety profile as it has only been isolated once (in 1955 in Saudi Arabia) and unlike most Ad serotypes it is not associated with any disease (Arnberg *et al.*, 1997). In the general population neutralising antibodies

to Ad19p occur at a much lower frequency compared to Ad5 neutralising antibodies, as they have been found in less than 20% and 80% of people respectively (Vigne *et al.*, 2003). Therefore although the Ad19p receptor has not yet been identified, it has many features that suggest it may be a useful serotype for providing a vector naturally detargeted from the liver which can be modified to be retargeted to the vasculature.

AAV2 has many features that suggest it could be developed into an effective plaque targeted vector. It has been shown to produce sustained transgene expression, it is not thought to be associated with any human pathology and it is less immunogenic than Ad vectors (Monahan and Samulski, 2000a). However, following systemic administration of AAV2 a very low level of transduction of vascular cells is achieved (White *et al.*, 2004, Work *et al.*, 2006) and the majority of the virus is found in the liver and spleen (Nathwani *et al.*, 2001, Koeberl *et al.*, 1997). Insertion of targeting peptides into the HSPG binding site of the virus capsid has been shown to reduce its natural tropism and retarget it to alternative cell types (Girod *et al.*, 1999). For example, peptide insertion has been shown to enable targeting to endothelial cells (Nicklin *et al.*, 2001a, White *et al.*, 2004), smooth muscle cells (Work *et al.*, 2004a) and the vasculature of the lung and brain (Work *et al.*, 2006). Therefore if plaque-targeting peptides can be identified and incorporated into the AAV2 capsid it may result in a highly efficient gene delivery vector.

3.1.5 Aims

The aims of this chapter are:

- To produce Ad and AAV vectors with the 3 plaque targeting peptide CAPGPSKSC, CNHRYMQC and CQEPTRLKC displayed on the capsid surface.
- To investigate the tropism of these vectors by performing *in vitro* transduction assays in a range of vascular and non-vascular cell types.
- To determine the biodistribution profile of selected vectors in a mouse model of atherosclerosis to investigate whether the vectors selectively target areas of atherosclerotic vasculature.

3.2 Results

3.2.1 ApoE-/- mouse model of atherosclerosis and plaque stability

One of the most reliable and established animal models of atherosclerosis uses ApoE-/- mice on a C57/Bl6 background. Maintaining these mice on a high fat diet supplemented with cholesterol causes the development of complex atherosclerotic lesions in the brachiocephalic artery (BCA) after a relatively short period of time (Johnson and Jackson, 2001). A new colony of mice was established for this study, so histology was performed to confirm that mice developed advanced lesions. From the age of 8 weeks mice were fed a high fat diet for 10 or 12 weeks before histological analysis was performed on the BCAs. Animals maintained on a normal chow diet showed no signs of atherosclerosis (13.2A), whereas mice fed a high fat diet for 10 weeks developed large stable lesions with thick fibrous caps (Figure 3.1B). Mice fed a high fat diet for 12 weeks generally developed more advanced lesions (Figure 3.1C). To further the development of advanced plaques with an unstable phenotype, mice received the high fat diet from 6 weeks old (Figure 3.1D). One mouse (from 3 examined) had a plaque with a buried fibrous cap indicative of an earlier plaque rupture and another animal had a very large almost totally occlusive plaque. Although a very small sample number has been used in this study, it suggests the mice consistently developed advanced plaques, but not plaque ruptures, therefore this model can be used for development of vectors that target advanced plaques.

The majority of animals used in this work were fed a high fat diet for 12 weeks from the age of 6 weeks and had visible lesions in the BCA at the time of dissection. Where stated, age matched mice maintained on a normal chow diet were used.

3.2.2.1 Production and *in vitro* characterisation of plaque targeted vectors based on the CAR binding mutant AdKO1

To initially test the ability of the peptides CAP, CNH and CQE to alter the *in vitro* tropism of Ad5, the peptides were inserted into the III loop of the AdKO1 vector, which has previously been shown not to bind CAR due to the knob mutations S408E and P409A (Nicklin *et al.*, 2001c, Jakubczak *et al.*, 2001). The AdKO1 vector provides a simple system in which the *in vitro* targeting capacity of the peptides can be assessed in the context of the Ad vector. The resulting virus particle titres (Table

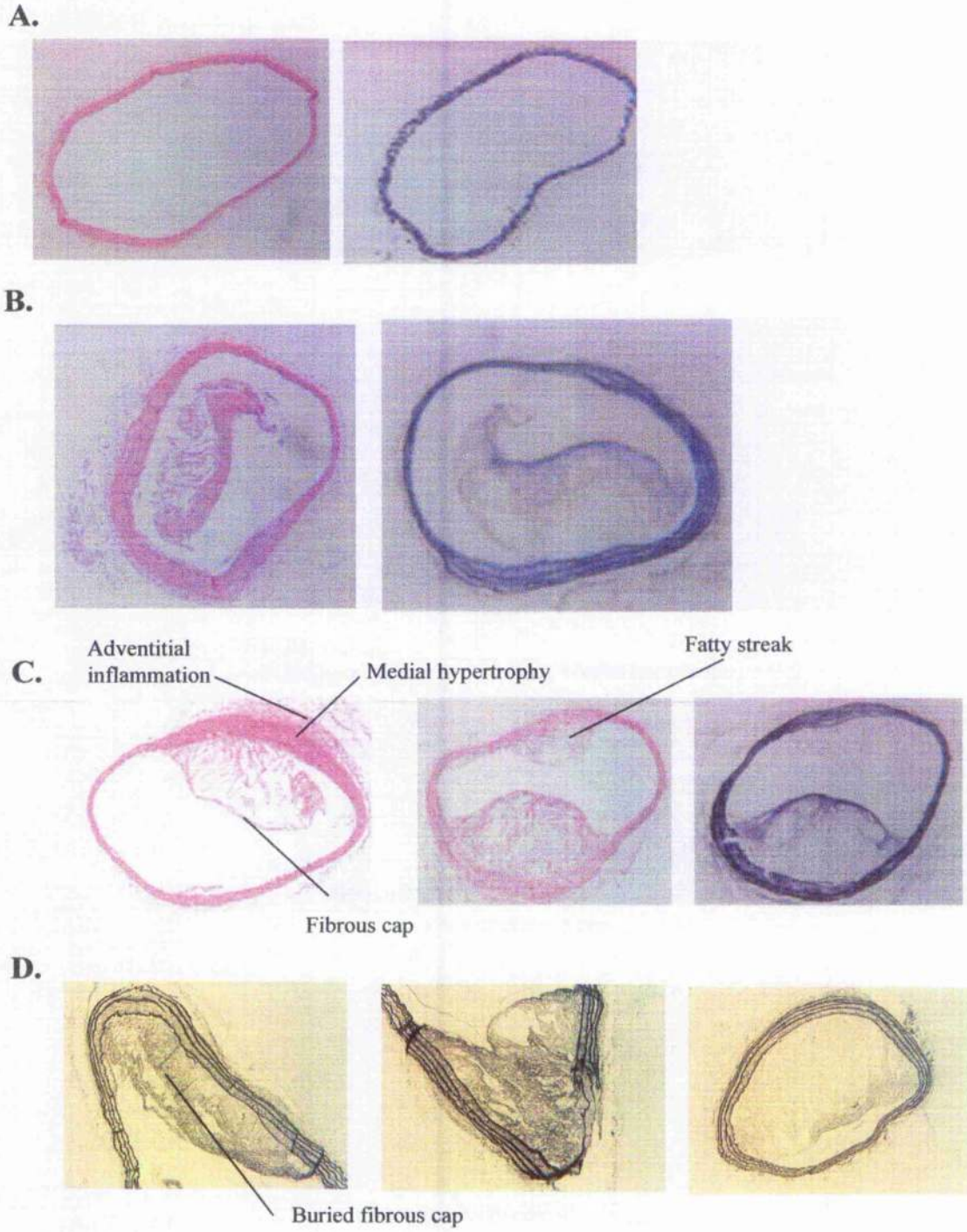


Figure 3.1 Example cross sections of BCAs from ApoE-/- mice stained with H and E or EVG. Mice were maintained on **A.** a normal chow diet **B.** a Western diet of of 21% beef lard supplemented with 0.15% cholesterol for 10 weeks from the age of 8 weeks **C.** a Western diet for 12 weeks from the age of 8 weeks. **D.** a Western diet for 12 weeks from the age of 6 weeks.

3.1) were all in the expected range, suggesting peptide insertion did not have a detrimental affect on capsid formation. Western blot analysis of the purified virus was able to detect the recombinant monomer fiber (60 kDa) under denatured conditions and fiber trimers (180 kDa) under semi-native conditions, indicating that peptide insertion did not affect capsid formation (Figure 3.2).

To examine the effects of the capsid modifications on virus infectivity, infections of several vascular cell lines were performed with the AdKO1-CAP, AdKO1-CNH and AdKO1-CQE. In all the vascular cell types tested all 3 peptide-modified viruses (except AdKO1-CAP in SVEC 4-10) produced significantly higher transduction than the parental AdKO1 virus ($p<0.05$) (Figure 3.3). As a control, infections were also carried out in a non-target cell line, HeLa. HeLa cells were chosen since they are a non-vascular cell line and do not express the putative receptors for the CAP peptide (Grp78) (Chae *et al.*, 2004) and CNH peptide (MT1-MMP) (Zhai *et al.*, 2005). There was no significant difference in transduction between any of the peptide-modified viruses and the AdKO1 virus (Figure 3.3). These results suggest that all 3 peptide-modified AdKO1 viruses have an increased tropism for vascular cells.

3.2.2.2 Infection of AdKO1 viruses in the presence of anti-CAR antibody

To test whether the peptide-modified vectors still require binding to CAR to infect cells, or whether the peptide insertion enables them to use an alternative receptor, infection of the mouse endothelial cells was repeated in the presence of a CAR-neutralising antibody that is known to reduce AdCTL infectivity (Cohen *et al.*, 2001) or control rabbit serum (Figure 3.4). Compared to cells incubated with control serum, only the transduction of AdCTL was significantly reduced ($p<0.05$) by the presence of the anti-CAR antibody whereas AdKO1, AdKO1-CAP, AdKO1-CNH and AdKO1-CQE transduction was unaffected, suggesting that the AdKO1 viruses do not use CAR for cellular attachment.

3.2.3 Production and *in vitro* characterisation of AdKO1S* viruses

The *in vitro* results using the AdKO1 viruses indicate the CAP, CNH and CQE peptides may efficiently target the vectors to cells of the vasculature, but it is important to determine whether this vascular transduction can be replicated *in vivo* following systemic injection of the viruses. However, it is well known that the

Virus	Particle titre (vp/ml)
AdCTL	3.75×10^{12}
AdKO1	3.75×10^{12}
AdKO1-CAP	5.03×10^{12}
AdKO1-CNH	4.59×10^{12}
AdKO1-CQE	2.32×10^{12}

Table 3.1 Example particle titres of genetically engineered Ad vectors.

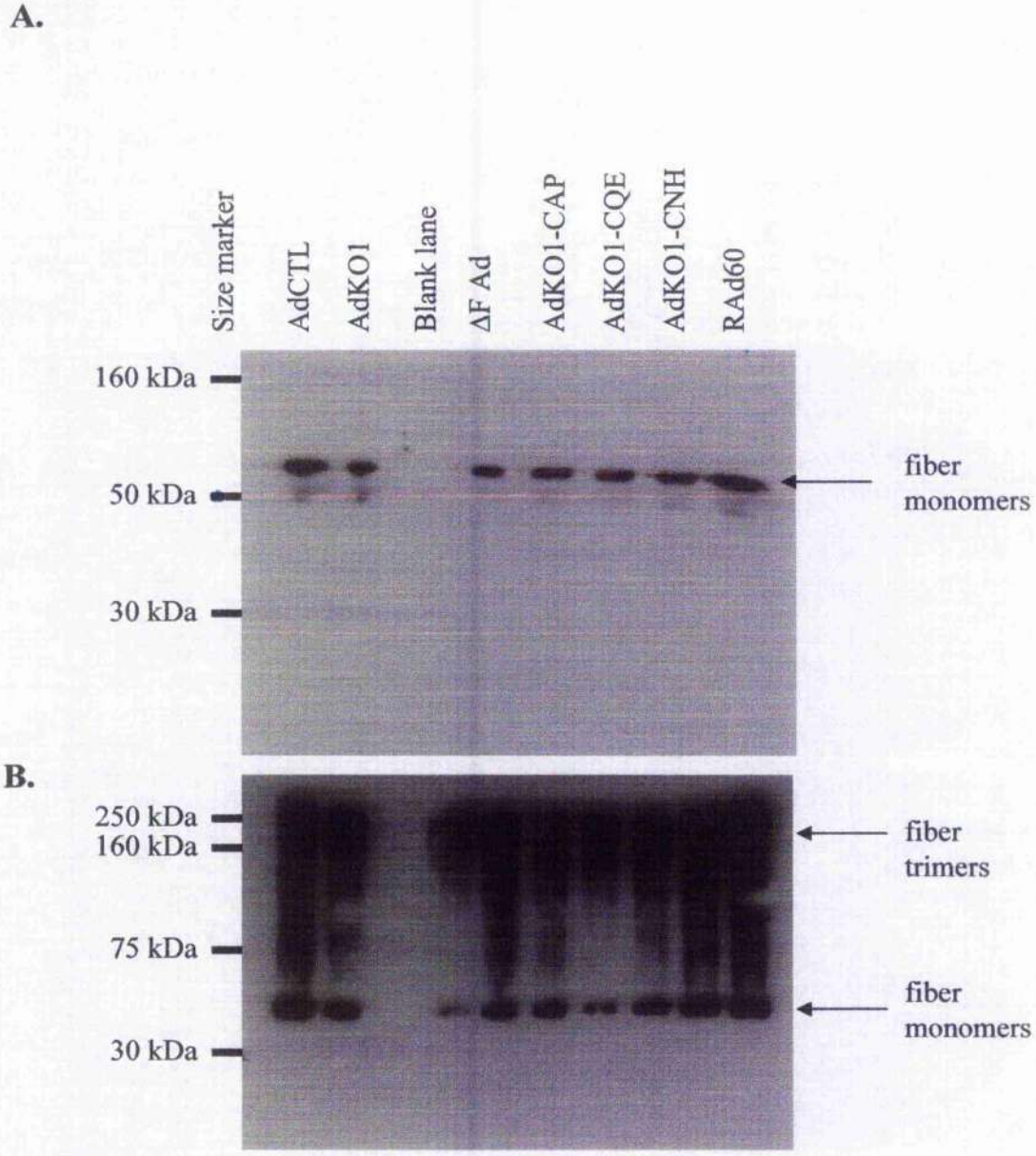


Figure 3.2 Western blot of fiber-modified Ad vectors. 2 x 10^{10} vp of each virus was used in western blotting to detect the virus fiber **A.** under reducing conditions **B.** under non-reducing conditions. Full range rainbow marker (Amersham Biosciences, Bucks, UK) was used to determine the size of proteins detected. Fiber monomer = 60 kDa. Fiber trimer = 180 kDa.

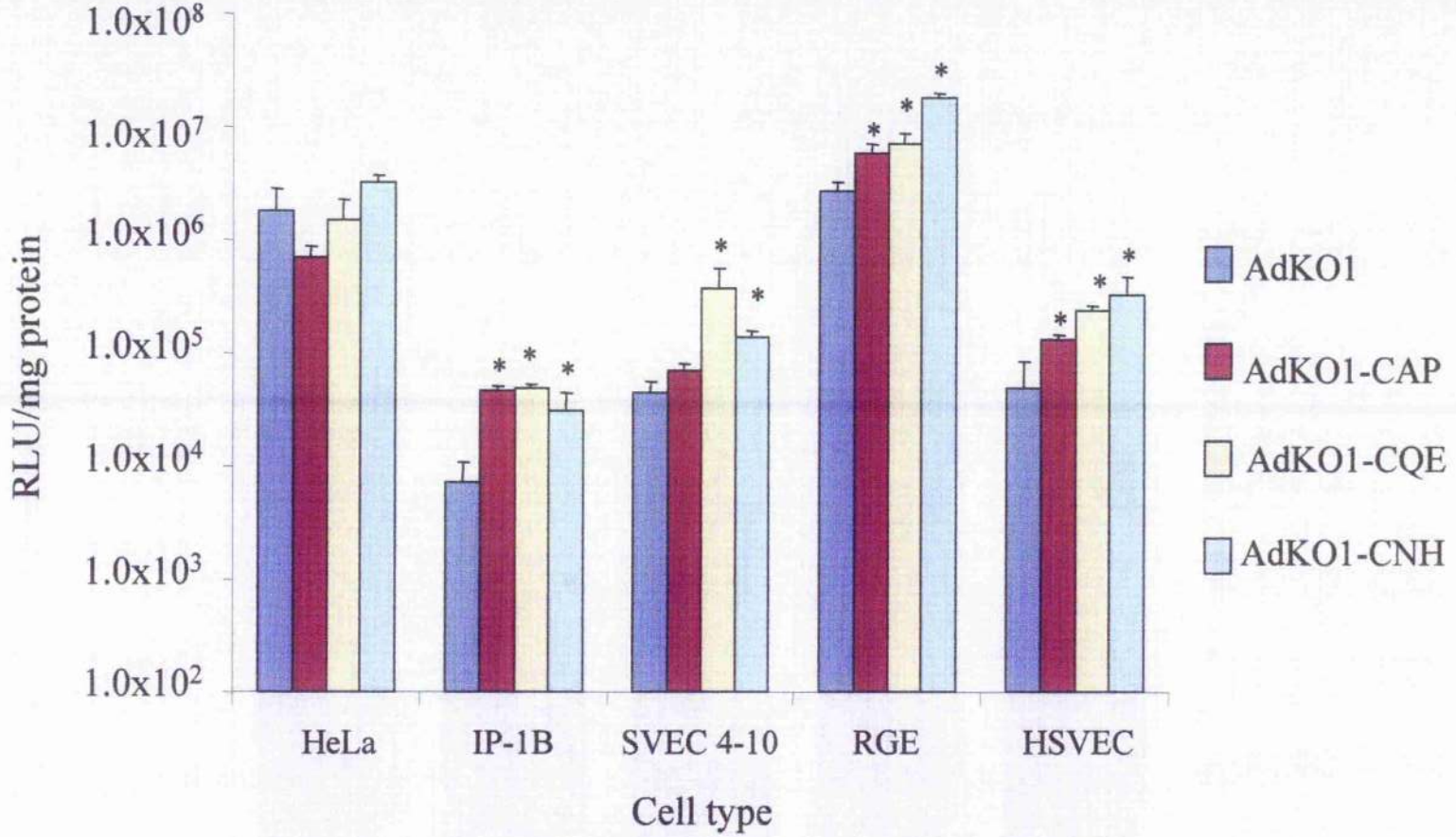
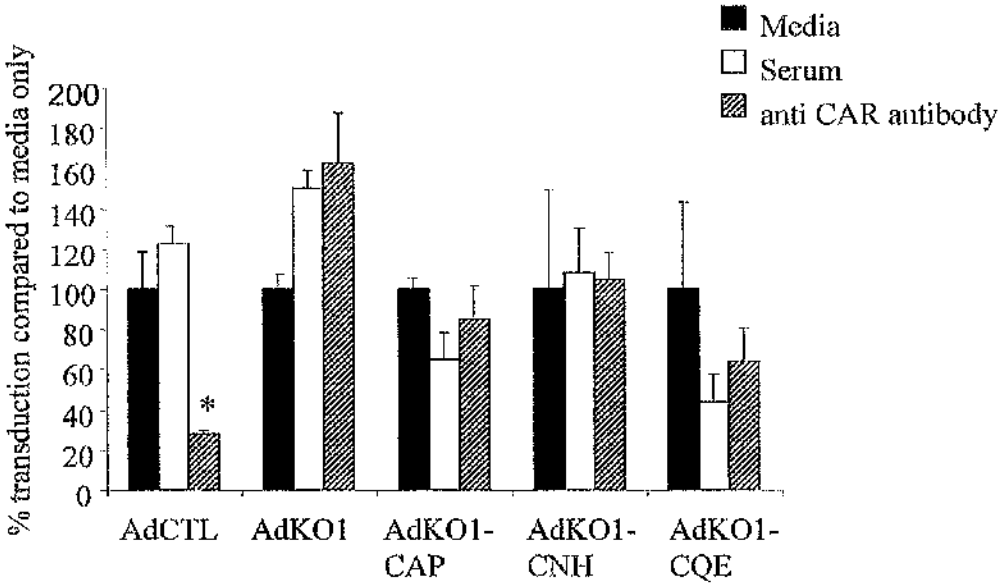


Figure 3.3 ***In vitro*** **infection of peptide-modified AdKO1 viruses.** Cells were infected with an MOI of 10,000 for 3 hours, then washed and incubated in fresh media for 48 hours. β-galactosidase (β-gal) activity in the cell lysates was quantified and levels were normalised to the total protein content. * $p < 0.05$ vs AdKO1.

A.



B.

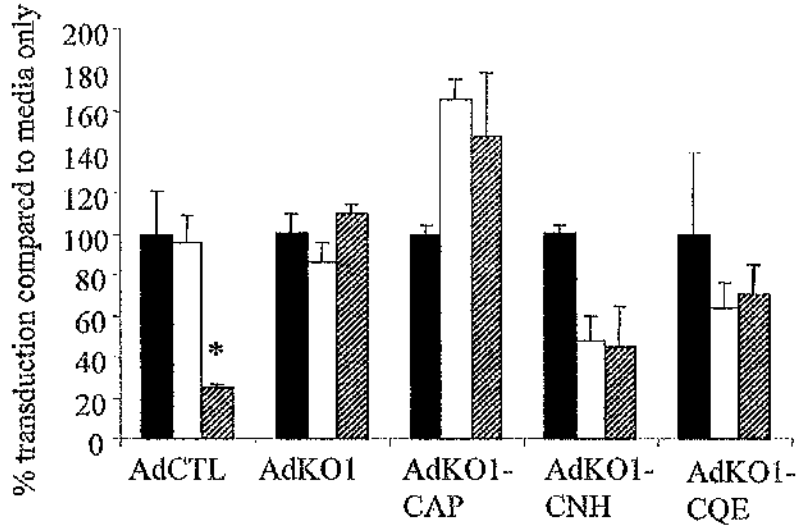


Figure 3.4 Infection of AdKO1 vectors in mouse endothelial cells in the presence of an anti-CAR antibody. A. IP-1B cells **B.** SVEC 4-10 cells. Cells were pre-incubated on ice for 30 minutes in serum free media, media containing control serum or anti-CAR antibody diluted 1 in 200 in serum free media. Cells were infected with an MOI of 10,000 for 3 hours, washed and incubated in fresh media for 48 hours. Cell lysate was used in a β-gal assay to quantify the transgene expression, which was normalised to the total protein content of the sample. * $p < 0.05$ vs serum.

AdKO1 mutation alone is insufficient for detargeting Ad5 from the liver (Smith *et al.*, 2003b, Einfeld *et al.*, 2001, Leissner *et al.*, 2001, Smith *et al.*, 2002, Nicol *et al.*, 2004). A previous study with peptide-modified AdKO1 viruses targeted to ECs showed that although the viruses were effective *in vitro* they failed to have any retargeting effect *in vivo* (Nicklin *et al.*, 2004). The CAP, CNH and CQE peptides were therefore incorporated into the fiber of AdKO1S* which has been shown to produce an approximately 1000-fold decrease in liver transduction compared to AdCTL following systemic administration in rats, mice and non-human primates (Nicol *et al.*, 2004, Smith *et al.*, 2003b, Smith *et al.*, 2003a). Therefore AdKO1S* may provide a potentially useful platform for *in vivo* retargeting.

The AdKO1S* vectors were generated using the same transient transfection/infection method as the AdKO1 viruses. All particle titres were in a similar range (between 1.5 x 10^{12} vp/ml and 9 x 10^{12} vp/ml) suggesting the capsid modifications had not affected virus formation.

Initially, the AdKO1S* viruses were tested *in vitro* using the same method as for the AdKO1 viruses. This showed that in all cell lines tested (RGE, IP-1B, SVEC 4-10, HSVEC, human coronary artery endothelial cells (HCAEC), HeLa cells and mouse hepatocytes the amount of transgene expression was never significantly higher than the background levels measured in uninfected cells (data not shown). Staining of cells infected with MOI 10000 confirmed the lack of transgene expression (data not shown). We therefore sought an alternative vector platform for *in vivo* testing of the peptides.

3.2.4 Production and *in vitro* characterisation of Ad5/19p viruses

Due to the lack of infectivity of the AdKO1S* viruses, it was decided to investigate using Ad5 pseudotyped with the Ad19p fiber (Ad5/19p) as an alternative platform (Denby *et al.*, 2004). The 3 plaque targeting peptides were inserted into the HI loop of Ad5/19p and pseudotyped viruses were produced using the transient transfection/infection method (Jakubczak *et al.*, 2001, Nicklin *et al.*, 2001c). Viruses were titred using the BCA particle titre method (Von Seggern *et al.*, 1998) (Table 3.2) and confirmed by Taqman analysis (Figure 3.5). Western blotting was used to confirm that the viruses had fibers (data not shown).

Virus	Titre vp/ml
Ad5/19p	3.45×10^{12}
Ad5/19p-CAP	4.22×10^{12}
Ad5/19p-CNH	3.05×10^{12}
Ad5/19p-CQE	1.7×10^{12}

Table 3.2 Particle titres of the Ad5/Ad19p viruses.

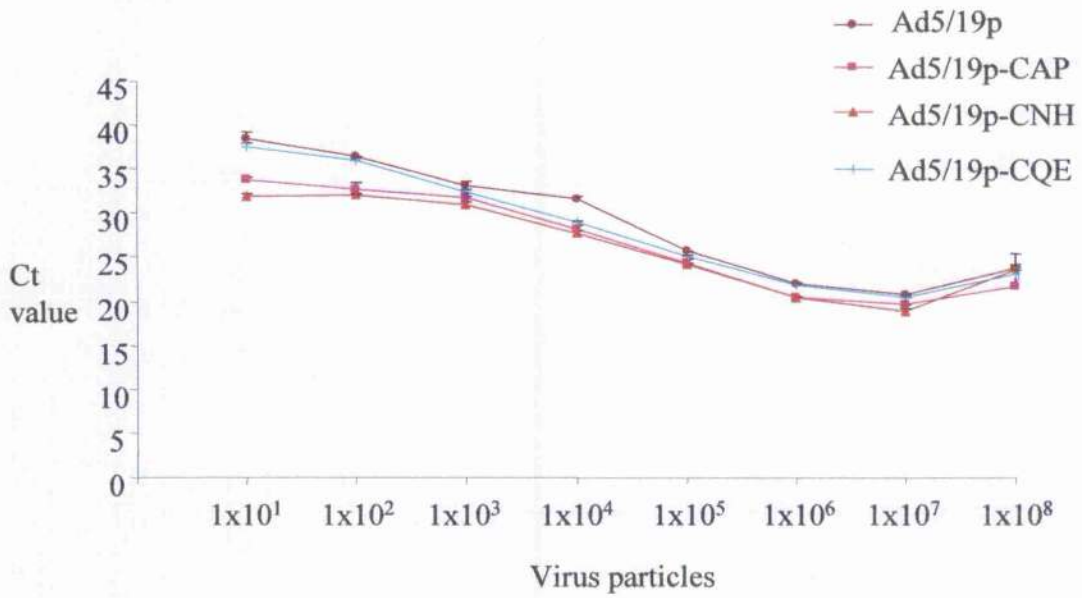


Figure 3.5 Taqman real time PCR to confirm the particle titres of the Ad5/19p viruses. Serial dilutions of the viruses based on the particle titre were used as a template for Taqman PCR using primers that bind the *lac Z* transgene.

In vitro infections were performed in the vascular cell lines and HeLa cells. In all the cell lines tested, the 3 peptide-modified viruses produced significantly higher levels of infection compared to Ad5/19p. (Figure 3.6) However, unmodified Ad5/19p only infected RGE, HSVECs and HCAECs at a low level and did not infect any of the other cell types tested. In all the cell lines and with all 3 peptide-modified viruses a dose response was seen and in all cases (except Ad5/19p-CNH MOI 1000 in IP-1B and SVEC 4-10, Ad5/19p-CQE MOI 10000 in HSVECs) significantly higher levels ($p<0.05$) of transduction compared to unmodified Ad5/19p were measured. X-gal staining of cells infected with 10000 vp/cell shows the same pattern of results as the β-gal assay (Figure 3.7). This suggests that peptide insertion enables a non-specific interaction between the vectors and cells, which results in cellular uptake of the vectors.

3.2.5.1 Production and *in vitro* characterisation of peptide-modified AAV2 vectors

In parallel with the adenovirus studies, the CAP and CNH peptides were incorporated into the capsid of AAV2 after residue 587, as described previously (Girod *et al.*, 1999, Nicklin *et al.*, 2001a). Although both the CAP and CNH peptides could be incorporated into the capsid to produce functional viruses, AAV2-CNH was difficult to produce at titres equivalent to AAV2-RC and AAV2-CAP, with the titres of preparations being approximately 10 fold lower.

Initial *in vitro* experiments were carried out with the plaque targeted AAV2 vectors to determine if the transduction profiles were similar to that seen with the AdKO1 vectors. As with the AdKO1 based viruses, the peptide-modified AAVs did not produce significantly higher transduction of non-vascular HeLa cells compared to the control virus, in fact AAV2-CAP transduction was significantly lower than control virus. In the mouse, human and rat endothelial cell lines, AAV2-CNH produced significantly higher levels of transduction (Figure 3.8). However, AAV2-CAP only produced a significantly higher level of transduction in RGE cells (Figure 3.8).

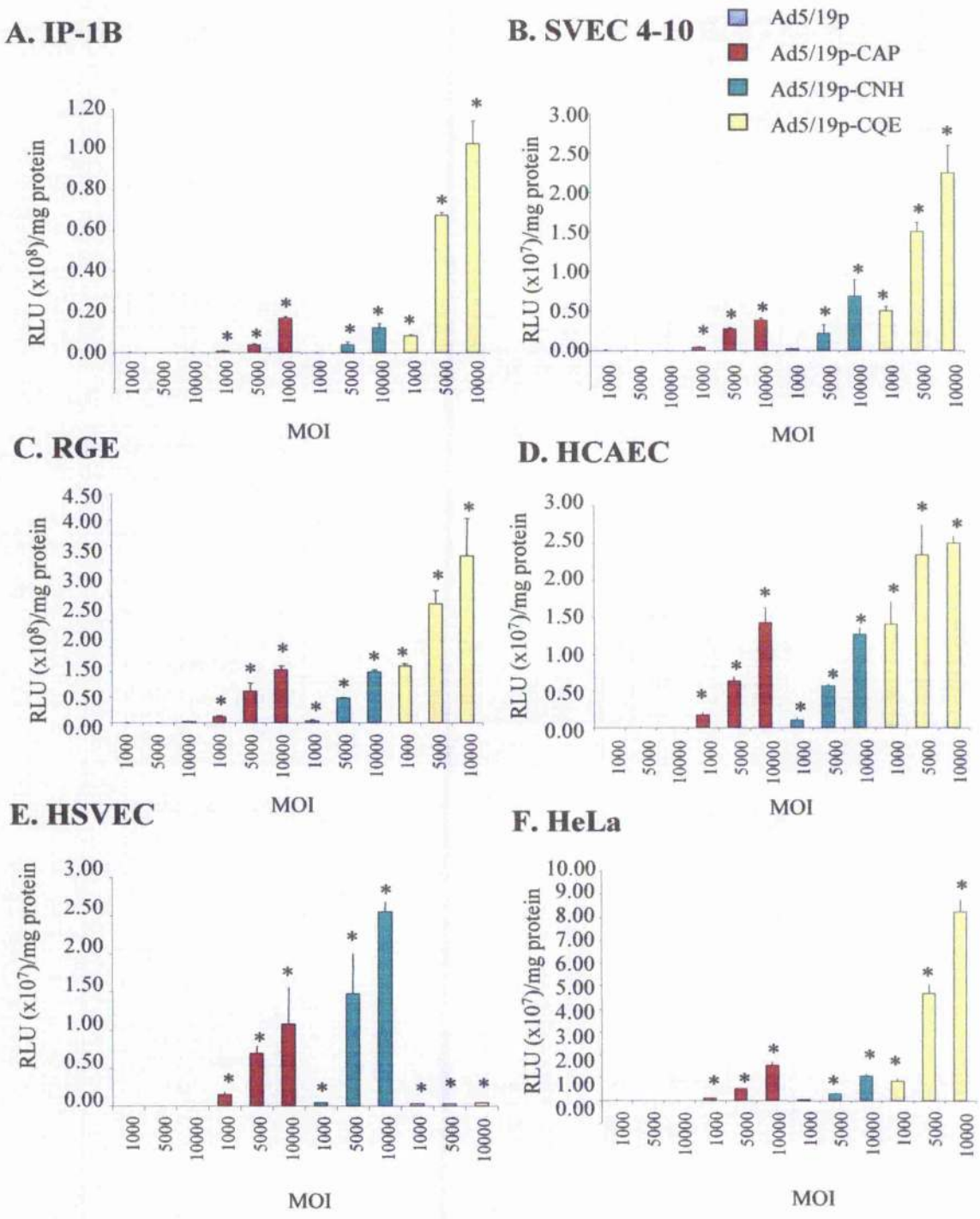


Figure 3.6 *In vitro* infection of peptide-modified Ad5/19p viruses. A. IP-1B, **B.** SVEC 4-10, **C.** RGE, **D.** HCAEC, **E.** HSVEC, **F.** HeLa. Cells were infected with the MOI stated for 3 hours, then washed and incubated in fresh media for 48 hours. β-gal activity in the cell lysates was quantified and levels were normalised to the total protein content. * $p < 0.05$ vs Ad5/19p.

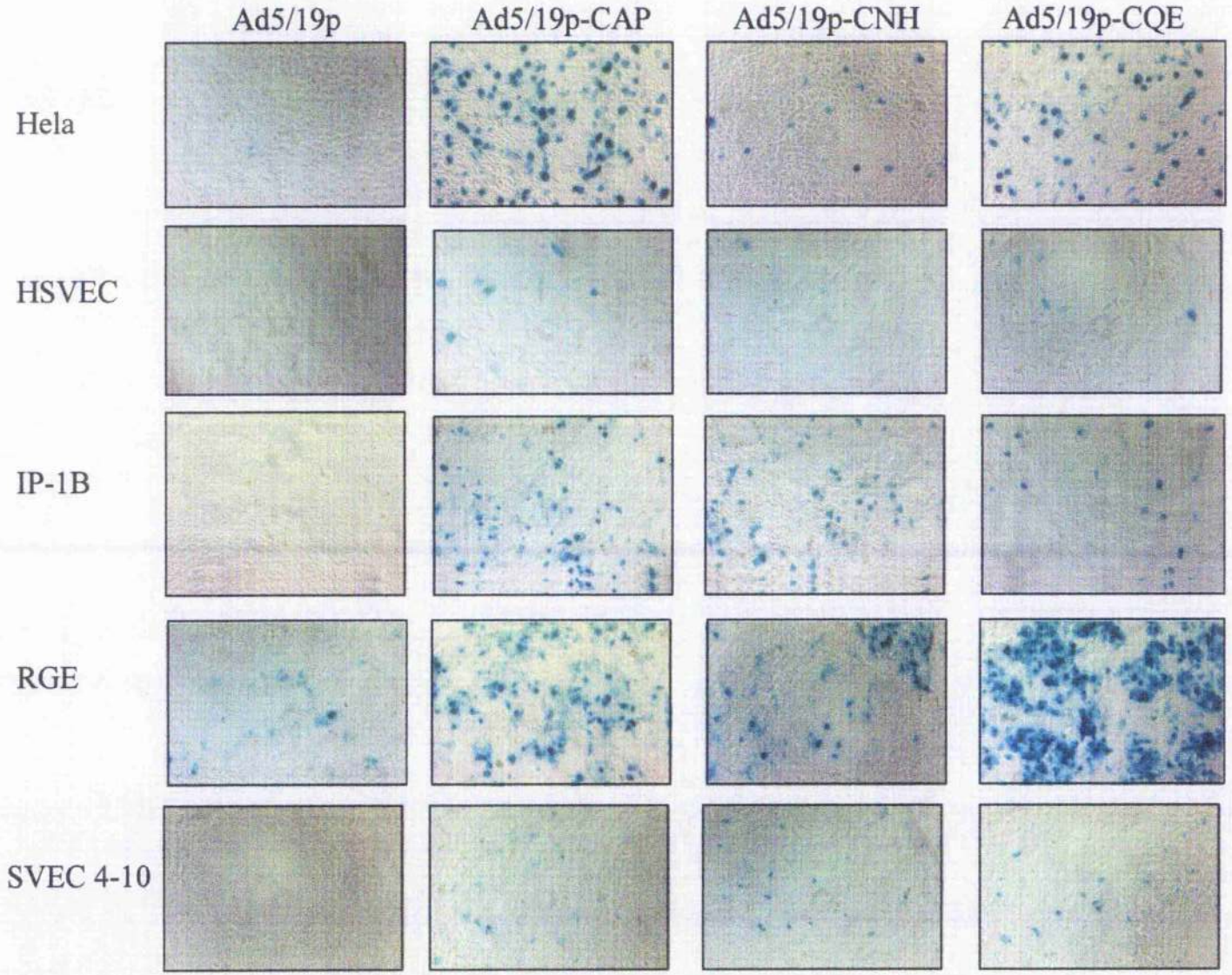


Figure 3.7 Infection of peptide-modified Ad5/19p viruses. Cells were infected with an MOI of 10,000 for 3 hours, then washed and incubated in fresh media for 48 hours. Cells were fixed and stained with X-gal.

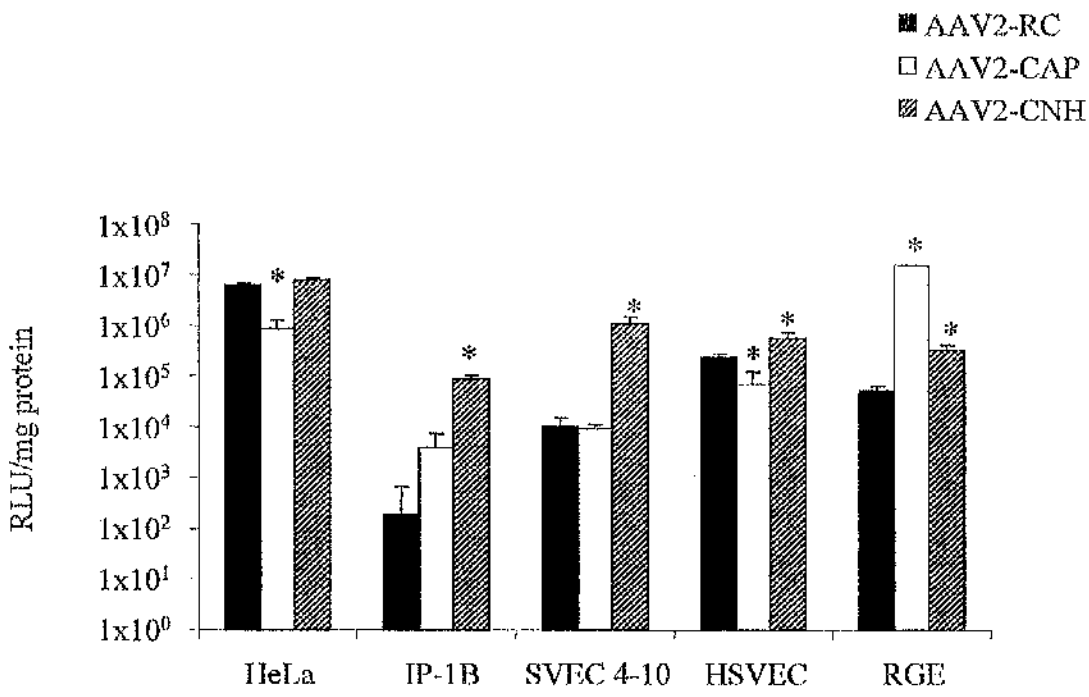


Figure 3.8 Infection of peptide-modified AAV2 vectors. Cells were infected with an MOI of 1000 for 24 hours, then washed and incubated in fresh media for 48 hours. Cell lysate was used in a β-gal assay to measure the amount of transgene which was normalised to the total protein content of the sample. * $p < 0.05$ vs AAV2-RC.

3.2.5.2 Heparin binding of peptide-modified AAV2 based vectors

To determine whether insertion of the peptides into the capsid of AAV2 affects the ability of the virus to bind HSPG, binding to the receptor analogue heparin was evaluated. The viruses were loaded onto a heparin column, which was then washed before bound virus was eluted in a high salt solution. Quantitative PCR of the resulting fractions was used to analyse the extent of heparin binding (Performed by H. Buening). Both peptide-modified viruses and control AAV2 bound to the column and were eluted in the first elution with 1 M NaCl (Figure 3.9), suggesting both modified vectors still bind heparin. However the majority of AAV2-CAP and AAV2-CNH was not detected in any of the fractions whereas 100% of AAV2-RC was recovered. This may be due to the remaining virus still being bound to the column and requiring a higher concentration of salt solution to elute it.

To determine whether this interaction with heparin affects infectivity of the viruses, infection of the mouse endothelial cell lines was repeated in the presence of soluble heparin (Figure 3.10). In both cell lines both peptide-modified viruses and AAV2-RC showed reduced levels of infection in the presence of heparin, suggesting both AAV2-CAP and AAV2-CNH maintain the ability to bind HSPG.

3.2.5.3 Affect of proteasome inhibitors on AAV2 transduction

The low level of transduction of endothelial cells by AAV2 is thought to be due to both the sequestration of the vector in the extracellular matrix caused by HSPG binding (Pajusola *et al.*, 2002) and due to degradation of internalised virions by the proteasome (Nicklin *et al.*, 2001a, Denby *et al.*, 2005). It has previously been demonstrated that the peptide aldehyde proteasome inhibitors N-acetyl-L-leucyl-L-leucyl-norleucine (LnLL) and MG132 can enhance transduction of wild type AAV2 but can have little affect on some peptide-modified AAV2 vectors (peptide dependent) as they are thought to traffic to the nucleus via an alternative mechanism (Work *et al.*, 2004a, Nicklin *et al.*, 2001a). To determine whether this pathway affects AAV2-CAP and AAV2-CNH, infections of SVEC 4-10 and IP-1B cells were performed in the presence of the proteasome inhibitors MG132 and LnLL (Figure 3.11). Both AAV2-RC and AAV2-CAP transduction was significantly enhanced by both proteasome inhibitors in both cell lines, whereas AAV2-CNH transduction was only significantly increased by MG132 in SVEC 4-10 cells.

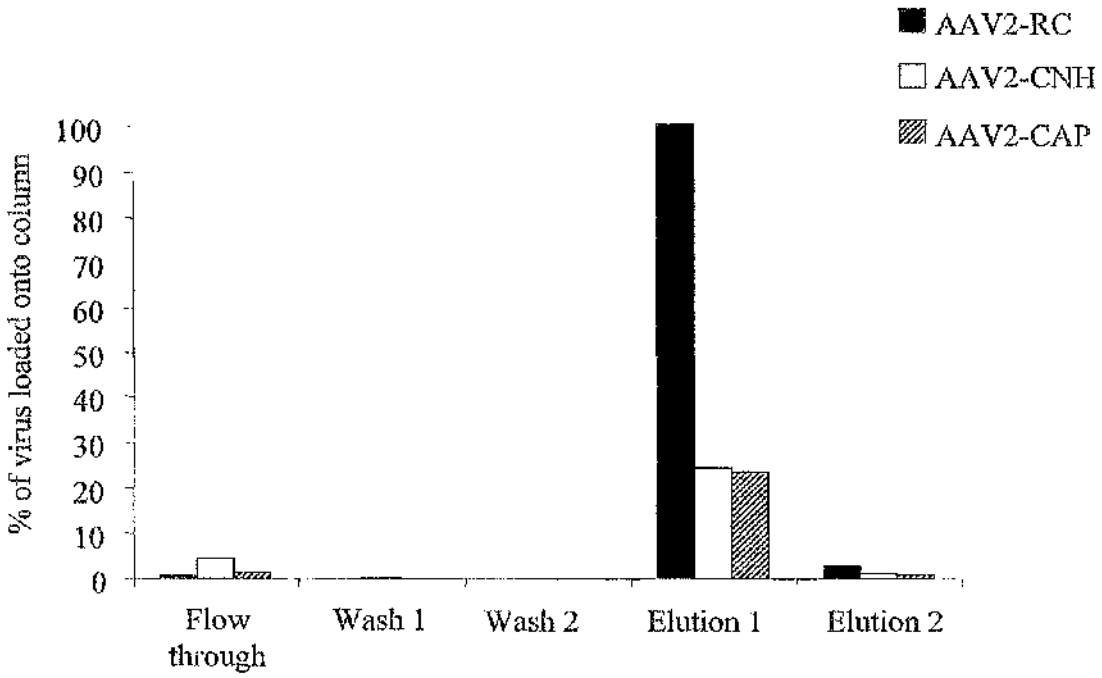


Figure 3.9 Binding of AAV2 vectors to a heparin column. The viruses were applied to a heparin column, which was washed with 1 mM $MgCl_2$, 2.5 mM KCl and then heparin bound virus was eluted in 1mM $MgCl_2$, 2.5 mM KCl, 1M NaCl. Collected fractions were analysed by quantitative PCR and the percentage of total virus loaded onto the column was calculated for each fraction.

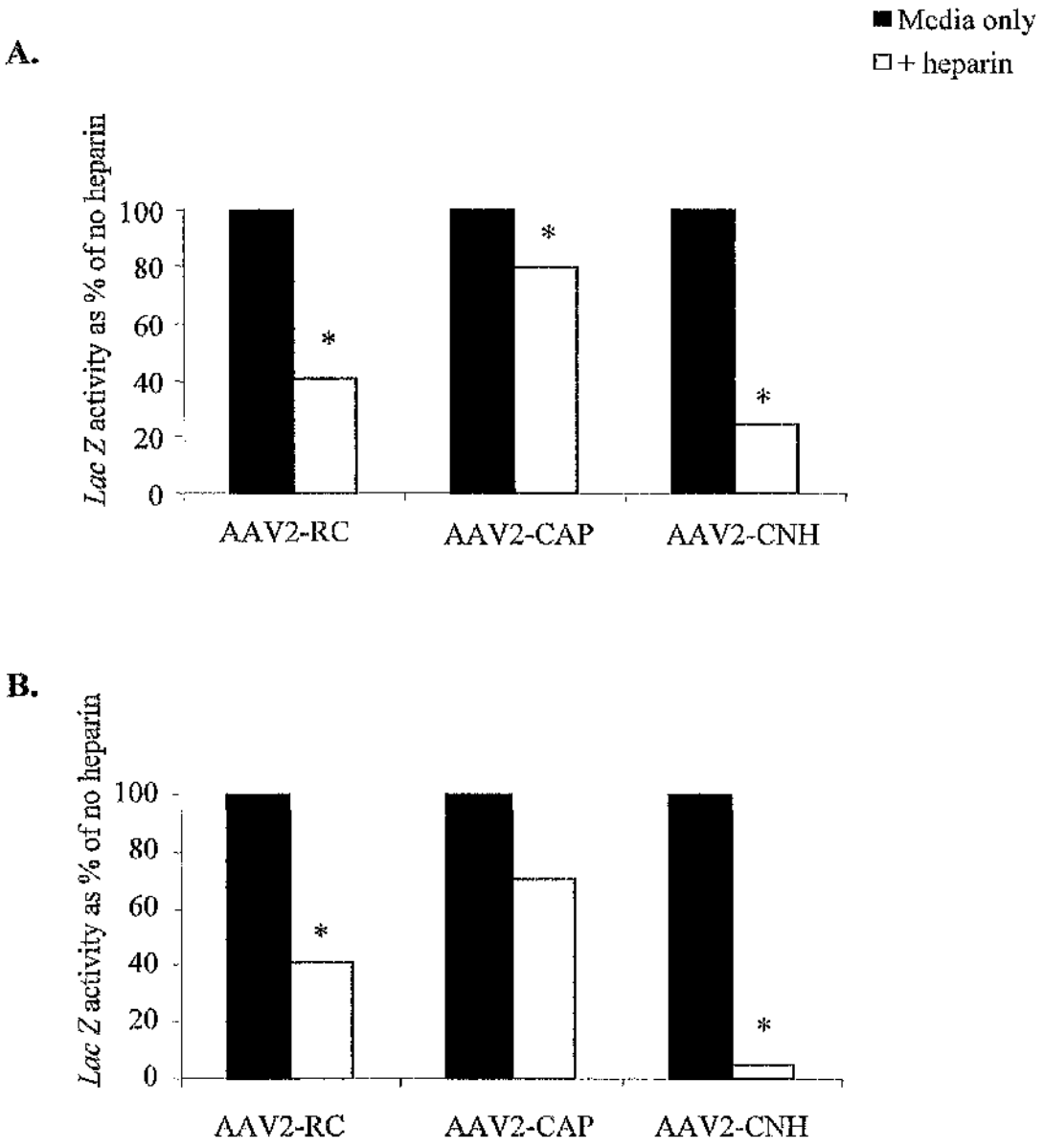


Figure 3.10 Heparin competition experiment. A. IP-1B **B.** SVEC 4-10. Cells were infected with an MOI of 1000 for 24 hours in the presence or absence of 1 IU of heparin per 1 x 10^6 gp, then washed and incubated in fresh media for 48 hours. Cell lysate was used in β-gal assay to measure the amount of transgene and normalised to the total protein content of the sample. * $p < 0.05$ vs no heparin.

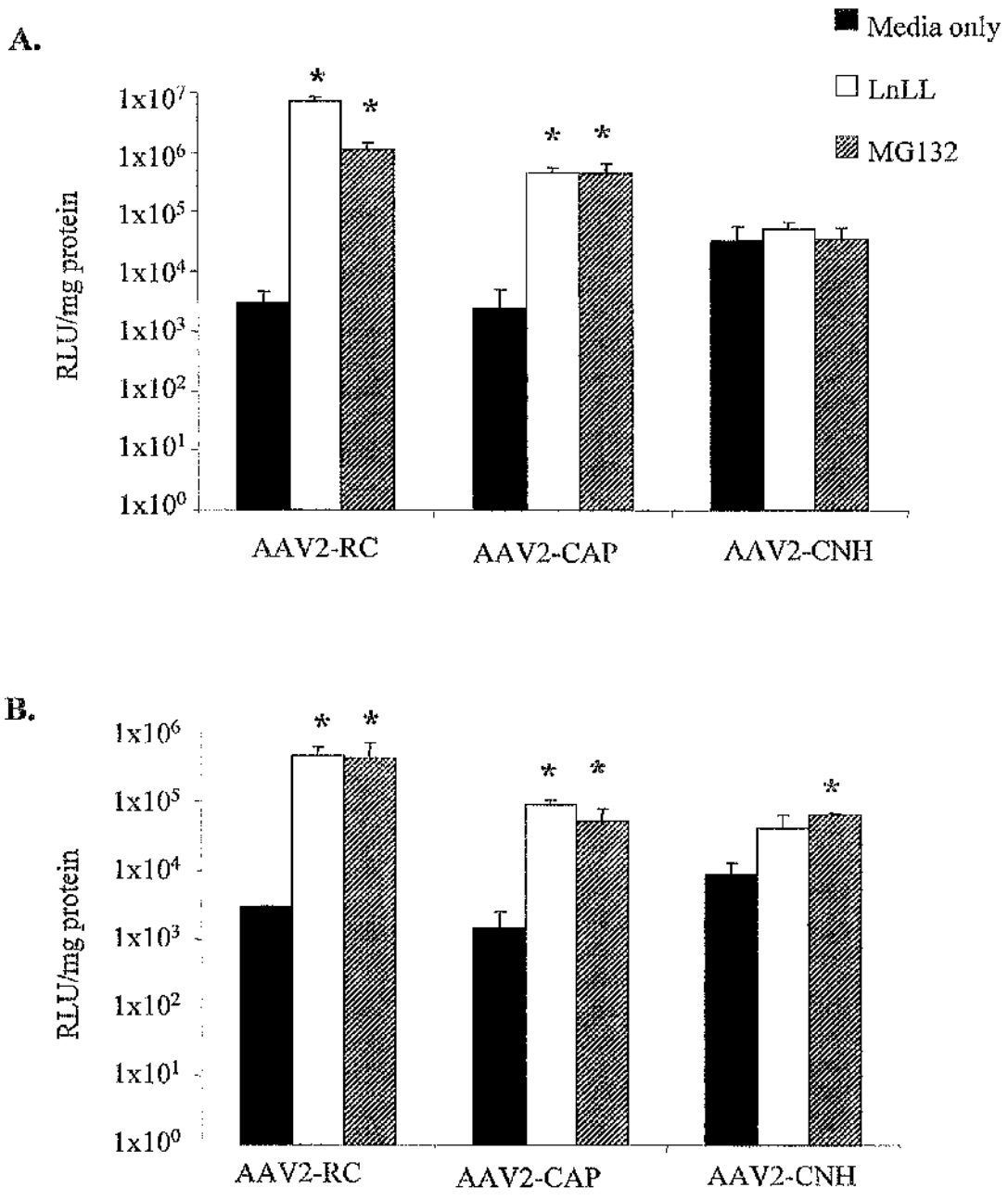


Figure 3.11 Affect of proteasome inhibitors on transduction of AAV2 vectors. A. IP-1B **B.** SVEC 4-10 cells were infected with an MOI of 1000 for 24 hours in the presence or absence of proteasome inhibitors LnLL (40 μM) or MG132 (4 μM), then washed and incubated in fresh media for 48 hours. Cell lysate was used in a β-gal assay to measure the amount of transgene expression, which was normalised to the total protein content of the sample. *$p<0.05$ vs media only.

3.2.6 Peptide competition assay

To attempt to assess whether the viruses infect cells via interactions between the inserted peptide and a novel receptor, peptide competition assays were performed with the AAV2 based vectors. The infection of SVEC 4-10 cells was repeated in the presence of soluble peptides with the same sequence as the inserted peptide or a different peptide as a control (Figure 3.12). There was no significant affect on transduction of either AAV2-CAP or AAV2-CNH with either the matched or control non-specific peptide.

3.2.7.1 MT1-MMP may act as the receptor for CNH peptide

BLAST searching the CNH peptide showed it had significant sequence homology to TIMP2 and it was shown that the binding of this peptide to endothelial cells could be inhibited by TIMP2 (Liu *et al.*, 2003). As TIMP2 forms a complex with MT1-MMP at the cell membrane, it is possible that the CNH peptide may target MT1-MMP. To determine if CNH uses MT1-MMP as a receptor, infections of AAV2-CNH were carried out in the human fibrosarcoma cell line HT1080 stably transfected with MT1-MMP and control untransfected cells that express a low level of MT1-MMP (Green *et al.*, 1994). AAV2-CNH produced significantly higher transduction of the cells transfected with MT1-MMP, whereas there was no significant difference in the transduction of the control vector AAV2-RC (Figure 3.13A). This suggests that AAV2-CNH uses MT1-MMP as a receptor. To provide further evidence that the increase in transduction was due to the CNH peptide interacting with MT1-MMP the experiment was performed with the AdKO1-CNH and Ad5/19p-CNH vectors. A similar pattern of results was seen with AdKO1-CNH, as transduction of the MT1-MMP expressing cells was 17-fold higher than the untransfected cell line (Figure 3.13B). However, transduction of Ad5/19p-CNH was not significantly higher in the MT1-MMP expressing cell line compared to untransfected HT1080 cells, although in both cell lines Ad5/19p-CNH produced significantly higher levels of transduction than the unmodified virus (Figure 3.13C). This supports the earlier results with the Ad5/19p peptide-modified vectors, which suggested that peptide insertion mediates non-specific uptake of the virus.

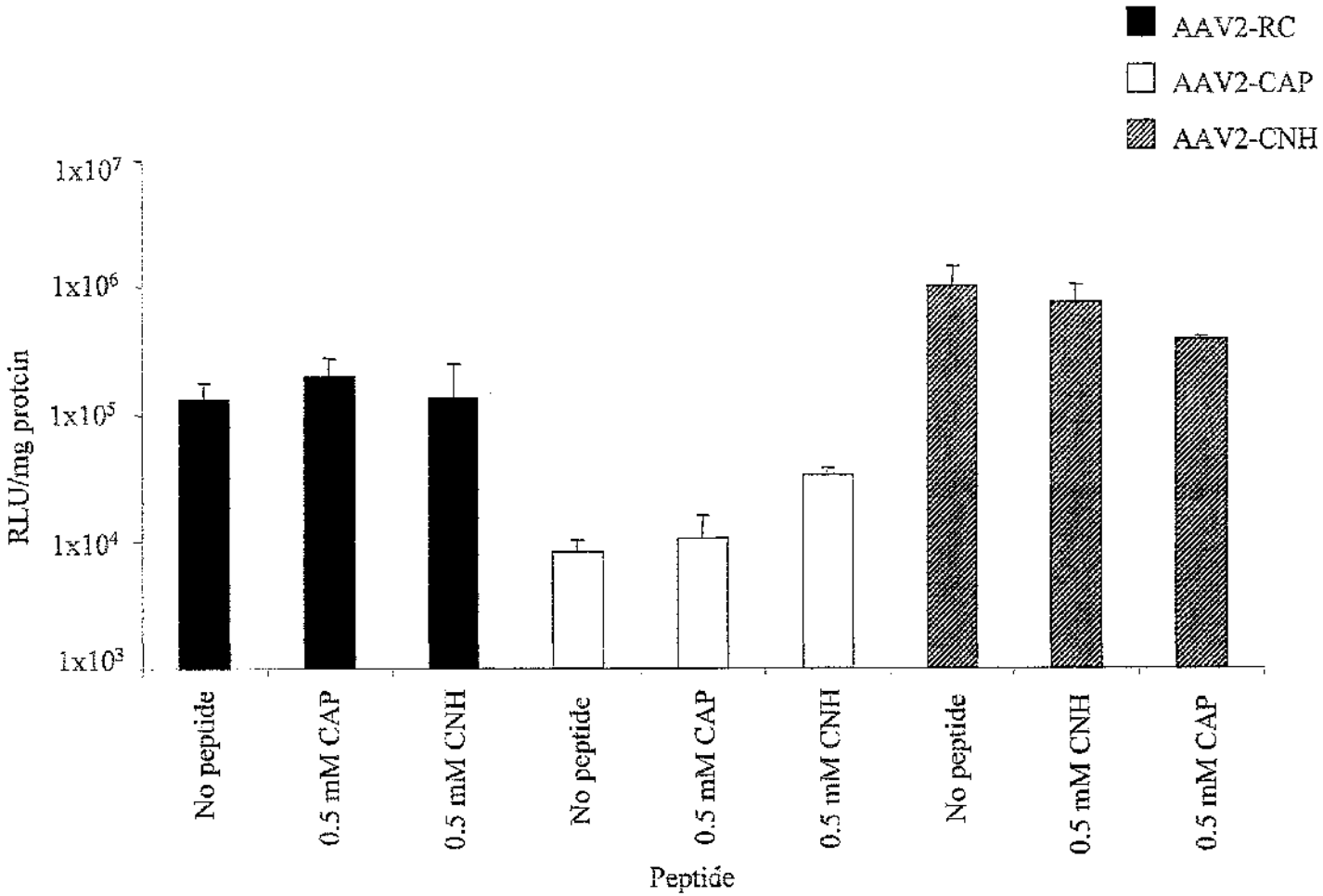


Figure 3.12 Peptide competition. SVEC4-10 cells were infected with AAV viruses (MOI 1000) for 24 hours in the presence or absence of sequence matched or control peptide, then washed and incubated in fresh media for 48 hours Cell lysate was used in a β-gal assay to measure the amount of transgene expression, which was normalised to the total protein content of the sample. *$p<0.05$ v no peptide.

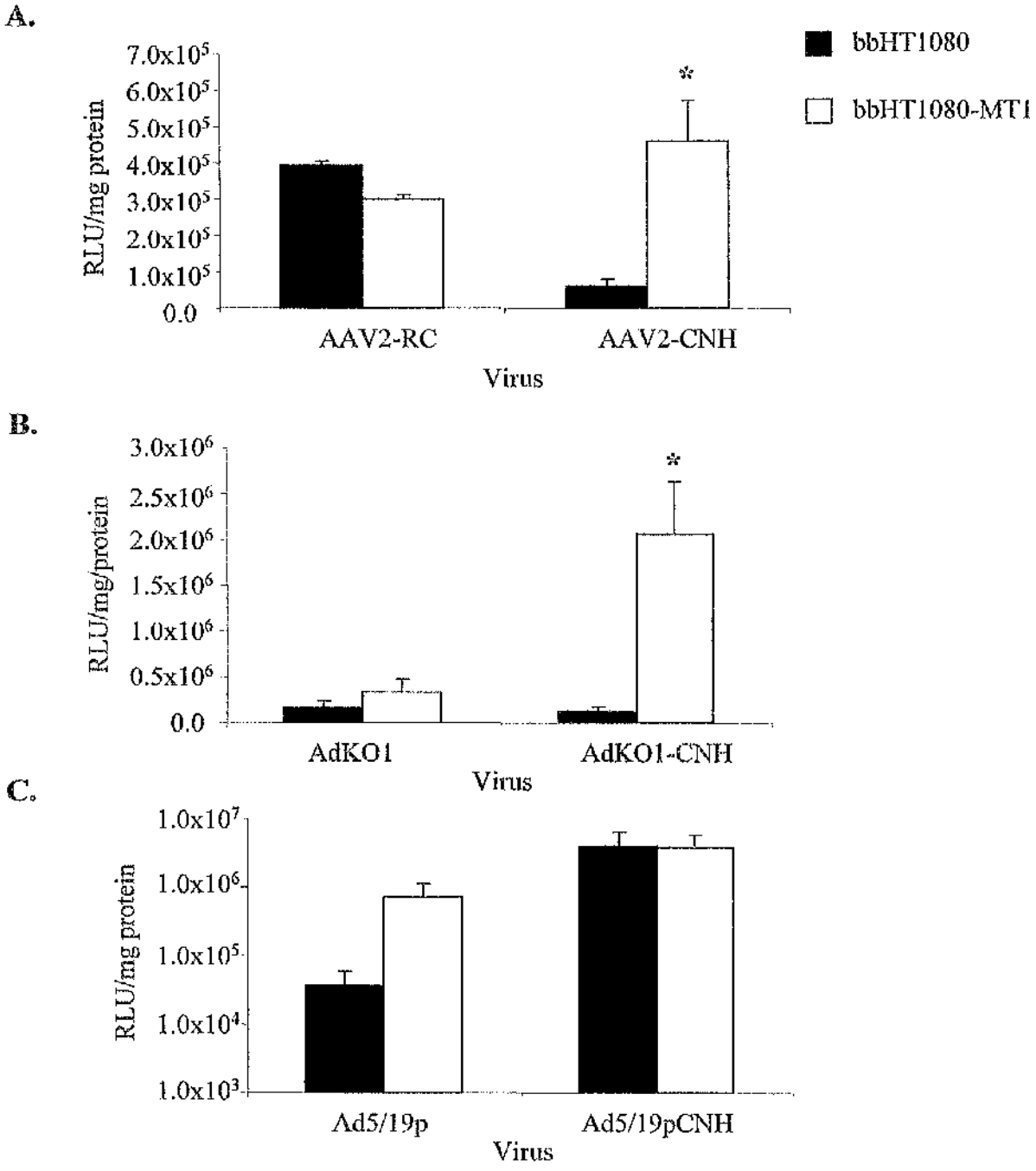


Figure 3.13 Infection of HT1080 cells expressing MT1-MMP. **A.** Cells were infected with AAV vectors MOI 1000 for 24 hours, then washed and incubated in fresh media for 48 hours. **B.** Cells were infected with AdKO1 viruses, MOI for 3 hours, then washed and incubated in fresh media for 48 hours. **C.** Cells were infected with Ad5/19p viruses, MOI 100,00 for 3 hours, then washed and incubated in fresh media for 48 hours. Cell lysate was used in a β-gal assay to measure the amount of transgene expression, which was normalised to the total protein content of the sample. *$p < 0.05$ v HT1080 cells.

3.2.7.2 Surface plasmon resonance (SPR)

Although the above experiments suggest that the CNH modified viruses are interacting with MT1-MMP, due to the complexity of the virus:cell interaction this needs to be confirmed by utilising an alternative technique. Hence, the interaction was tested by surface plasmon resonance (SPR) experiments using a Biacore system. Biacore is a cell free system that investigates the direct interaction between a protein and another molecule and can provide information on their rate of association, dissociation and affinity, in real time. MT1-MMP was immobilised on a biosensor chip and TIMP2, AAV2-RC, AAV2-CNH, Ad5/19p or Ad5/19p-CNH were injected over the chip (performed by Dr J. McVey) (Figure 3.14). A dose-dependent interaction with AAV2-CNH was observed but none of the other viruses tested bound to MT1-MMP, suggesting that AAV2-CNH can bind to MT1-MMP. The positive control TIMP2 was also shown to bind MT1-MMP (Figure 3.14). This supports the previous evidence that suggests that the CNH peptide binds to MT1-MMP and that the AAV2 vector provides an optimal platform for this interaction to occur.

3.2.8 *In vivo* characterisation of Ad5/19p vectors in ApoE-/- mice maintained on a high fat diet

The Ad5/19p vectors were tested *in vivo* to determine if the viruses had any tissue specificity. Biodistribution profiles were produced by Taqman analysis (Figure 3.15A) and immunohistochemistry was used to detect transgene expression. Taqman analysis detected higher levels of the unmodified Ad5/19p in all tissues except the BCA, spleen and muscle of mice that received Ad5/19p-CAP. (Figure 3.15B)

Immunohistochemistry for the *lacZ* transgene was carried out on sections of the BCA, aorta and liver. *LacZ* expression could only be detected in the liver of mice that received Ad5/19p (Figure 3.16). These results suggest that in the context of the Ad5/19p fiber, the 3 plaque-targeting peptides do not produce retargeting to areas of atherosclerosis.

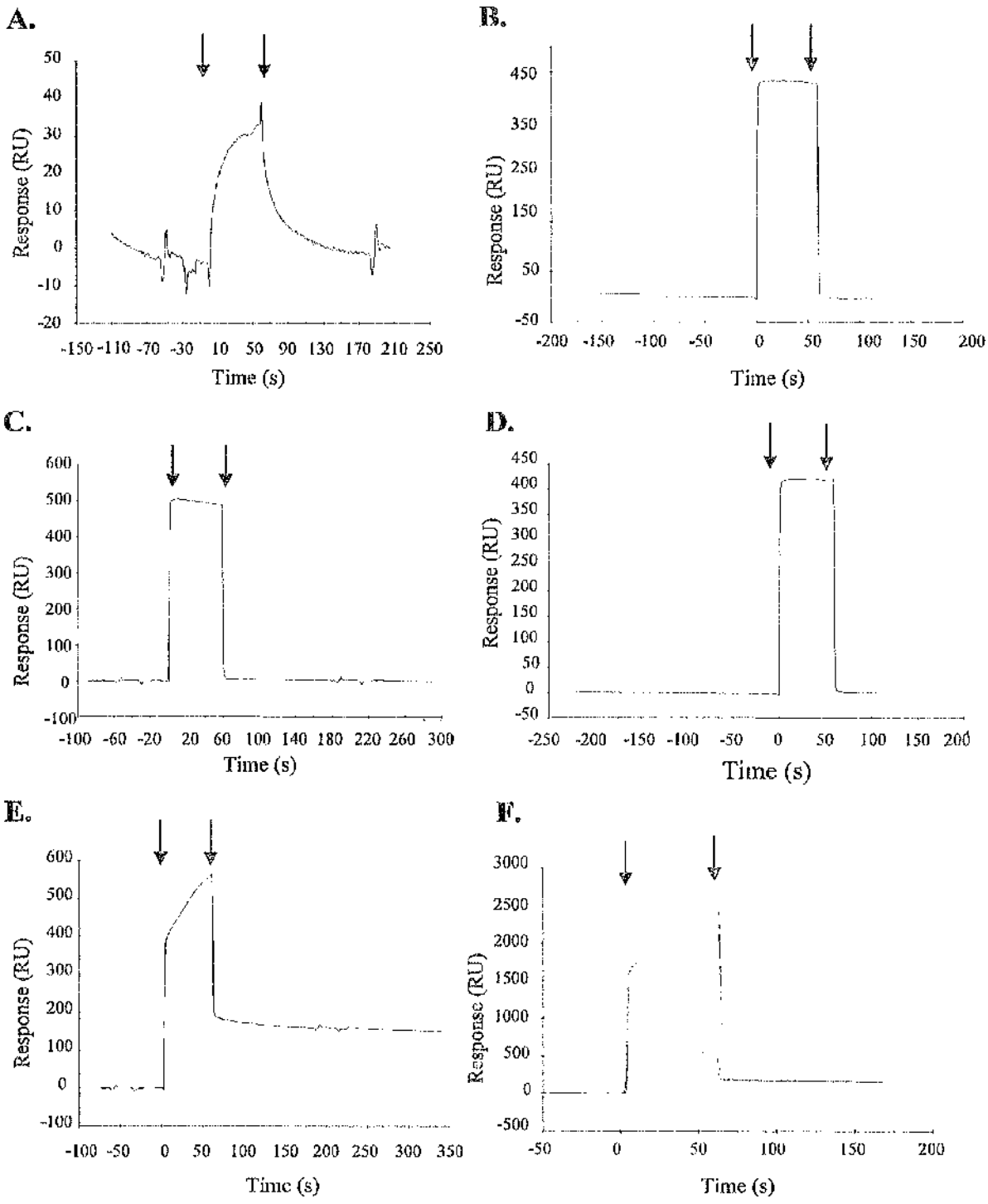


Figure 3.14 SPR using an MT1-MMP coated chip. A. TIMP2 (1.3 μM) **B.** Ad5/19p. **C.** Ad5/19p-CNH. **D.** AAV2-RC. **E.** AAV2-CNH were injected over the chip for the timeframe marked by the arrows. **F.** Dose response of AAV2-CNH.

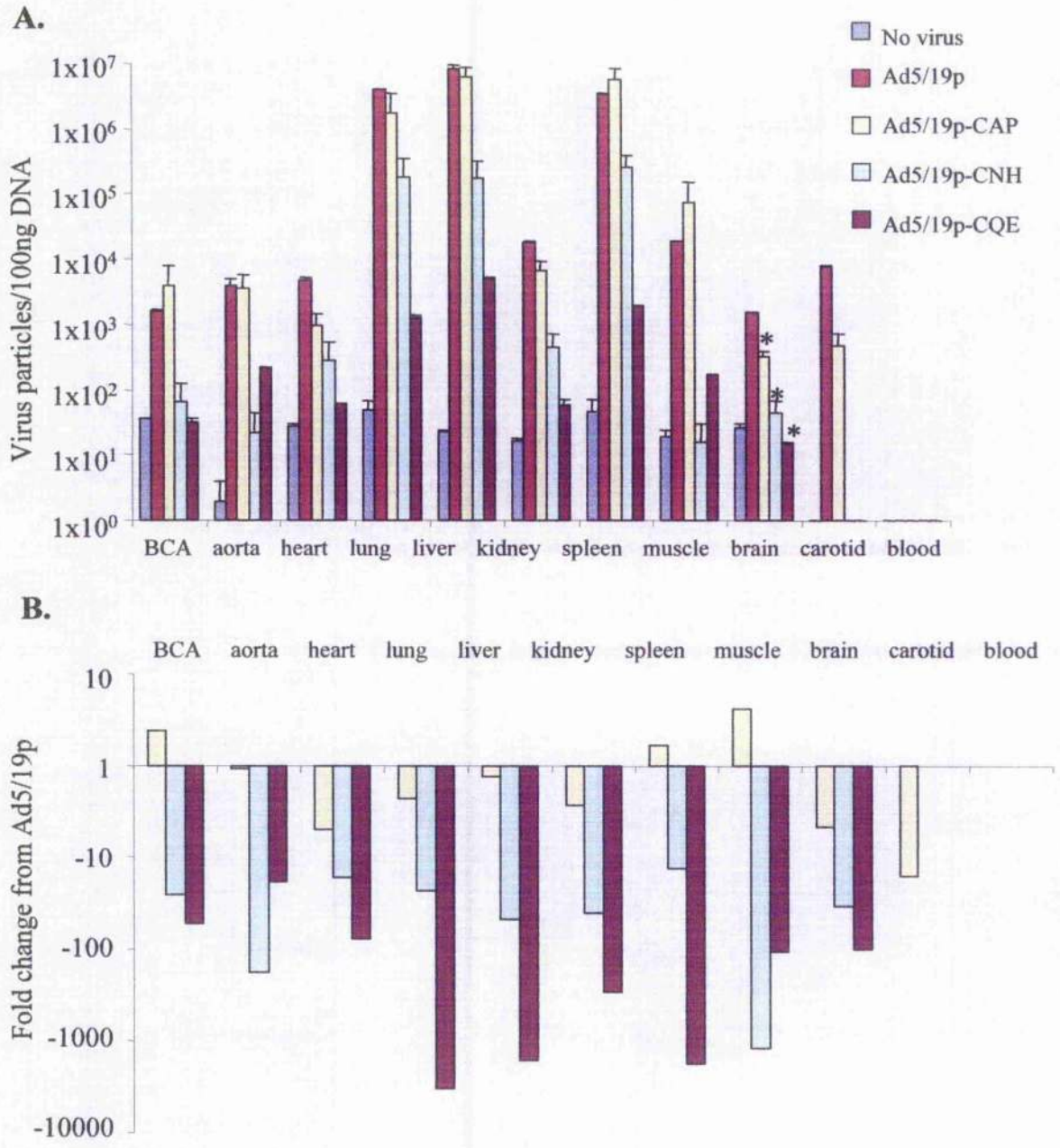


Figure 3.15 Biodistribution of peptide-modified Ad5/19p viruses in ApoE-/- mice fed a high fat diet. 1 x 10^{11} vp were injected into the tail vein of mice. Mice were sacrificed 1 hour later. **A.** DNA was extracted from tissues and used as a template for real time PCR using primers that detect the *lac Z* transgene. **B.** Fold change in the amount of virus detected compared to Ad5/19p. n=3 per group. *p<0.05 vs Ad5/19p.

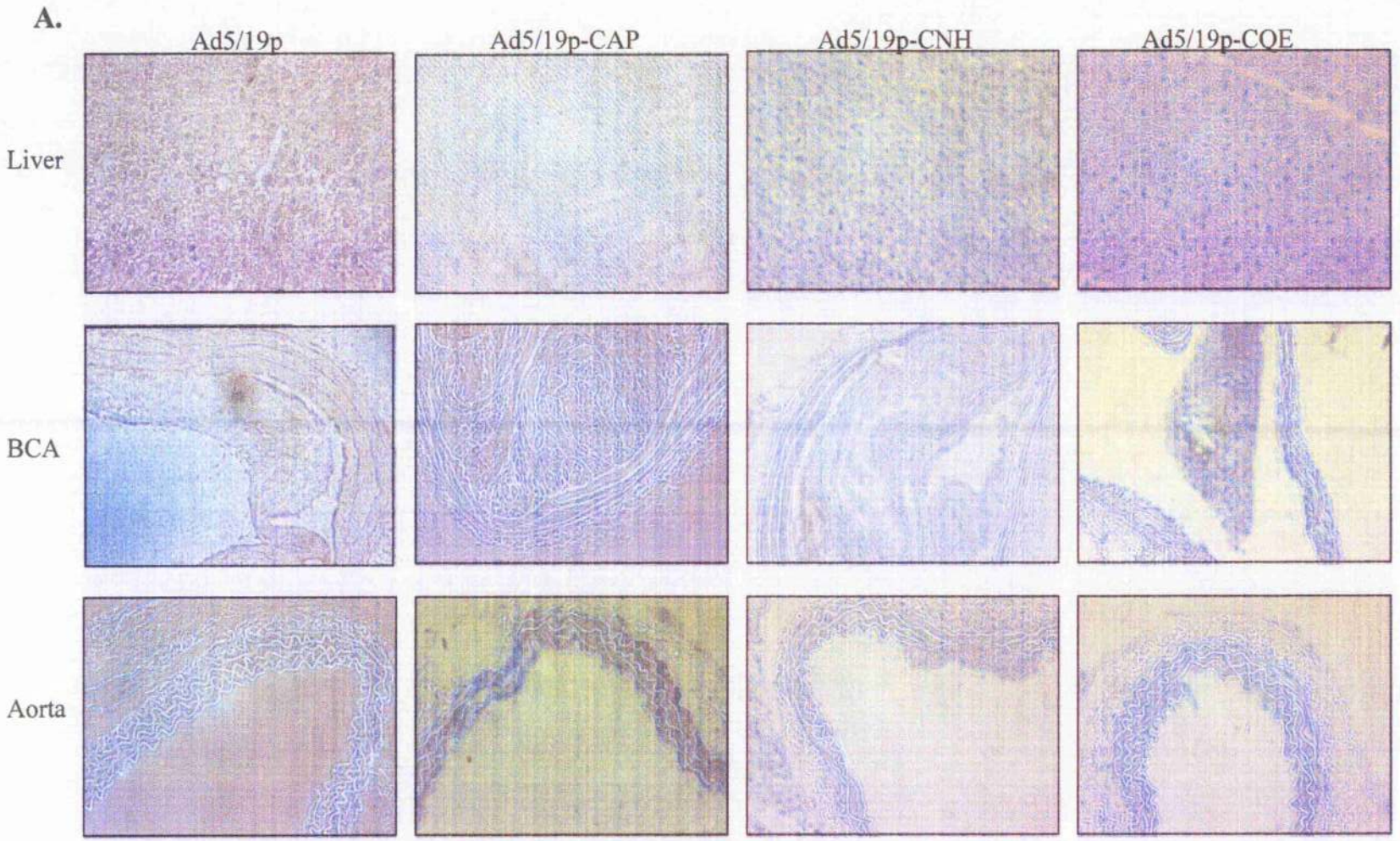
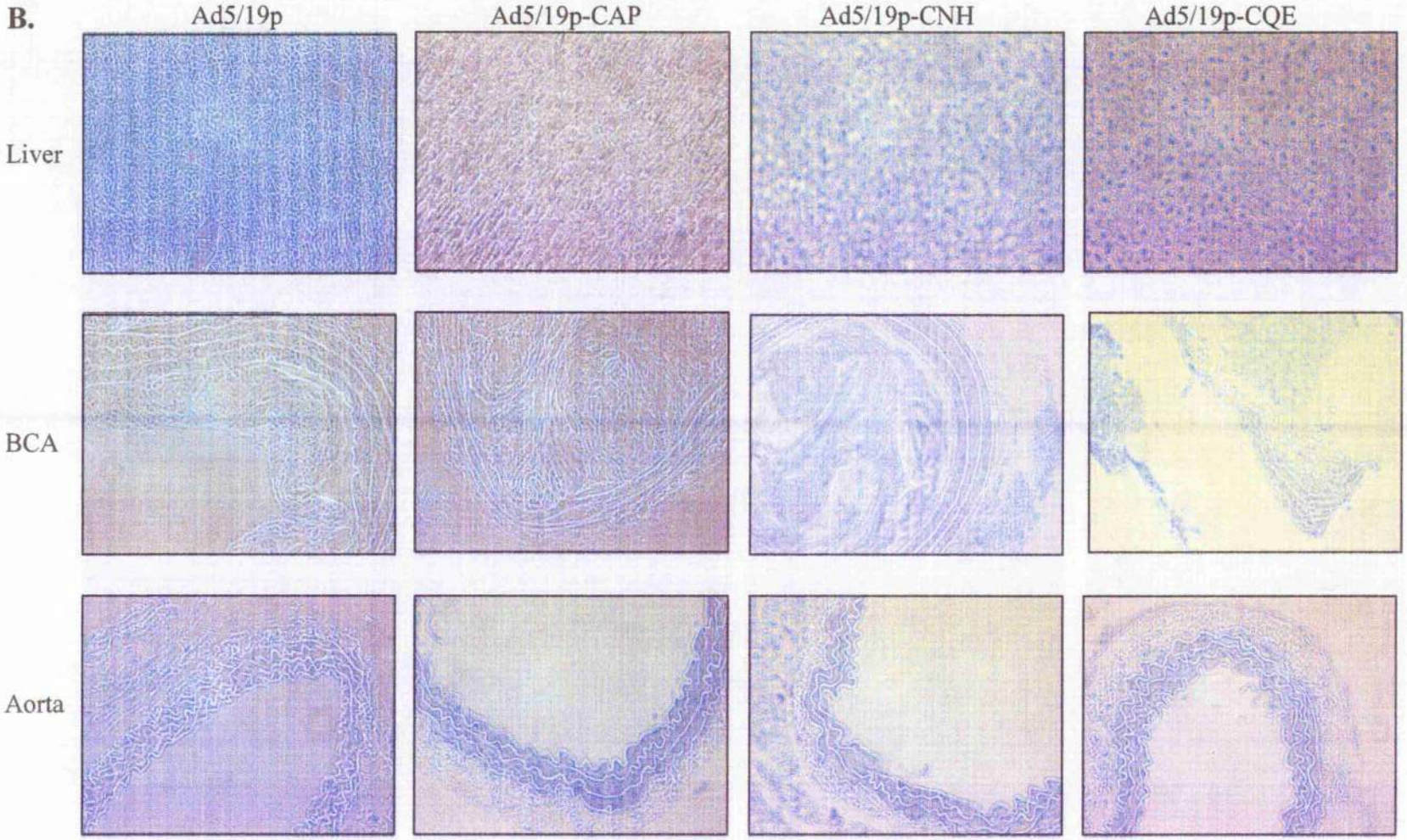


Figure 3.16 Immunohistochemistry to detect expression of the *lac Z* transgene. ApoE-/- mice fed a high fat diet received 1 x 10^{11} vp of the peptide modified Ad5/19p viruses and were sacrificed 5 days later. Immunohistochemistry was carried out to detect **A.** *Lac Z* expression and **B.** an IgG control (shown overleaf).


B.
Ad5/19p
Ad5/19p-CAP
Ad5/19p-CNH
Ad5/19p-CQE
Liver
BCA
Aorta

3.2.9 Biodistribution profiles of the AAV2 based vectors in ApoE-/- mice maintained on a high fat diet

As the *in vitro* results with the peptide modified AAV2 vectors were encouraging, the tropism of the vectors was analysed *in vivo* in ApoE-/- mice. 6×10^9 gp of the AAV2 vectors was injected into the tail vein of the mice. 28 days later DNA was extracted from tissues and used as a template for real-time PCR to produce biodistribution profiles of the viruses (Figure 3.17). The results showed that for both AAV2-CAP and AAV2-CNH there was a 10-100 fold increase in the amount of vector detected in the BCA and aorta compared to the amount of unmodified AAV2 detected (Figure 17B). In addition to this retargeting, both modified vectors were detected at a lower level than control virus in all other tissues examined, suggesting that the peptides also have a detargeting affect on the natural tropism of the virus.

3.2.10 Biodistribution profiles of the AAV2 based vectors in ApoE-/- mice maintained on a normal rodent chow diet

The biodistribution profiles of AAV2-RC and AAV2-CAP were analysd in ApoE-/- mice fed a normal rodent chow diet, which have been shown not to develop atherosclerosis by the age of 18 weeks (See section 3.2.1). The protocol was identical to that used with the mice fed a high fat diet. The results show that unlike in the fat fed mice, AAV2-CAP was not detected at a higher level than AAV2-RC in the BCA or aorta of healthy mice (Figure 3.18A and 3.18B). Compared to AAV2-RC there was a slightly higher level of AAV2-CAP detected in the spleen and brain of mice maintained on a healthy diet, but the results suggest that in healthy mice AAV2-CAP is not efficiently targeted to any of the tissues investigated (Figure 3.18A and 3.18B). Comparing the biodistribution profiles of AAV2-CAP in healthy and fat fed mice showed that there was over a 100-fold decrease in the amount of vector detected in the BCA and aorta of healthy mice (Figure 3.18C). There was also a relatively large decrease in the amount of vector detected in the kidney (Figure 3.18C), a common site for atherosclerosis in humans (Krumme and Donauer, 2006). These results suggest that AAV2-CAP may be targeted to areas of atherosclerotic vasculature and not to healthy vasculature.

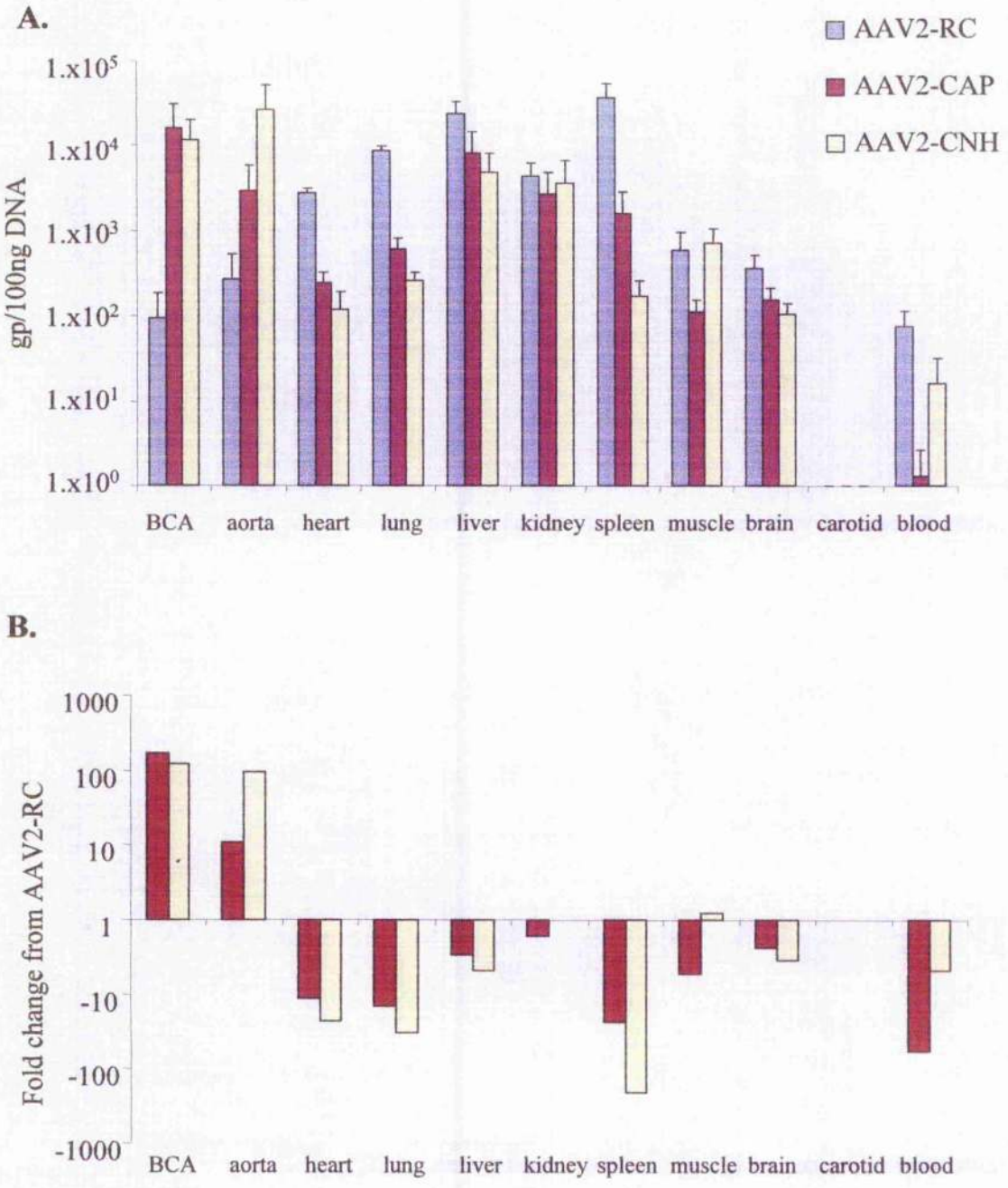


Figure 3.17 Biodistribution of peptide-modified AAV2 viruses in ApoE-/- mice fed a high fat diet. 6 x 10^9 gp were injected into the tail vein of mice, that were sacrificed 28 days later. **A.** DNA was extracted from tissues and used as a template for real time PCR with primers that bind the eGFP transgene. **B.** Fold change in number of gp/100 ng DNA compared to AAV2-RC. n=3 per group

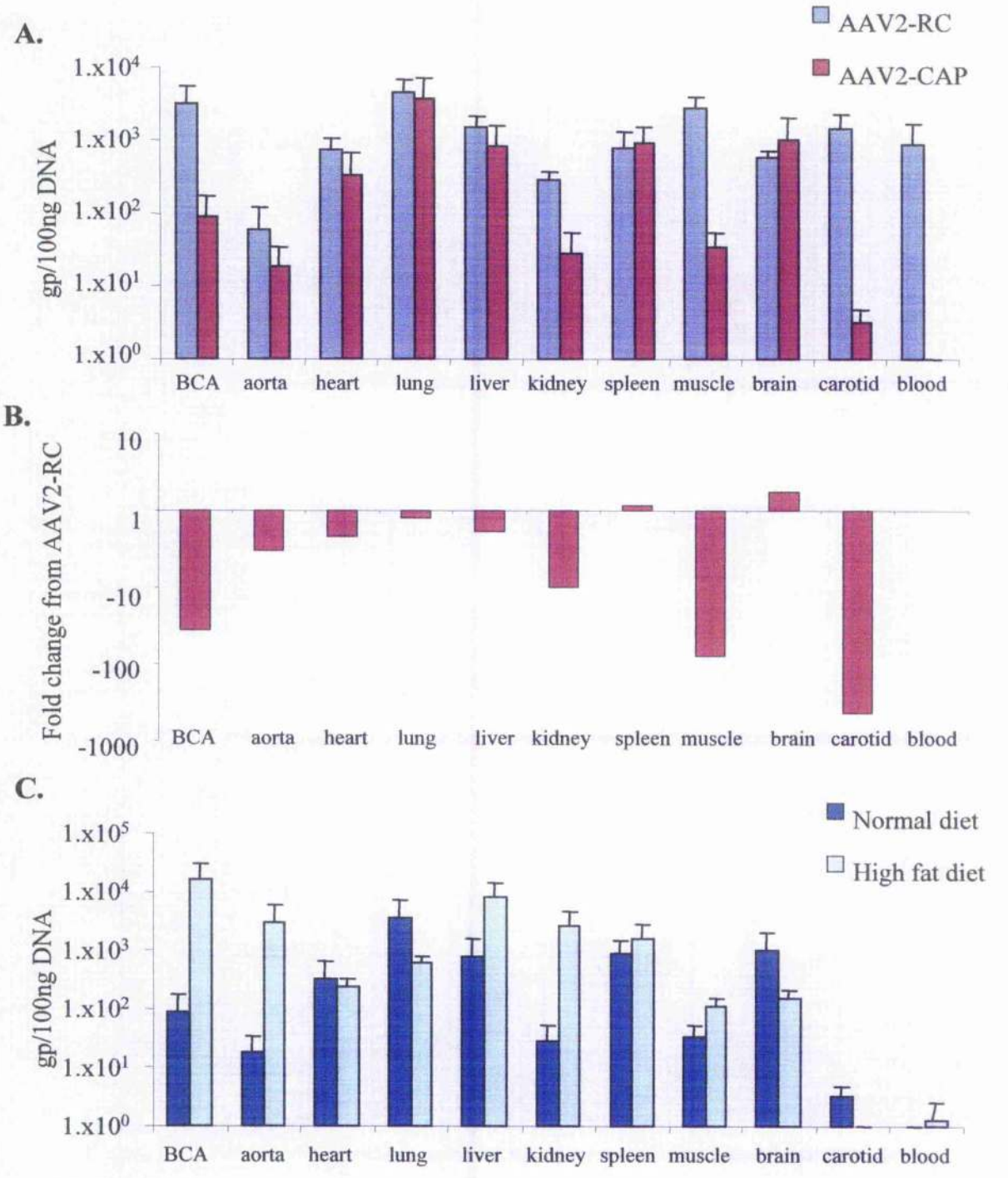


Figure 3.18 Biodistribution of AAV2 vectors in ApoE-/- mice fed a normal chow diet. 6 x 10^9 gp were injected into the tail vein of mice, that were sacrificed 28 days later. **A.** DNA was extracted from tissues and used as a template for real time PCR with primers that bind the eGFP transgene. **B.** Fold change in number of gp/100 ng DNA compared to AAV2-RC. **C.** Comparison of AAV2-CAP biodistribution in mice fed a normal diet and mice fed a high fat diet. n=4 per group for normal diet, n=3 per group for high fat diet

3.3 Discussion

This chapter describes the production and characterisation of viral vectors containing atherosclerotic plaque targeting peptides that were identified by phage display (Liu *et al.*, 2003). The peptides were successfully incorporated into AdKO1, AdKO1S*, Ad5/19p and AAV2 vector platforms to identify the optimum system for exposure of the peptides. *In vitro* the peptide-modified AdKO1, Ad5/19p and AAV2 vectors generally produced higher levels of transduction of endothelial cells compared to the control viruses. However the AdKO1S* viruses failed to efficiently transduce any of the cell types tested. *In vivo* the most promising results were achieved with the AAV2-based vectors, as compared to control virus higher levels of AAV2-CAP and AAV2-CNH were found in the BCA and aorta of atherosclerotic mice. A decrease in the amount of the peptide-modified viruses was found in all other tissues suggesting that the vectors had been detargeted from their native tropism and retargeted to areas of atherosclerotic vasculature. No retargeting of AAV2-CAP was detected in healthy mice, supporting the hypothesis that the vector is targeted specifically to atherosclerotic plaques and not healthy vasculature.

3.3.1 Production of peptide-modified viruses

All 3 plaque targeting peptides (CAP, CQE and CNH) were successfully inserted into the capsid of AdKO1, AdKO1S* and Ad5/19p vectors. The CAP and CNH peptides were incorporated into the capsid of AAV2 to produce functional viruses, however AAV2-CNH was difficult to produce at titres equivalent to AAV2-RC and AAV2-CAP, suggesting that the CNH peptide may have some affect on the efficiency of AAV2 capsid assembly and packaging. The CNH peptide contains a tyrosine residue, which as a large amino acid might cause a greater alteration in capsid structure and therefore could affect the efficiency of capsid formation (see chapter 5). Also, peptides containing multiple cysteine residues that can form a constrained conformation have in some cases been shown to be less well tolerated by the AAV2 capsid during packaging (Grifman *et al.*, 2001).

3.3.2. Analysis of the targeting capacity of the peptides *in vitro*

The basic transduction profiles of the AdKO1 and AAV2 peptide-modified vectors suggest that the peptide insertions are targeting the viruses to endothelial cells, as in

the majority of cases the peptide-modified viruses gave significantly higher levels of transduction than the appropriate control virus. Although the peptides were identified in a mouse model, these results suggest the viruses are not species-specific. Therefore, in the AdKO1 and AAV2 platforms the peptides are exposed on the vector surface in a location and conformation that enables binding to a receptor expressed on the surface of the vascular cell types investigated. However, in the Ad5/19p and AdKO1S* platforms, the peptides did not target the vectors to vascular cells. Therefore, the results suggest that the CAP, CNH and CQE peptides are able to target vectors to vascular cells, but the efficiency of this is dependent on the vector platform. The differences seen may be due to differences in platform vector tropism, the degree of surface exposure of the peptide or influences of the capsid on the peptide structure, as surrounding amino acids in the virus capsid may affect the conformation of the peptide.

3.3.3 Comparison of the vector platforms

3.3.3.1 AdKO1 based vectors

Unlike the other vector backgrounds the transduction profiles produced by the AdKO1 viruses show that all 3 peptide-modified viruses and unmodified AdKO1 have similar transduction profiles as they all gave the highest level of transduction in RGE cells and lowest in IP-1B cells. Importantly all 3 peptides enhanced the transduction of AdKO1 in vascular cells. Although the *in vitro* results were promising, the AdKO1 vectors were not tested *in vivo* as previous studies have shown the vector platform is not sufficiently detargeted from the liver (Smith *et al.*, 2003b, Einfeld *et al.*, 2001, Leissner *et al.*, 2001, Smith *et al.*, 2002, Nicol *et al.*, 2004, Nicklin *et al.*, 2004).

3.3.3.2 AdKO1S* based vectors

In vitro testing showed that unmodified AdKO1S* and the peptide-modified AdKO1S* viruses inefficiently infected all cell lines tested. This is despite the peptides being inserted into the capsid in the same position as in the AdKO1 vectors, suggesting that incorrect peptide exposure may not be the cause of the reduced level of infectivity. The lack of infectivity is likely to be due to the AdKO1S* mutations reducing virus infectivity and not the inability of the peptides to retarget the virus. To

determine if this is the case, cell-binding assays could be carried out *in vitro* to examine whether the viruses still binds target cells but are unable to infect them.

The S* mutation was originally proposed to block fiber binding to the putative Ad5 co-receptor HSPG (Dechecchi *et al.*, 2000, Dechecchi *et al.*, 2001, Smith *et al.*, 2003b) but a direct interaction has not been demonstrated. So it could be affecting other properties of the shaft such as fiber flexibility, or intracellular trafficking of the virus. Since this work was begun, other studies have also been performed with peptide-modified AdKO1S* viruses (A. Kritz, in press) (Bayo-Puxan *et al.*, 2006). The integrin targeting peptide CDCRGDCFC (RGD4C) (Pasqualini *et al.*, 1995) that has previously been shown to enhance transduction of both AdKO1 and AAV2 based vectors (Reynolds *et al.*, 1999, Shi and Bartlett, 2003) was inserted into the HI loop of the AdKO1S* vector. This vector was shown to produce no significant increase in transduction of integrin expressing cells such as HSVECs, although it was demonstrated to bind to cells at a significantly higher level than unmodified AdKO1S* (A. Kritz, in press) suggesting that the mutation inhibits cellular entry. Brain and lung targeting peptides that have been used to retarget AAV2 (Work *et al.*, 2006) were also tested in the AdKO1S* virus *in vitro* and *in vivo* and no retargeting was seen compared to unmodified AdKO1S* (A. Kritz, in press). A similar study that combined mutating the fiber shaft $_{91}$KK$_{92}$ to GA and the insertion of an RGD containing peptide in the III loop also reported reduced transduction levels and lack of retargeting both *in vitro* and *in vivo*, although efficient detargeting was achieved (Bayo-Puxan *et al.*, 2006). This suggests that the lack of retargeting and transduction of the plaque targeted AdKO1S* vectors is not unique to the plaque targeting peptides but has so far been shown with all peptides tested. Also, mutational ablation of HSPG binding has been shown to cause a greater reduction in transduction than the presence of soluble heparin, so it has been suggested that the HSPG mutation must be having additional affects on the virus:cell interaction (Bayo-Puxan *et al.*, 2006). Together these results indicate that the AdKO1S* mutation may actually be inhibiting a later stage in the transduction pathway following receptor binding, such as virus internalisation, endosome disruption or nuclear trafficking.

The $_{91}$KKTK$_{94}$ motif that is substituted for GAGA in the S* virus is located in a linker region of the 3rd repeat of the fiber shaft, and is just 3 residues away from a 4 amino

acid sequence that is thought to enable the fiber to bend (Chiu *et al.*, 2001, Chroboczek *et al.*, 1995). This fiber flexibility is thought to have an important role in mediating cell entry as when the flexible 3rd repeat of Ad5 was exchanged for the rigid 3rd repeat of Ad37, a significant decrease in receptor binding and infection was seen (Wu *et al.*, 2000). It is thought the fiber bend enables the virus to bind to co-receptors such as integrins by allowing the virus capsid close proximity to the cell surface whilst avoiding steric hindrances between the cell and virus (Chiu *et al.*, 2001, Wu, 2004). It may also be required to orientate the virus so it can contact multiple receptors simultaneously (Wu, 2004). It has also been proposed that the fiber bend could serve to expose the KKTK motif to enable interactions with HSPG (Smith *et al.*, 2003b). However, only subgroup C viruses have the KKTK motif but other viruses such as Ad12 in subgroup A have a flexible fiber (Chiu *et al.*, 1999), so at least in these viruses the bend must have an alternative function. Although the KKTK motif does not directly produce the flexibility in the fiber, the substitution of it may affect the fiber flexibility due to structural changes (van Raaij *et al.*, 1999) in such close proximity to the fiber bend. This could restrict interaction between the RGD sequence in the penton base and α_v integrins and so reduce virus internalisation. To determine the exact role of the KKTK motif further work is required to determine the structural and functional affect of mutating it.

3.3.3.3 Ad5/19p based vectors

The *in vitro* results show unmodified Ad5/19p only infects RGE, HSVECs and HCAECs at a low level and does not infect either of the mouse endothelial cell lines, suggesting they do not express the receptor for Ad19p, which is yet to be identified, although soluble Ad19p knob has been shown to bind sialic acid (Burmeister *et al.*, 2004) so this may be involved in the virus:cell interaction.

Although the *in vitro* results show the peptide-modified Ad5/19p vectors transduce vascular cells more efficiently than Ad5/19p, they also produce significantly higher levels of transduction of the non-target HeLa cells that do not express the putative receptors for the CAP and CNH peptides. When comparing the transduction of the vascular cells with non-target HeLa cells, only transduction of RGE cells was significantly higher ($p<0.05$). There was also no difference in the transduction of

Ad5/19p-CNH in HT1080 and HT1080 MT1-MMP expressing cells. These results suggest that the incorporation of the peptides may be enabling non-specific uptake of the viruses into cells, however to fully test this transduction of a wider range of cells, including more primary cells needs to be investigated.

Taqman results from the *in vivo* study detected reduced levels of Ad5/19p-CNH and Ad5/19p-CQE in all tissues compared to Ad5/19p, suggesting that peptide insertion has had a detargeting affect on the virus, but no retargeting effect. Ad5/19p-CAP does show some enhancement in transduction of BCA, spleen and muscle, suggesting that to some extent retargeting to areas of atherosclerosis and other tissues may be occurring. Interestingly all the Ad5/19p viruses are cleared from the blood very rapidly as no virus could be detected in any of the blood samples taken just one hour after vector administration. The total amount of virus detectable by Taqman from all organs examined appears to be greatly reduced for the Ad5/19p-CNH and Ad5/19p-CQE compared to Ad5/19p, with only 0.1% and 0.002% of the total amount of unmodified virus detected. In contrast to this, the total amount of Ad5/19p-CAP detected was 92% of the control virus. Although the viruses may have been retargeted to other tissues that were not analysed in this biodistribution study, these findings suggest that insertion of the CNH and CQE peptides into the Ad5/19p background appears to create viruses that are very unstable *in vivo* as they are quickly cleared from the body.

Interestingly, the *in vivo* results appear to show conflicting results to the *in vitro* results. *In vitro* unmodified Ad5/19p produced only very low levels of transduction in all cell lines tested and Ad5/19p-CQE gave the highest level of transduction in 5 of the 6 cell lines. Whereas *in vivo* Ad5/19p-CQE was virtually undetectable and Ad5/19p virus was detected at the highest level. As the *in vitro* results suggest a degree of non-specific cellular uptake of the peptide-modified viruses, *in vivo* this same mechanism may be resulting in the very rapid clearance of the peptide-modified viruses. The stability of Ad5/19p *in vivo* has not previously been assessed, however Ad5 has been shown to have a half-life of less than 3 minutes in mice (Alemany *et al.*, 2000, Koizumi *et al.*, 2003, Sakurai *et al.*, 2003), mainly due to the rapid uptake and degradation of the virus by Kupffer cells (Lieber *et al.*, 1997, Wolff *et al.*, 1997,

Worgall *et al.*, 1997a). It is possible that Ad5/19p viruses may also be cleared by this mechanism.

Overall these results suggest that the peptide-modified Ad5/19p vectors do not specifically transduce vascular cells or areas of atherosclerotic vasculature. As the peptides have been shown to function in other vector platforms, it may be possible to improve the retargeting of Ad5/19p by inserting the peptides into a different region of the fiber knob protein. The HI loop is normally used for targeting Ad5 viruses as it forms a flexible loop and it is one of the most exposed regions of the fiber protein (Xia *et al.*, 1994), so peptide insertion can be tolerated and the inserted peptide is accessible for binding to potential receptors (Krasnykh *et al.*, 1998). The peptides have also been inserted into the HI loop of the Ad19p fiber, but the Ad19p fiber structure (Burmeister *et al.*, 2004) shows that the HI loop may not be the most suitable position for peptide insertion and that the FG loop is more exposed so may provide a more efficient insertion site (Figure 3.19). However there is no structural evidence that shows whether the fiber can tolerate insertions at this site, so this requires further investigation. Although, it has been demonstrated that peptides can be successfully incorporated into the less flexible CD and IJ loops of Ad5 fiber knob (Lord *et al.*, 2006). Insertion of an RGD peptide into these 3 loops showed that the HI loop and CD loop modified viruses bound to integrins with a similar efficiency but the IJ loop modified virus bound with slower association and faster dissociation rates as the virus was only able to bind one integrin molecule per fiber trimer whereas the other viruses were able to bind three molecules per fiber trimer. This study highlights the fact that the peptide insertion site has an important role in determining the efficiency of retargeting.

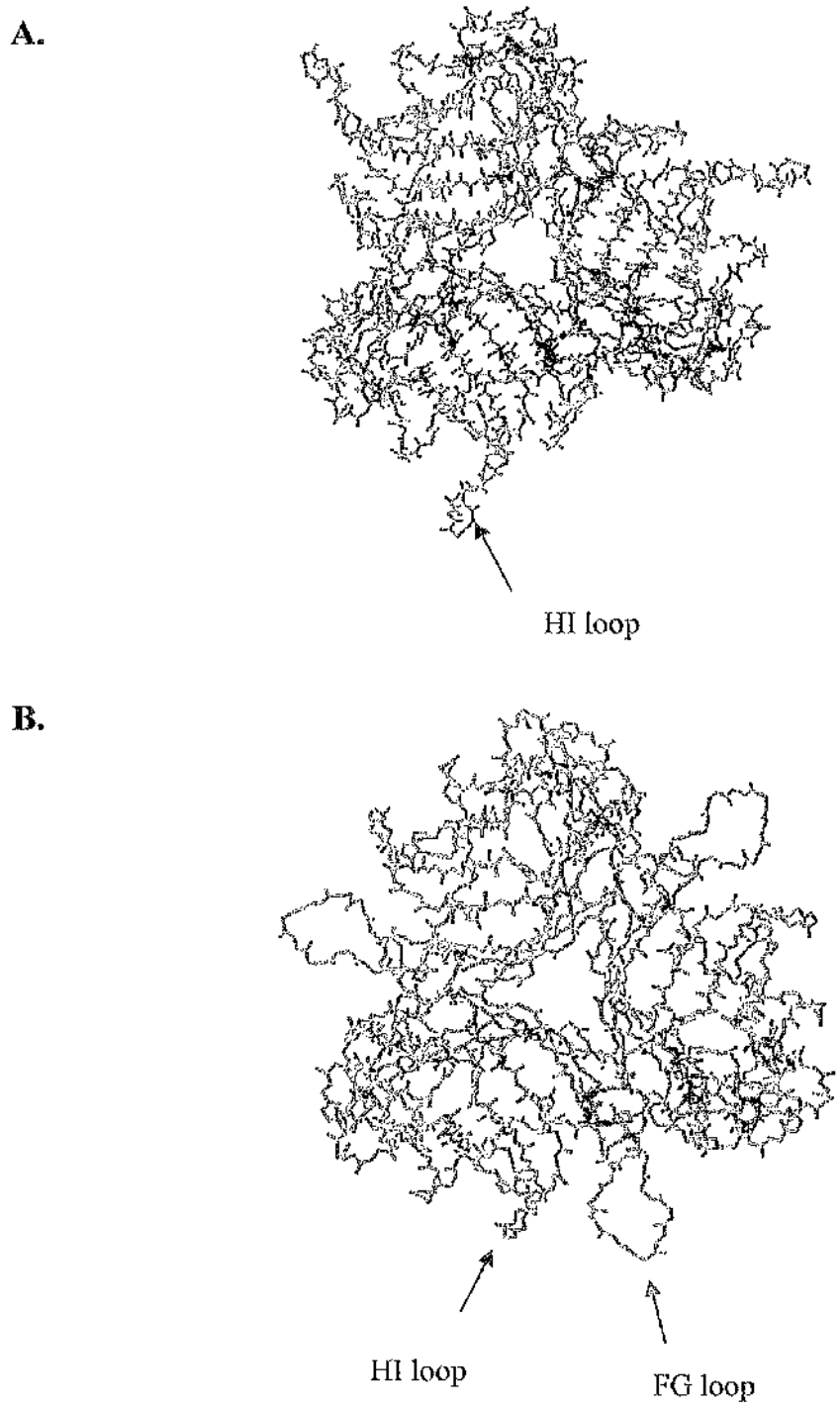


Figure 3.19 Structure of Ad fiber proteins. A. Ad5 **B.** Ad19p (Burmeister *et al.*, 2004).

3.3.3.4 AAV2 based vectors

The basic transduction profiles of the AAV2 vectors show that AAV2-CNH seems to target vascular cells whereas AAV2-CAP was the only peptide-modified virus (excluding the AdKO1S* viruses) that did not give higher transduction levels than the control virus in all vascular cell types tested. AAV2-CAP transduction was only higher than control virus in RGE and IP-1B cells. As in other vector backgrounds the CAP peptide has been shown to be capable of mediating enhanced transduction of vascular cells, further work was carried out to investigate the inefficiency of AAV2-CAP.

Infection of the mouse endothelial cells in the presence of the proteasome inhibitors MG132 and LnLL showed significant enhancement of the transduction of both control AAV2-RC and AAV2-CAP, whereas AAV2-CNH was less affected. This suggests that AAV2-CNH might use an alternative mechanism for trafficking to the nucleus that reduces virus degradation by the ubiquitin-proteasome pathway and therefore increases transgene expression. Whereas AAV2-CAP may be entering cells but producing limited transgene expression due to degradation of the virus before it reaches the nucleus. Other vascular cell targeted AAV2 vectors have also shown altered trafficking mechanisms, for example, the smooth muscle cell targeting AAV2-EYH is unaffected by the proteasome (Work *et al.*, 2004a), and the EC targeted AAV2-SIG showed increased transduction in the presence of bafilomycin A_2 an inhibitor of endosomal acidification that reduces wild type AAV2 transduction (Nicklin *et al.*, 2001a). These demonstrate not only the importance of the initial receptor binding interaction, but also the effect of capsid modifications on the later stages of transduction.

Despite the insertion of the peptides into the HSPG binding site of the AAV2 capsid, binding of the viruses to heparin columns and heparin competition experiments *in vitro* suggest that the ability of the viruses to bind HSPG and utilise it as a receptor has not been ablated. Recent studies have shown that the nature of the mutations made and peptides inserted into the heparin binding domain at residue 587 affect whether heparin binding is maintained or ablated. Binding is due to an electrostatic interaction between positively charged residues in the heparin binding site and negatively charged HSPG (Xie *et al.*, 2002), so the charge of the inserted peptide is

thought to be an important determinant of whether heparin binding is maintained (Opie *et al.*, 2003, Kern *et al.*, 2003, Perabo *et al.*, 2006b, Work *et al.*, 2006). Characterisation of an AAV2 library with random 7-mer peptides inserted after residue 587 suggests that positively charged peptides do not prevent the formation of an HSPG binding site whereas negatively charged and neutral peptides ablate HSPG binding (Perabo *et al.*, 2006b) (See chapter 5). This is supported by results using other targeted viruses, for example, the insertion of a SMC targeting peptide with 3 positively charged residues (EYHHYNK) maintained heparin binding (Work *et al.*, 2004a), whereas the insertion of the uncharged endothelial cell targeting peptide SIGYPLP ablated binding to heparin (Nicklin *et al.*, 2001a). Both the CAP (CAPGPSKSC) and CNH (CNHRYMQMC) peptides have a net positive charge as they contain 1 and 2 basic amino acids respectively. Modeling to predict the conformation of the peptides within the AAV2 capsid, suggests ways in which the heparin binding site could be formed with and without the formation of a disulphide bond between the 2 cysteine residues of the peptides (Figure 3.20). This may enable both of these AAV2 vectors to maintain an ability to bind heparin despite the disruption of the original binding site in the capsid. The CQE peptide (CQEPTRLKC) also has a net positive charge, so although it was not tested in the AAV2 background, it can be predicted that it would also produce a vector with some ability to bind HSPG.

To further enhance the detargeting of these vectors, the peptide insertion could have been combined with making 3 point mutations (the A3 vector) in the heparin binding site (R585A, N587A and R588A) that have been shown to decrease vector binding to HSPG (Wu *et al.*, 2000, Perabo *et al.*, 2006b). Based on the model of how peptides restore HSPG binding (Perabo *et al.*, 2006b), it can be speculated that making these additional mutations in combination with peptide insertion would prevent HSPG binding. There are also other residues in the capsid that are thought to contribute to HSPG binding. Structural and mutational analysis of AAV2 capsid indicates that amino acids R484 and K532 may also form part of the heparin binding site (Opie *et al.*, 2003) Kern, 2003 #5214}. The mutations R484A and K532A cause a partial decrease in heparin binding, but cause a greater decrease in transduction than expected, so they are thought to be important for a later stage in the infection process (Opie *et al.*, 2003, Kern *et al.*, 2003). To try and overcome the

A.

B.

Figure 3.20 Possible structures formed by the insertion of plaque targeting peptides after residue 587 of the AAV2 capsid. **A.** CNH peptide **B.** CAP peptide. Residues potentially involved in forming a heparin binding site are highlighted in red and possible disulphide bonds are shown in blue.

partial ablation of HSPG binding seen by inserting peptides in the 580-590 region, Shi *et al.* (Shi *et al.*, 2006) have simultaneously inserted the integrin targeting RGD4C peptide after residues 520 and 584. They showed that the double insertion had no inhibitory effect on capsid formation and caused complete inhibition of heparin binding and retargeting to cell lines expressing integrins (Shi *et al.*, 2006). But, this peptide has a net negative charge, so detargeting may be easier to achieve. With further development it may be possible to improve the detargeting of AAV2-CNH and AAV2-CAP and produce more efficient and specifically targeted vectors.

Peptide competition experiments failed to demonstrate that the infectivity of the peptide-modified AAV2 viruses is due to a specific interaction between the inserted peptide and the target cell as the presence of competing peptide had no specific effect on virus transduction. One possible explanation for this is that the synthetic peptides are produced in a linear conformation whereas the peptides were identified from a constrained phage library where the peptides are flanked by cystine residues that form a di-sulphide bond. Therefore, the structural conformation formed by the peptides in the context of the phage and viral backbone is likely to be different from the structure of the synthetic peptides. The synthetic peptides may therefore not have the same affinity for the corresponding receptor. Instead of using synthetic peptides to try and block virus transduction, it may have been more successful if the original phage containing the peptides were used as they have been shown to bind to the receptors and are more likely to contain the peptide in a similar conformation to that in the virus. However in previous studies variable results have also been seen with this method as the extent to which the secondary structure of the peptide changes in the phage and viral background is peptide dependent. For example, for 2 peptide-modified AdKO1 vectors targeted to ECs competitive inhibition was seen for only one of the viruses (Nicklin *et al.*, 2004).

Despite the possible inefficiency of AAV2-CAP *in vitro*, *in vivo* both AAV2-CAP and AAV2-CNH seem to be retargeted to the BCA and aorta of ApoE-/- mice with atherosclerosis and compared to AAV2-RC they were detargeted from all other tissues examined. The *in vitro* results may not accurately reflect the *in vivo* results as *in vitro* levels of receptor expression on healthy cell lines in tissue culture are likely to differ from that seen *in vivo*.

The biodistribution pattern of AAV2-CAP and AAV2-CNH show different results to that seen with the Ad5/19p form of the vectors. Whereas both the AAV2 vectors gave 10- 100-fold increase in transduction of both the BCA and aorta, with the Ad5/19p viruses CAP showed only a small increase (3.5-fold) in the BCA and there was actually a decrease seen with Ad5/19p-CNH. Also, the detargeting of Ad5/19p-CAP from other tissues was not as complete as that seen with AAV2-CAP as higher levels of the virus was also detected in the spleen and muscle. These results highlight the importance of both the platform vector and peptide in determining the tropism of the virus.

The *in vivo* results therefore suggest that both AAV2-CAP and AAV2-CNH provide vectors that are detargeted from the native AAV2 tropism and retargeted to areas of the vasculature in mice with atherosclerosis. The lack of retargeting of AAV2-CAP detected in mice fed a normal diet (that are thought to not develop atherosclerosis) suggests the vector is selectively targeting atherosclerotic plaques and not healthy vasculature. The study in healthy mice is due to be repeated with the AAV2-CNH vector to more precisely determine its selectivity. IHC is also being performed to determine which regions of the plaque or surrounding vasculature are transduced by the vectors. With further work these AAV2 based vectors may be developed into vectors for atherosclerotic plaque targeted gene therapy.

3.3.4 Determining whether MT1-MMP acts as the receptor for CNH modified viruses

Although one of the main advantages of biopanning is that it can be used to identify targeting peptides without the requirement for prior knowledge of a specific molecular target, this has created problems with the further development and characterisation of the peptides used in targeting viral vectors. In most published examples where peptides have been used to retarget viral vectors for gene therapy, there has been very little attempt to elucidate the novel receptor that the virus is utilising. Identifying the receptors for these vectors may be advantageous in the development of the vector by providing additional information about the tropism and specificity of the vector and may highlight any potential problems with species

specificity of the vector. It would also be advantageous for developing an accurate safety profile of modified viruses.

As the CNH peptide has homology to TIMP2 (Liu *et al.*, 2003), it was thought that MT1-MMP could act as a receptor for the CNH modified viruses. *In vitro* results suggest AAV2-CNH and AdKO1-CNH bind MT1-MMP, and for AAV2-CNH this was confirmed by SPR. However, both the SPR results and *in vitro* infections with Ad5/19p-CNH suggest that although the virus has increased transduction compared to unmodified Ad5/19p it is unlikely to be caused by the virus using MT1-MMP as a receptor. The SPR results show Ad5/19p-CNH does not bind MT1-MMP. This could be because in the Ad5/19p background the CNH peptide might form an altered conformation that has lower affinity for MT1-MMP, or it could be due to the peptide being less well exposed on the surface of the virus capsid, making it inaccessible to receptor binding. This is in agreement with the transduction data that showed there was no difference in the transduction of HT1080 cells and HT1080 cells over-expressing MT1-MMP.

The CNH peptide is homologous to a region of the N-terminus of TIMP2, which spans 2 α-helices (helices II and III). This region has high homology between human and bovine TIMP2 and human TIMP -3 and -4, but it is not conserved in TIMP1 (Fernandez-Catalan *et al.*, 1998). According to the crystal structure of the MT1-MMP:TIMP2 complex, this region of TIMP2 does not form any direct contact with MT1-MMP (Fernandez-Catalan *et al.*, 1998, Lee *et al.*, 2004a) (Figure 3.21) (Liu *et al.*, 2003). The critical residues for TIMP2 binding to MT1-MMP are 4 residues at the N-terminus, the AB-loop and the CD- and EF-connecting loops (Lee *et al.*, 2004a), so it is not known how CNH peptide may be binding to MT1-MMP, therefore the exact nature of the interaction requires further investigation.

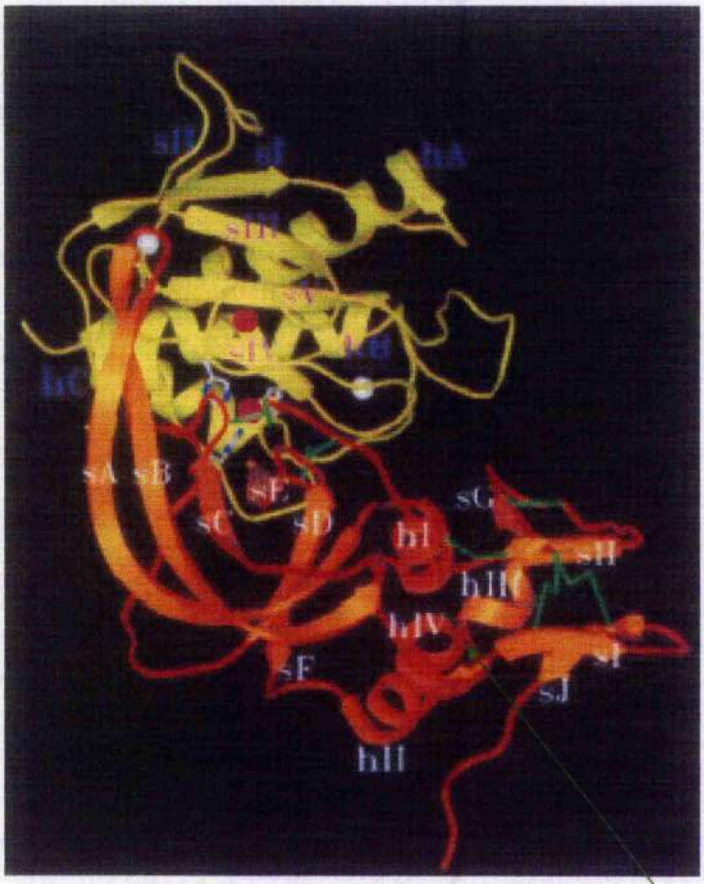


Figure 3.21 Ribbon diagram of MMP-TIMP complex. MT1-MMP is shown in yellow and TIMP2 in orange (Fernandez-Catalan *et al.*, 1998). The region with homology to CNH peptide is marked.

3.3.5 Summary

The 3 atherosclerotic plaque targeting peptides CAP, CNH and CQE have been successful incorporated into the capsid of viral vectors AdKO1, AdKO1S*, Ad5/19p and AAV2. Although there are noticeable differences in the *in vitro* transduction profiles of the peptides in the different platform vectors, the results suggest that the peptides are able to mediate virus transduction of vascular cells. As an exception to this, in the AdKO1S* background only low levels of transduction were detectable both with the unmodified virus and all 3 peptide viruses, suggesting that in some way the viruses all had significantly reduced efficiency of infection. It is therefore unlikely that the AdKO1S* background will provide a useful platform for any gene therapy vector as the level of transgene expression achieved is too low to have any therapeutic effect.

In vitro, the Ad5/19p peptide-modified viruses did give significantly higher levels of transduction of the vascular cells, but this was also seen in HeLa cells, which suggests that the increase may not simply be due to peptide:receptor interaction and peptide incorporation may have activated non-specific viral uptake. *In vivo* Taqman data suggests that Ad5/19p-CNH and Ad5/19p-CQE were cleared from the body very quickly as only very low levels of virus could be detected in any of the tissues tested. Ad5/19p-CAP which generally gave the lowest levels of transduction of the 3 peptide-modified viruses *in vitro* was detectable *in vivo* and did demonstrate some degree of retargeting to the BCA, but also to other organs such as the spleen.

The most promising results were achieved with the AAV2-CAP and AAV2-CNH. The *in vitro* results were particularly good for AAV2-CNH, which produced enhanced transduction of all the vascular cells, but not HeLa cells. The results also suggest it may traffic to the nucleus via a pathway which avoids degradation by the ubiquitin-proteasome pathway. The *in vivo* results with both AAV2-CAP and AAV2-CNH were very encouraging, as they suggest the native tropism of the virus has been greatly reduced and the viruses have been retargeted to areas of the vasculature where atherosclerosis is known to occur. However, for both AAV2-CAP and AAV2-CNH some ability to bind heparin has been maintained, although by incorporation of further detargeting mutations it may be possible to reduce this and produce efficiently retargeted vectors.

Chapter 4:
Phage Display to Identify Peptides That Target Unstable Atherosclerotic Plaques

4.1 Introduction

Rupture of unstable atherosclerotic plaques is one of the major causes of mortality due to CVD (Falk *et al.*, 1995). Although there are several treatments available for atherosclerosis e.g. statins, there is currently a lack of treatments which aim to directly stabilise rupture prone plaques by altering their composition. Gene delivery could potentially be used to stabilise vulnerable atherosclerotic plaques and thereby prevent plaque rupture, if a vector that can selectively target unstable plaques can be developed. For this to be possible, molecules expressed at a high level on the surface of unstable plaques need to be identified and exploited as receptors for novel vectors.

4.1.1 Molecular characterisation of atherosclerotic plaques

Unstable plaques have been well characterised at the cellular level, but at the molecular level they are less well understood. There are few molecular markers that are known to be expressed at a high level, selectively on unstable atherosclerotic plaques. There have been several microarray and proteomics studies carried out to try and improve this knowledge, however the majority of studies have compared plaques with healthy vasculature (Seo *et al.*, 2004, Martinet *et al.*, 2003, Duran *et al.*, 2002) and not specifically focused on unstable plaques. A recent study, however, has investigated the gene expression profile of unstable and stable plaques from the same patient and identified many genes that have upregulated expression levels in unstable plaques (Papaspyridonos *et al.*, 2006). Some of these have previously been associated with plaque rupture e.g. MMP1 and MMP9 whilst others such as legumain (possibly involved in controlling matrix degradation) and vinculin (involved in attaching actin filaments to the plasma membrane) have no previous association (Papaspyridonos *et al.*, 2006). In another study genetic profiling of stable and ruptured human atherosclerotic plaques found perilipin was expressed in 80% of ruptured human plaques but not in any stable plaques. It is involved in lipid hydrolysis and may have a role in increasing the size of the lipid core (Faber *et al.*, 2001). A further study has also been carried out using an ApoE-/- mouse model of atherosclerosis. Proteomic and metabolomic analysis of aortas from 10 week, 12 month and 18 month old ApoE-/- mice was used to look at changes that

occur in different stages of plaque development (Mayr *et al.*, 2005). Many changes in immune activation, oxidative stress and energy metabolism were identified (Mayr *et al.*, 2005).

Although these studies have provided further information about the disease, their relevance for developing gene therapy vectors targeted to unstable plaques is limited. To target a gene therapy vector to unstable atherosclerotic plaques, the vector needs to bind efficiently to a receptor that is expressed at a relatively high level on the plaque surface and this expression must be selective. Many of the proteins identified in the studies described above are intracellular so could not be used as receptors for vectors as they would not be accessible for binding. Also, the proteins are not exclusively expressed in plaques but are also found in other tissues so may not provide the required level of specificity.

4.1.2.1 Phage display

Biopanning with a phage display library provides a method of identifying peptides that bind to a specific target without any prior knowledge of either the target receptor or the targeting ligand. Since complete molecular profiling of atherosclerotic plaques has not yet been performed, biopanning is likely to be the most effective way to identify potential atherosclerotic plaque specific receptors.

4.1.2.2 Phage libraries

There are 2 types of phage that are commonly used for phage display, M13 and T7. M13 is a single-stranded filamentous DNA bacteriophage. It enters bacteria by binding to the F pilus, then uncoats and its DNA is converted to a double stranded form, so that DNA replication can occur. From each infected cell about 200 copies of the phage are produced. When M13 phage is released from the bacterium it does not kill the cell, so M13 plaques are areas of reduced growth and not clear areas due to lysis of the bacteria. M13 is a very stable phage and can survive temperatures as high as 55°C and pH as low as 3. The M13 phage capsid consists of about 2700 copies of the major coat-protein pVIII and about 3-5 copies of the minor protein pIII. In most M13-based libraries the

peptides are inserted into the minor coat protein gene III protein (g3p) so that the peptides are displayed on the surface of the phage without significantly affecting its ability to infect *E.coli*.

T7 is a double stranded DNA, lytic phage. Like M13 phage T7 can withstand harsh conditions such as extremes of temperature. Proteins of up to 1200 amino acids can be displayed on the phage capsid using the low or mid copy number display systems (0.1-15 copies per phage) and peptides up to 50 amino acids long can be displayed in the high copy number system (415 copies per phage) (Novagen, 2002).

Phage libraries normally contain peptides that are between 6 and 15 amino acids long. They can be in a linear conformation or they can be flanked by cysteine residues that form a disulphide bond under physiologically conditions, which forces the peptides into a constrained conformation. The configuration of linear peptides can be affected by the surrounding amino acids in the phage capsid to a greater degree than constrained peptides. Constrained peptides are thought to more closely represent the configuration of the peptides when they are inserted into viral vectors as both termini of the peptides will be bound to residues in the capsid. For example, peptides inserted into the HI loop of Ad vectors are constrained at both the N and C terminus, whereas peptides in filamentous phage libraries are constrained at only the C terminus.

Originally phage display used M13 based libraries but T7 libraries are becoming more common as they have several advantages over M13 phage. T7 phage replicate faster than M13 phage, so plaques form in 3 hours and cultures lyse within 1-2 hours whereas plaques take 10-12 hours to form with M13 phage.

A recent study used a bioinformatics approach to compare the diversity of 12-mer and 7-mer libraries of T7 and M13 phage. It was found that the T7 libraries had a higher diversity (up to 14-fold higher for a 12-mer library) and fewer amino acid biases in their peptide composition (Krumpe *et al.*, 2006). The restricted diversity of M13 libraries is in part due to the peptides having to be compatible with the phage assembly, secretion and

infection process. T7 phage are not secreted through the host cell membrane so do not have to be compatible with the bacterial cell membrane secretory complex, therefore a larger range of peptides should be compatible with the phage biology (Krumpe *et al.*, 2006). M13 libraries have also been shown to have an amino acid bias caused by some peptides reducing the efficiency of phage assembly and infection whereas other peptides seem to have a selective advantage (Rodi *et al.*, 2002).

One potential problem with *in vivo* biopanning with T7 phage is that it has been shown in mice to be rapidly removed from the blood due to an immune response against the phage (Srivastava *et al.*, 2004). Just 5 minutes after systemic administration into C57/Bl6 mice less than 50% of phage remains detectable in the blood and after 1 hour this is reduced to less than 1% (Srivastava *et al.*, 2004). Using immunocompromised strains of mice it was demonstrated that this neutralising effect is mediated mainly by B-cells (Srivastava *et al.*, 2004). This rapid neutralisation of phage may reduce the efficiency of biopanning as it might prevent potential targeting phage reaching the tissue of interest. However this means that any phage identified are likely to be highly efficient and stable *in vivo*.

4.1.3 Vascular targeted phage display

There are many examples of studies where *in vitro* phage display has been used to identify vascular targeting peptides. If the molecular target is known then biopanning can be carried out on either an immobilised form of the protein or cells overexpressing the protein. For example, White *et al.* (White *et al.*, 2005) carried out phage display on endothelial cell protein C receptor (EPCR) as it is known to be expressed at relatively high levels on the vascular endothelium of arteries and veins, so could potentially be a good target receptor for endothelium targeted gene therapy. Cells that do not express EPCR were transfected with an EPCR plasmid so that it was over-expressed. Negative selection steps were carried out on untransfected cell lines to remove phage that bound to naturally occurring receptors and then biopanning was carried out to select EPCR binding phage. Several targeting motifs were identified which had homology to functionally significant proteins such as an MMP (White *et al.*, 2005).

If no receptor is known then *in vitro* biopanning can be performed on a target cell type such as cardiomyocytes (McGuire *et al.*, 2004), to identify both the targeting ligand and potential receptors. To increase the efficiency of *in vitro* biopanning a pre-clearing step on another cell type can be used. For example, preclearing steps using vascular SMC, HepG2 and peripheral blood mononuclear cells was used to create a restricted library for biopanning on human umbilical vein ECs, which identified the endothelial cell targeting peptide SIGYPLP (Nicklin *et al.*, 2000). Similar preclearing steps on HepG2 and peripheral blood mononuclear cells were used to identify peptides that target human saphenous vein SMC (Work *et al.*, 2004a).

4.1.4 Phage display targeting atherosclerosis

In vitro biopanning has also been performed to identify phage that bind to molecules known to be expressed in atherosclerosis. For example, biopanning has been carried out on cells overexpressing lectin-like oxidized LDL receptor (LOX-1) (White *et al.*, 2000). LOX-1 is a receptor for oxidized LDL that is expressed on endothelial cells, SMC, monocytes and macrophages. Its expression is up regulated by ox-LDL, angiotensin II, endothelin, cytokines and shear stress, so it is found in high levels in atherosclerotic plaques (Mehta *et al.*, 2006). It has also been found to be expressed on platelets and has a role in platelet activation and thrombus formation (Chen *et al.*, 2001). Its expression is upregulated from the early stages of plaque development so it may be a useful target for gene therapy to prevent plaque development.

Phage display has also been performed on immobilised murine P-selectin, which is expressed on the surface of both early and advanced plaques (Molenaar *et al.*, 2003). After 10 rounds of biopanning one peptide was found in more than 80% of phage sequenced. This peptide was found to be highly specific for murine P-selectin as its binding was found to be 100-fold higher than binding to either E-selectin or human P-selectin (Molenaar *et al.*, 2003). Due to the high species specificity of the isolated peptide it is unlikely to be useful for developing clinically relevant gene therapy vectors.

All these examples of *in vitro* biopanning are targeting potential receptors expressed in the early stages of atherosclerotic development and not more advanced plaques. Further studies are required to determine their selectivity for atherosclerotic vasculature *in vivo*.

Several groups have performed *in vivo* phage display to identify plaque-targeting peptides. The first study used a 12-mer constrained library in LDLR-/- mice (Houston *et al.*, 2001). For the first round of biopanning mice received 1 x 10^{10} pfu phage and in rounds 2 and 3 mice received 1 x 10^{9} pfu phage. After round 1 only 250 phage were picked and amplified for the next round and after round 2 this was reduced to just 100 phage. The low dose used and the severe restriction of the library diversity after one round of biopanning will have limited the results of this study. Also, only one mouse was used for each round of the biopanning. The study identified 10 peptides, one of which (CLVEAYPGL-Stop-VRSC) was then investigated further. IHC performed on frozen sections of plaques incubated with the selected phage showed the phage mainly bound to the plaque, although some was localised to the surrounding endothelium. When this was repeated using vasculature from a healthy C57/Bl6 mouse no phage binding was detected. However, when the phage was administered systemically high levels of phage were found to have bound to the liver, spleen, kidney and lung as well as the vasculature. Therefore there is some question about the specificity of the selected phage. The biopanning may have been improved by using a predose of non-peptide expressing phage, to reduce the chances of identifying a phage that binds to non-specific receptors. Also, due to the variability in the extent of disease in different mice in all models of atherosclerosis, it may have been better if more than one mouse was used at each round. In this study there was no attempt to determine what the potential receptor for the selected peptide could be.

Liu *et al.* (Liu *et al.*, 2003) carried out biopanning with a T7 7-mer constrained library in fat fed ApoE-/- mice to identify phage that bound to plaques in the aorta (Liu *et al.*, 2003). A predose of UV irradiated phage was administered 30 minutes before injection of the phage library. After 4 rounds of biopanning several consensus motifs were identified and some of these were found to have homology to proteins associated with

atherosclerosis e.g. TIMP2 (Liu *et al.*, 2003) (See chapter 3). Further work showed one of the selected phage also targeted plaques isolated from humans (Liu *et al.*, 2003).

Recently another group have performed biopanning in ApoE-/- mice to identify atherosclerotic plaque targeted peptides (Kelly *et al.*, 2006). 3 rounds of biopanning using an M13 7-mer linear library were carried out. The aim of the study was to identify peptides that could be used for targeted imaging techniques so the biopanning was designed to identify phage that were efficiently internalised into cells. To achieve this phage bound to the vessel surface were removed by an acidic glycine wash before the cells were lysed and internalised phage were isolated. After 3 rounds of panning, 96 phage were sequenced and very few repeated sequences were found, so peptides were divided into 30 different groups based on common motifs found within the peptides. Two groups were said to have homology to proteins associated with atherosclerosis. One of these peptide motifs was said to have homology to Very Late Antigen-4 (VLA-4), a ligand for VCAM-1, however only 2 out of the 7 amino acids were the same and the other 5 amino acids were not conservative differences (VHPKQHR peptide homologous to VNPGAIY). The peptide was shown to bind to murine coronary artery endothelial cells, and its uptake could be inhibited by soluble VCAM-1. Further *in vivo* testing is required to determine the specificity of this peptide.

Previously the same group had performed four rounds of biopanning on mouse endothelium under physiological flow conditions and identified another peptide that was thought to bind to VCAM-1 (CVHSPNKKC) (Kelly *et al.*, 2005). For plaque imaging studies the peptide was conjugated to a magnetofluorescent nanoparticle to enhance the accumulation of the nanoparticle in endothelial cells to improve target to background ratios (Kelly *et al.*, 2005). The complex was shown to rapidly internalise into murine cardiac endothelial cells by binding to VCAM-1. It was also found to accumulate in areas of atherosclerosis in ApoE-/- mice. But, because a constrained peptide was used, only 4 peptides could be attached to each nanoparticle, therefore to increase this they attempted to identify a linear peptide to use instead (Kelly *et al.*, 2006). VCAM-1 is expressed on the endothelium under inflammatory conditions and has been detected in athero-prone

areas before any visible atherosclerosis has developed (Cybulsky and Gimbrone, 1991), so it could be used as a marker of early atherosclerotic development. However VCAM-1 is expressed by cells other than ECs (Minami and Aird, 2005) so may not provide a highly specific target.

Although there has been some success in identifying peptides that target to atherosclerotic plaques using *in vivo* biopanning, none of the studies have focused on identifying peptides that specifically target advanced plaques. Following identification of plaque targeting peptides none of the groups have shown that systemic administration of the targeted phage results in a high level of specific binding to areas of atherosclerosis.

4.1.5 Aims

The aim of this chapter was to use *in vivo* phage display to identify peptides that target to unstable atherosclerotic plaques. This should provide more information about the molecular composition of plaques by identifying proteins that are expressed on the plaque surface and identify peptides that can be incorporated into the capsid of viral vectors to produce an atherosclerotic plaque targeted vector which could be used as a biological tool and as a potential therapy.

4.2 Results

4.2.1 Determining the diversity of the T7 library

Constrained (CX8C) and linear (X8) T7 phage libraries with random 8-mer peptides displayed at the C-terminal of the phage capsid protein 10B were used in this study (gifts from E. Ruoslahti, Burnham Institute, CA, USA). Both libraries and non-recombinant (NR) control phage without a peptide insertion were amplified using the standard liquid culture method and the phage samples were purified by PEG precipitation. To examine the diversity of the libraries, individual plaques were picked then the peptide insertion region was amplified by PCR and sequenced. 49 and 58 phage peptides from the X8 and CX8C libraries respectively were sequenced. All were found to contain different peptides (sequences not shown). Within these peptides there were 14 that were not 8-mers but had inserts of between 5 and 11 amino acids long. There were also 36 peptides that encoded stop codons (sequences not shown).

Analysis of the amino acid diversity showed the peptides contained approximately the expected amounts of the majority of amino acids (Figure 4.1). The results suggest that amino acids D and R were over-represented in the linear library where as K and T were under-represented. In the constrained library C and N were over-represented and P, Q and R were under-represented. Based on the amino acid distribution of the 2 libraries the diversities of the linear and constrained libraries were calculated to be 9.06×10^8 and 2.20×10^9, respectively.

4.2.2 UV irradiation of control phage

Following systemic injection phage naturally accumulate in the liver and spleen due to uptake by the reticulo-endothelial system (RES) (Clackson and Lowman, 2004). To prevent a large proportion of the library being taken up by the RES, 'control' phage that have no inserted peptide can be pre-injected to block these non-specific binding sites and saturate the RES before the library phage are administered. In these experiments NR phage inactivated by UV irradiation was used as a 'predose'. The inactivation is required to prevent the control phage from being able to replicate and therefore affect the titering

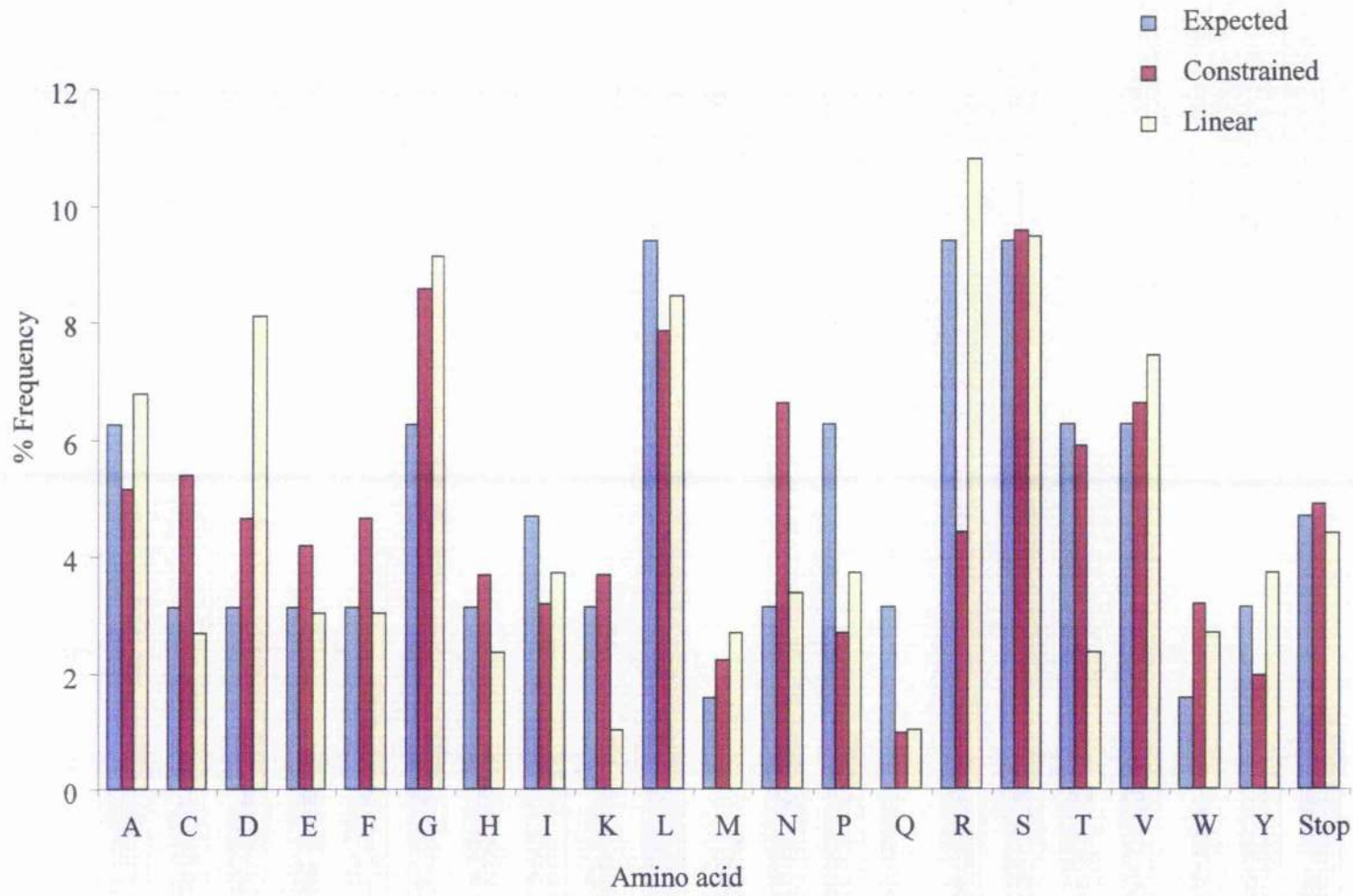


Figure 4.1 Amino acid composition of the peptides from phage libraries. Based on sequencing results of 49 peptides from the linear library and 58 peptides from the constrained library. Alanine (A), Cysteine (C), Aspartic acid (D), Glutamic acid (E), Phenylalanine (F), Glycine (G), Histidine (H), Isoleucine (I), Lysine (K), Leucine (L), Methionine (M), Asparagine (N), Proline (P), Glutamine (Q), Arginine (R), Serine, (S), Threonine (T), Valine (V), Tryptophan (W), Tyrosine (Y).

results. A timecourse experiment was performed to determine the level of irradiation required to completely inactivate the NR phage (Figure 4.2). This showed that 9 hours UV irradiation was sufficient to render the phage replication deficient.

4.2.3 *In vivo* biopanning

In vivo biopanning to identify peptides that target unstable atherosclerotic plaques was performed using ApoE-/- mice on a C57/Bl6/129SvJ background fed a high fat Western diet for 10 weeks. These animals have been shown to develop unstable plaques, as after 8 weeks of fat feeding over 60% of mice have plaques that appear to have ruptured (Johnson *et al.*, 2005b). This model was chosen as it is one of the most reliable models of spontaneous plaque rupture and has many of the features of the human disease.

Five different biopanning protocols were used (Table 4.1). In experiment 1, approximately 400 individual phage plaques were picked, sequenced and amplified at each round. Individual amplification of phage was used to try and reduce the potential bias created by individual peptides causing a sequence specific reduction in the efficiency of phage replication (Pasqualini and Ruoslahti, 1996). However, the majority of the biopanning was performed using bulk amplification of the phage directly from the tissue lysate so that the amplification process did not artificially limit the diversity of the phage used in subsequent rounds. The number of mice used for each round was between 3-5 depending on availability (Table 4.2). Although in many published biopanning experiments only one animal is used per round (Pasqualini and Ruoslahti, 1996, Arap *et al.*, 2002b, Arap *et al.*, 2002a, Kolonin *et al.*, 2001, Hoffman *et al.*, 2004, Laakkonen *et al.*, 2002), for this work it was thought using several animals would produce more consistent results as there is some variation on the degree of atherosclerosis and plaque instability in this model.

At each round of biopanning the amount of phage recovered from the BCA was calculated (Figure 4.3). In all 5 biopanning experiments the amount of phage recovered was higher in the final round compared to the first round, although this increase was only significant for protocols 2 and 5 (Figure 4.3). As it was not possible to use the same dose

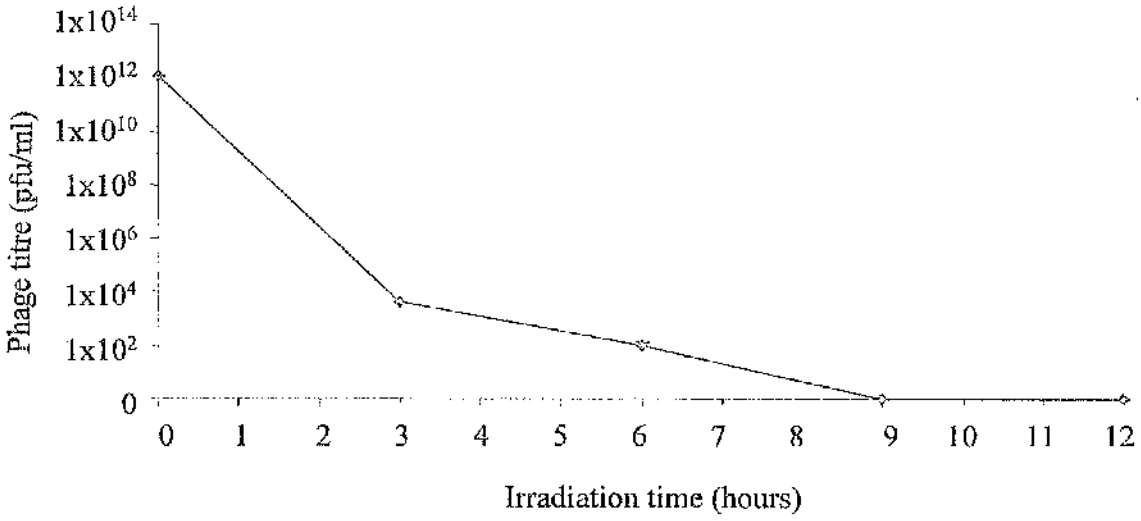


Figure 4.2 Effect of UV irradiation on phage titre. Aliquots of phage were irradiated in the UV Stratalinker (Stratagene) using 120 mJ for between 0-12 hours. At three hour intervals phage were titred to determine the amount of infectious phage remaining.

Protocol	Predose	Dose	Circulation time	Phage library	Dose	Circulation time	Amplification method
1	UV irradiated insertless phage	1 x 10^{11} pfu	15 mins	X8	1 x 10^{10} pfu	15 mins	Individually picked an amplified phage
2	UV irradiated insertless phage	1 x 10^{11} pfu	15 mins	X8	1 x 10^{10} pfu	15 mins	Bulk amplification of tissue lysate
3	None	-	-	X8	1 x 10^{11} pfu	15 mins	Bulk amplification of tissue lysate
4	UV irradiated insertless phage	1 x 10^{11} pfu	15 mins	CX8C	1 x 10^{10} pfu	15 mins	Bulk amplification of tissue lysate
5	None	-	-	CX8C	1 x 10^{11} pfu	15 mins	Bulk amplification of tissue lysate

Table 4.1 Protocols used for *in vivo* phage display. For round 2 of the bulk amplified biopanning, the titres were not high enough to give the dose stated. A dose of 5 x 10^{10} pfu and 2.6 x 10^{10} pfu of X8 and CX8C pools were used in the non-predosed animals. For all other rounds the stated doses were used.

Protocol	Round 1	Round 2	Round 3	Round 4
1. X8 picked + predose	4	4	3	--
2. X8 + predose	5	3	4	3
3. X8 no predose	5	3	4	4
4. CX8C + predose	5	3	4	4
5. CX8C no predose	5	3	4	4

Table 4.2 The number of animals used in each round of biopanning. From the age of 8 weeks all animals were fed a high fat diet containing 21% beef lard supplemented with 0.15% cholesterol for 10 weeks before being used in the study.

of phage for all rounds, the amount of phage recovered was calculated as a percentage of the total dose received (Figure 4.4). In 3 of the 4 biopanning experiments where bulk amplification of the libraries was used, a decrease in the amount of phage in the BCAs was seen over the first three rounds and then an increase was seen in the last round. As a control, the amount of phage in a non-target organ was also determined. The kidney was chosen for this as it is a highly vascularised tissue that has previously been used as a target for *in vivo* biopanning in mice and has been shown to express unique molecular markers (Pasqualini and Ruoslahti, 1996) that should be distinct from those upregulated in atherosclerotic BCAs. For experiments 2, 4 and 5 a decrease in the amount of phage in the kidney was seen from rounds 1 to 4 and by the final round there was no detectable phage (Figure 4.5). In the other two experiments there was a slight increase in the amount of phage recovered. In experiment 3 this was not significant and the amount of phage detected was lower than that found in the BCA.

4.2.4 Sequencing of phage recovered from the BCA

Analysis of the sequencing results from experiment 1 showed the library diversity was reduced as biopanning progressed (Table 4.3). After just one round there were very few repeated peptides. In the second round the number increased, but the majority of repeated peptides still had an occurrence of 1% or less. By the third round there were fewer repeated peptides, but they made up a larger percentage of the sequences. Of particular interest was the LSVISS peptide as this was found in 19.4% of phage sequenced.

After rounds 1, 3 and 4 of biopanning with the bulk amplified libraries, phage samples were picked and sequenced to monitor the progress of the biopanning. After 1 round of panning between 150-200 peptides from the X8 and CX8C pools were sequenced and no repeated sequences were found in any of the experiments (data not shown). Round 2 was not sequenced as it was thought it would still be too early to detect any targeting motifs within the peptide pool. After the third round of biopanning, sequencing showed that there were several repeated peptides within the libraries, suggesting that the library was becoming enriched in phage targeting the BCA, but some diversity still remained (Table 4.4). There was one peptide (CFGEGAEQSC) found in both CX8C biopanning

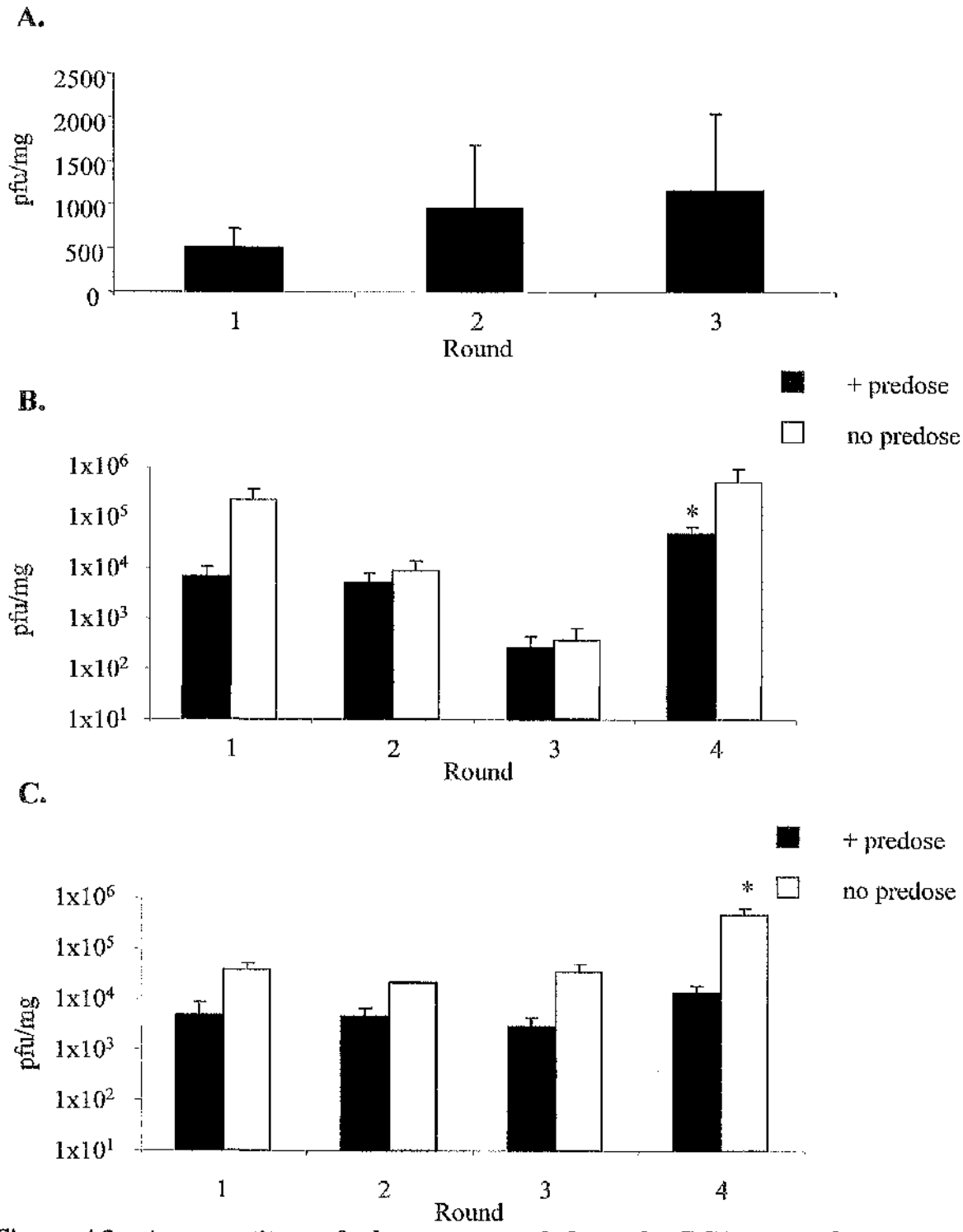


Figure 4.3 Average titres of phage recovered from the BCAs at each round of biopanning. A. Protocol 1. X8 picked phage, **B.** Protocols 2 and 3. X8 bulk amplified phage, **C.** Protocol 4 and 5. CX8C bulk amplified phage. * $p < 0.05$ vs round 1

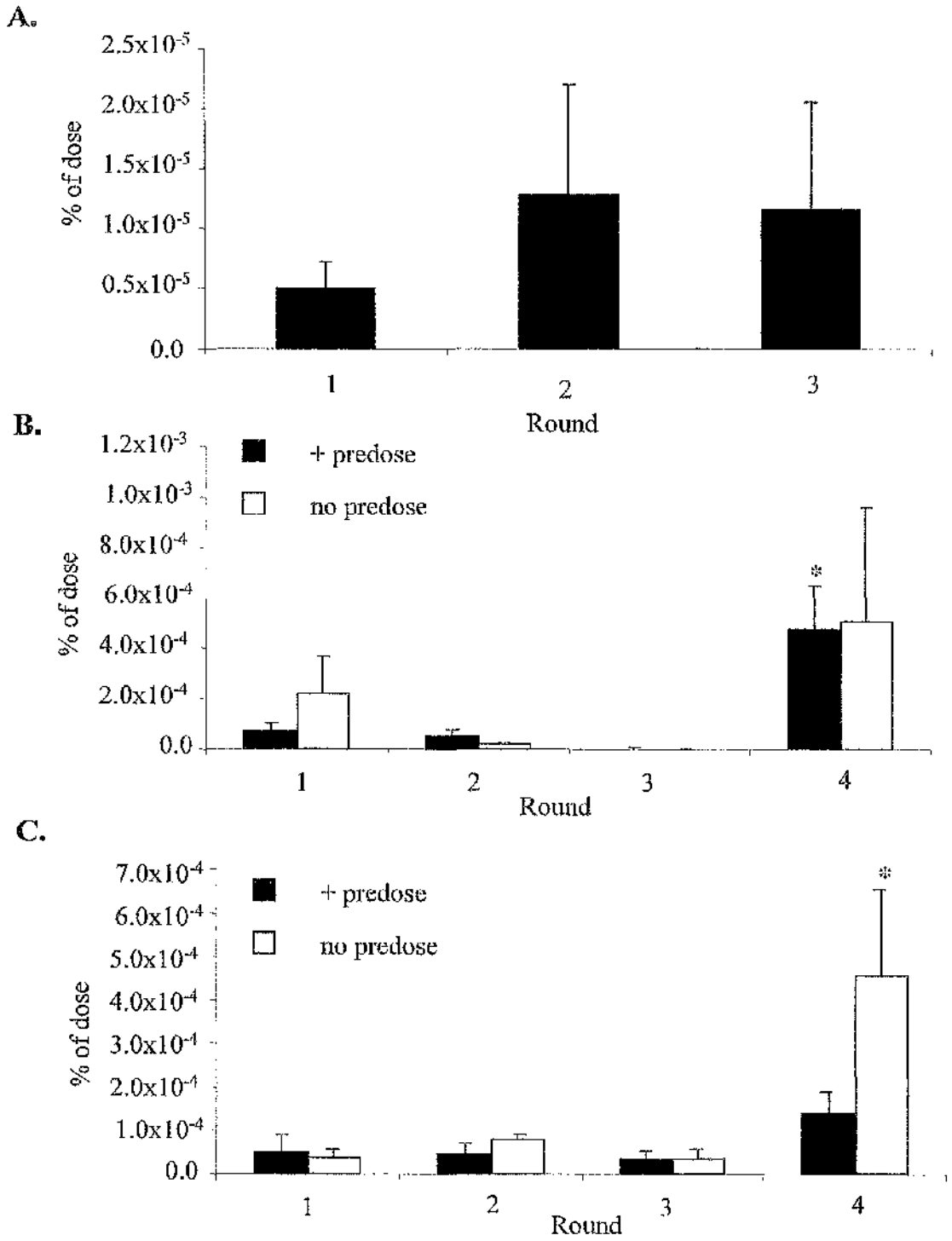


Figure 4.4 Percentage of phage recovered from the BCAs at each round of biopanning. Titres are shown as a percentage of the dose given. **A.** 1. X8 picked phage, **B.** Protocols 2 and 3 X8 bulk amplified phage, **C.** Protocols 4 and 5 CX8C bulk amplified phage. * $p < 0.05$ vs round 1

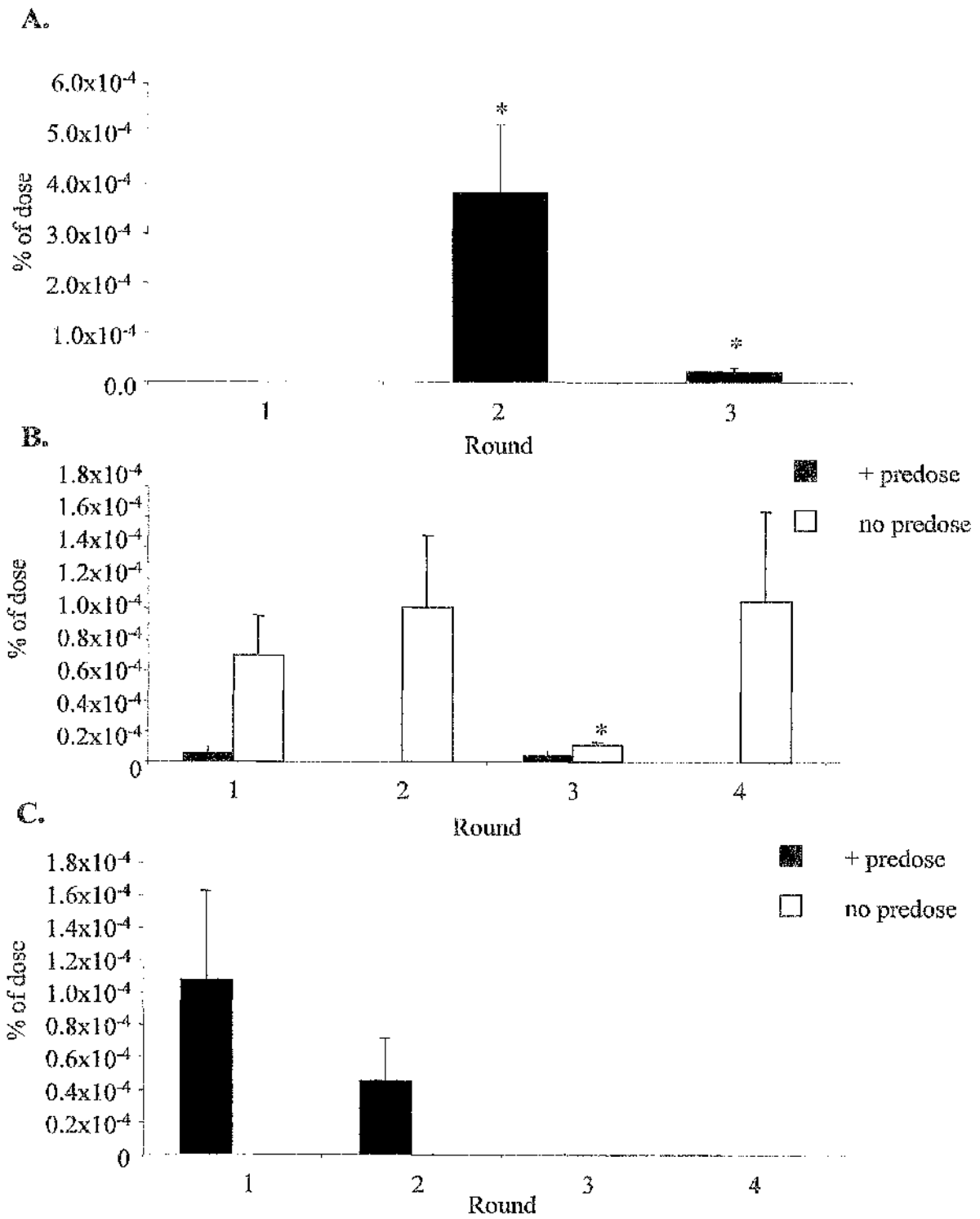


Figure 4.5 Average titres of phage recovered from the kidney at each round of biopanning. Titres are shown as a percentage of the dose given **A.** Protocol 1. X8 picked phage, **B.** Protocols 2 and 3, X8 bulk amplified phage, **C.** Protocols 4 and 5, CX8C bulk amplified. * $p < 0.05$ vs round 1

Peptide	% Occurrence Round 1	% Occurrence Round 2	% Occurrence Round 3
*GQCWLVT	-	0.5	-
A**YADAY	-	0.5	-
AK*LCDDG	-	0.5	2.1
ALRVY*LP	-	0.5	-
CTSRYNGL	-	0.5	-
CVTVSA	-	1.2	-
DLH*LVNR	-	0.5	4.2
DLVSYTAM	-	0.7	-
DTRQFICC	-	0.5	-
DWG*KDLY	-	0.7	-
FDK*QFAD	-	0.5	-
FSDSIVRQ	-	0.5	-
GICRGRNK	-	1.0	-
GITALCFN	0.5	-	-
GNS*V*HS	-	0.5	-
INPTNKLA	-	0.5	-
IRSLIYNT	-	0.5	-
ISWSHLLT	-	0.7	-
L*LVMMTG	-	1.2	-
LAPSRSAS	-	0.5	-
LATLAYA*	-	0.7	-
LLLRR*SWA	-	0.5	-
LMTCDASV	-	0.7	-
LPRLNSMV	-	1.7	2.1
LSTVKTYE	0.5	-	2.8
LSVISS	-	3.0	19.4
LVEMLLC	-	0.5	-
NEHGIINS	0.5	-	-
NFEIFNAS	-	1.0	-
NFLMR*LR	-	0.5	-
NTWIAYKA	-	-	1.4
PIET*KVH	-	1.0	2.8
PLNDCHSF	-	0.7	2.8
PTSMCGLV	-	0.5	-
PVRLDSDN	-	0.7	-
QIP*LFEI	-	0.7	2.8
RFQVFDT*	-	0.5	-
RGLDSYET	-	0.5	-
RSQDQLN*	-	1.0	-
RTEQHLPL	-	0.7	-
RVPH[illegible]VMI	-	0.7	2.1
SCGVSGLYY	-	0.5	-
SIESSSKR	-	1.5	4.2
SPVSKGRD	-	0.7	-
SQP*P[illegible]KT	0.5	-	-
SYPFRLSL	1.2	1.2	-
TALKNAEQ	0.5	0.5	-
TGGNVSYG	0.7	0.7	1.4
TMCGIHRK	0.7	0.7	-
TVDGNLWS	0.5	-	-
VS*RFV*I	-	1.0	-
VTSEKGQT	-	0.5	-
VYLTGRSP	-	0.7	-
VYWKSLI*	-	0.5	-
WLRDD*QYH	-	0.5	-
WW*RHYM	-	0.5	-
YRKRWTDM	-	0.5	-
YRPLDKSK	-	0.5	-
YRTRLISK	-	0.7	-

Table 4.3 Sequences of peptides from phage identified more than once from biopanning protocol 1. (X8 library with predose using individually picked phage). From rounds one, two and three 379, 404 and 144 phage peptides were sequenced respectively. Peptides recovered from more than one biopanning experiments are highlighted in bold. * = stop codon

A.

Peptide	% Occurrence Round 3 from 153 peptides	% Occurrence Round 4 From 142 peptides
*AHVNVNT	1.3	-
*GLSSMTN	-	1.4
*TSLSNID	-	1.4
AWMANKDF	-	1.4
FNLIMAQS	-	1.4
FYDMESVN	-	2.1
GNLCVQ*I	-	1.4
HPIIGLSY	-	2.8
LALQLGRD	-	1.4
LSVISS	-	2.8
LV*FISSS	-	2.1
PLYVEIDR	1.3	-
PSFDSGMN	1.3	-
PSSVDNTG	-	2.8
SCVTVM*F	2.0	-
SVTRHIMK	-	1.4
TLFKRGNQ	1.3	-
VNKPIHVF	-	2.1
VSIANNRL	-	4.2
VSNSYYTR	-	3.5
YMR*RSFF	-	2.1

B.

Peptide	% Occurrence Round 3 From 137 peptides	% Occurrence Round 4 From 118 peptides
*RMTSSII	-	2.5
ANDYSL*I	-	1.7
AVRHDYK*	-	1.7
F*SLLKKL	1.5	-
FFL*AKTF	-	1.7
FGDDLVSS	1.5	-
FISVRVLS	-	2.5
FNSRRSME	-	1.7
GMEVVIKM	-	1.7
HRGIFSNQ	-	3.4
IHRLFLTR	-	1.7
PSFDSGMN	-	6.8
QDHFPIVN	-	8.5
RIVRT*HF	-	-
SCVTVM*F	-	3.4
TYSQK*FY	-	1.7
YTWHAPVK	-	1.7
YVTMKHHP	-	3.4

Table 4.4 Sequences of peptides from phage identified more than once from the biopanning using the bulk amplification protocol. A. 2. X8 + predose. **B.** 3. X8 no predose. **C.** 4. CX8C + predose (overleaf). **D.** 5. CX8C no predose (overleaf). * = stop codon. Peptides recovered from more than one biopanning experiments are highlighted in bold.

C.

Peptide	% Occurrence Round 3 From 170 peptides	% Occurrence Round 4 From 243 peptides
C*C*RVTNTC	-	0.8
C*GRSRVRIC	-	0.8
C*IEANSLRC	-	1.2
C*NFHRTLNC	-	0.8
CADYCG*HRC	-	0.8
CAPSMNILPC	-	0.8
CCVAQVD*GC	1.2	-
CDASLWDYTC	-	0.8
CDGSVTVRFC	-	0.8
CE*GSADFFC	-	0.8
CGANHAITEC	-	1.2
CGIYNRDATC	-	0.8
CHFEVLQNLC	-	0.8
CHHSTGNKSC	1.2	-
CHQSTGNKSC	-	0.8
CIRTAHM*MC	-	1.2
CKSKLVDDTC	-	0.8
CN**RNYLC	-	0.8
CPNGVLYVGC	1.2	1.2
CQLYKERFSC	-	0.8
CQM*VVSSC	-	0.8
CRNDTHSWSC	1.7	1.6
CSFKAWHSTC	-	1.2
CSGWK*RNC	-	0.8
CSLNTTDYSC	-	0.8
CSNSLL*PNMC	-	1.6
CSQDVYIDQC	-	0.8
CSVAQVD*GC	-	1.6
CTIRSISDVC	1.7	-
CTRWGVGAPC	-	0.8
CTS*LNYTYC	-	1.2
CTVTPGYNDC	-	0.8

D.

Peptide	% Occurrence Round 3 From 140 peptides	% Occurrence Round 4 From 421 peptides
CAAARVIFRC	-	0.7
CFDLLTNLPC	-	0.5
CFSIRKKTSC	-	0.5
CFYLSMDKTC	-	0.5
CGANHAITEC	1.4	-
CGDYQTRKVC	-	0.5
CGGIQSKADC	-	0.7
CGHGSLHSTC	-	0.5
CGIYNRDATC	-	0.5
CGPHNLWDTC	-	0.5
CGTGSDNNYC	1.4	0.7
CHIMMVVSEC	-	0.5
CHVDRLD*YV	-	0.5
CHYNATLTLC	-	0.5
CKGILVG*SELAV	-	0.5
CKLELNDL*C	-	0.5
CLRDILV	-	0.5
CLYSGKPHLC	1.4	-
CMLNLWDSNC	-	0.5
CNTSLSMNNSC	-	0.7
CNYRDPTHRC	-	0.5
CPASKRNGLC	-	0.7
CPNGVLYVGC	-	0.5
CQIT*DFTSC	-	0.7
CRADANVYHC	1.4	-
CRSAKTDD*C	-	0.5
CSLKKSLDPC	-	0.5
CSRGAYSAPC	-	0.5
CTMRDTFKIC	-	0.5
CTS*LNYTYC	-	0.5
CWLEK*TPHC	-	0.5

experiments. After 4 rounds of biopanning there were more repeated peptides, but in most cases the percentage occurrence of these remained relatively low (Table 4.4). The results showed that the peptide pools from both CX8C biopanning experiments remained more diverse than those from the X8 biopanning (Table 4.4). Interestingly there were several peptides that were found to be repeated in more than one library. LSVISS, the most commonly occurring peptide from the individually amplified biopanning was also one of the more frequently occurring peptides from experiment 2. The peptides CGIYNRDATC, CPNGVLYVGC and CTS*LNYTYC were also found in both CX8C biopanning experiments.

38 peptides isolated from the BCA of mice fed a normal diet that received the X8 library, were found to share no common sequences with the peptides isolated from round 4 of the biopanning with the fat fed mice (data not shown).

4.2.5 BLAST searching peptides for homology to known proteins

To try and provide more information about the identified consensus motifs, BLAST searching for homologous proteins was performed. By searching for short nearly exact protein matches from the order Rodentia some homologous proteins were identified (Table 4.5). Interestingly many of the peptides have homology to proteins that have roles in inflammation, cholesterol metabolism or thrombus formation, so are associated with atherosclerosis.

Peptide and number of hits from BLAST search	Homologous region of protein	Protein homologue	Accession number	Relevance to atherosclerosis and additional notes
LSVISS 102	LSVISS	Dynein, axonemal, heavy chain 10 (murine)	XM_978566.1	Unknown.
LSTVKTYE 107	VKTYE	Leukocyte adhesion protein beta chain - Integrin β2 (murine)	A45839	Expressed on the surface of leukocytes and is known to bind to many molecules expressed on the surface of plaques.
PLNDCHSF 38	PLNDSQSF	ADAM6, a disintegrin and metalloproteinase domain 6-like (murine)	NM_00100954 5.1 AY158689.1	Metalloprotease-disintegrin with high similarity to human metargidin; may participate in dual proteolysis and integrin-mediated cell-cell and cell-matrix interactions.
QIFLFEIG 110	LFEIG	ApoB protein (murine)	BC038263.1	The main apolipoprotein found in LDL cholesterol.
SIESSSKR 130	SIESSTK	ApoB 100 [Atherurus africanus].	AF548416.1	Homologous to region near LDL receptor binding domain.
HPIIGLSY 100	IGLSY	Natural killer cell receptor protein NKR-P1B (murine)	AF354260.1	Transmembrane protein expressed by NK cells, is a negative regulator of NK cell activity (Carlyle *et al.*, 1999).
PSSVDNTG 105	PSSVD-TG	BRCA2 homolog. (murine)	U82270.1	BRCA2 is a tumour suppressor involved in DNA repair.
VSIANNRL 104	VSINNNR	laminin, alpha 1 (murine)	NM_008480.1	Laminins are the major non-collagenous component of basement membranes. They are secreted and incorporated into cell-associated extracellular matricies and they also bind to cell membranes through integrin receptors and other plasma membrane molecules.

Table 4.5 Selected results of BLAST searching the peptides identified from biopanning for atherosclerotic plaque targeting peptides.

VSNSYYTR 100	SNLSYYTR	COX-2 (murine)	NM_011198.2	COX-2 is an inducible enzyme produced by macrophages at sites of inflammation. COX-2 inhibitors increase the risk of atherothrombosis (Kearney *et al.*, 2006).
*RMTSSII 102	MTSSIV	vitamin K epoxide reductase complex, subunit 1-like 1 isoform 1 (VKORC1) (murine)	NM_027121.3	Catalytic subunit of the VKOR complex reduces inactive vitamin K 2,3-epoxide to active vitamin K. VKORC1 activity has also been linked with the extent of calcification within plaques (Wang *et al.*, 2006).
*RMTSSII 102	TSSII	plasminogen (murine)	NM_008877.2	Converted to plasmin, which breaks down blood clots.
FISVRLS 50	ISVRVMS	similar to fibronectin type III domain containing 1 isoform 2 (murine)	XM_974498.1	Large amounts of fibronectin have been detected in atherosclerotic plaques, suggesting that it may play a role in the pathogenesis of atherosclerosis.
HRGIFSNQ 104	RGIFSIQ	dachsous 1 Homologous to Cadherin repeat domain. (murine)	XM_194371.7	This gene is a member of the cadherin superfamily that has a role in cell adhesion. This particular cadherin family member is expressed in fibroblasts and may be involved in wound healing.
PSFDSGMN 100	PSFAPGMN	pericentriolar material-1 (murine)	AB029291.1	Unknown.
QDHFPIVN 104	EDHFPIV	BRCA2 (murine)	U65594.1	Unknown.
SCVTVM*F 100	SCVAVM	Heat shock protein (HSP) 9A and HSP70 (murine)	D17556.1 BC057343.1	Intracellular proteins involved in protein folding.
SCVTVM*F 100	SCVTV	TGF-beta induced apoptosis protein 2 (TAIP2) (murine)	AB091688.1	May play a role in apoptosis. Probably a nuclear protein.

Table 4.5 Selected results of BLAST searching the peptides identified from biopanning for atherosclerotic plaque targeting peptides.

YVTMKHHP 100	YRSMKHH	Fibroblast growth factor 15 (murine)	NM_008003.1	FGFs are involved in wound healing, are angiogenic and promote EC proliferation. They bind heparin. FGF15 is expressed in the developing mouse CNS (Saitsu *et al.*, 2006), the developing heart and (Vincentz *et al.*, 2005) the small intestine (Inagaki *et al.*, 2005)
C*IEANSLRC 101	ANSLRC	P-selectin (murine)	AAA40008	A cell adhesion molecule expressed on endothelial cells and platelets. Expression is upregulated in atherosclerosis and due to inflammation. Binds to leukocytes.
CGIYNRDATC 106	GIENRDAT	Isocitrate dehydrogenase 1 (NADP+), soluble (murine)	BC088986.1	Unknown
CIRTAHM*MC 100	CIRTMH	Platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit (PAF-AH) (murine)	AAH14831	PAF-AH secreted by macrophages, it degrades PAF and oxidation products of phosphatidylcholine produced by LDL oxidation and/or oxidative stress. May be an anti-inflammatory enzyme (Karabina and Ninio, 2006).
CPNGVLYVGC 109	NGVLYV	similar to death-inducing-protein (murine)	XM_989954.1	Unknown
CRNDTHSWSC 100	None found			
CSFKAWHTSC 100	SFKTWH	cardiotrophin 2 (murine)	NP_942155	Increases the platelet count associated with splenomegaly. May have an important role in neuronal precursor development and maturation Belongs to the IL-6 superfamily

Table 4.5 Selected results of BLAST searching the peptides identified from biopanning for atherosclerotic plaque targeting peptides.

CSNSLL*PNMC 103	CSNSL	Oxysterol-binding protein (OSBP) (murine)	AAH22908	Involved in lipid metabolism. Bind oxysterols (biologically active derivatives of cholesterol). Oxysterols are found in the plasma membrane so could potentially act as a receptor. They are found at higher concentrations in atherosclerotic vessels, but whether they have a pro- or anti-atherogenic effect is uncertain (Björkhem and Diczfalusy, 2002).
CSVAQVD*GC 103	VAQVD	Interferon regulatory factor 6 (murine)	BC008515.1	A DNA binding protein with no known association to atherosclerosis.
CTS*LNYTYC 100	NYSYC	leukocyte adhesion protein beta chain - Integrin β2 (murine)	CAA32563	Expressed on the surface of leukocytes and is known to bind to many molecules expressed on the surface of plaques.
CQIT*DFTSC 112	CQIT	TIMP4	AAT46411	TIMP4 has been associated with inflammatory disorders such as arthritis (Celiker *et al.*, 2002) and has been shown to be upregulated in human cardiovascular disorders. It is expressed by lymphocytes, mast cells monocytes and macrophages (Koskivirta *et al.*, 2006).

Table 4.5 Selected results of BLAST searching the peptides identified from biopanning for atherosclerotic plaque targeting peptides.

4.2.6 Developing a protocol for testing the targeting capacity of individual phage populations

Some of the most frequently occurring phage from the final round of biopanning were chosen for further testing to analyse their specificity for atherosclerotic plaques in more detail. High titre populations of individual phage were amplified for systemic administration into ApoE-/- mice so their biodistribution profile could be determined. Initially the peptide CPNGVLYVGC (CPN) that was identified from both the CX8C biopanning experiments was tested both with and without a predose of UV irradiated NR phage (Figure 4.6). The results showed that a 55-fold higher level of phage was detected in the BCA when no predose was used and that the ratio of phage recovered from the blood:BCA and liver:BCA were 4.3 and 2.5 fold higher when the predose was used, suggesting that the results without the predose were more favorable. Therefore it was decided to test all the homogeneous phage without administering a predose.

4.2.7 Analysis of the biodistribution of individual phage populations

To enable a relatively large number of phage to be tested rapidly, the individual phage were each tested in two ApoE-/- mice on a C57/Bl6 background and an average titre for each tissue was calculated. The titres from the peptide-modified phage were compared to that of 6 mice that received NR phage (Figure 4.7). The results showed that only 2 of the peptide modified phage (CPN and CQl) had higher levels of phage in the BCA than mice that received NR phage, and for the majority of the phage tested, higher levels were found in the liver compared to NR phage.

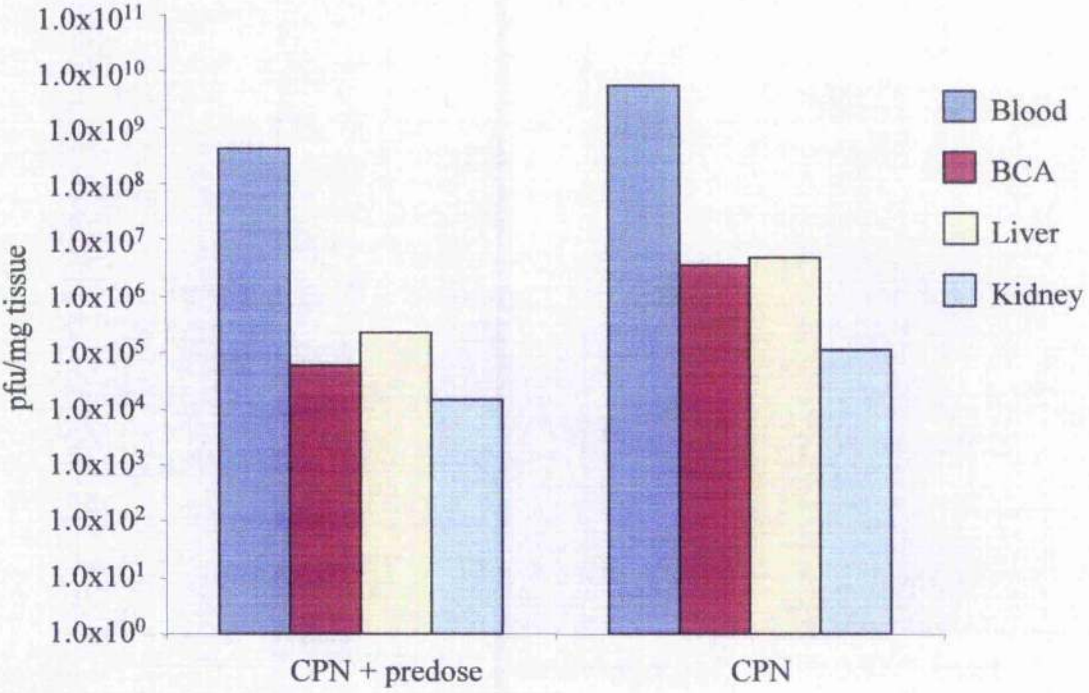


Figure 4.6 Comparing biodistribution profiles of phage administered with and without a predose. ApoE-/- mice received either a predose of 1 x 10^{11}pfu UV irradiated phage followed by 1 x 10^{10} pfu of phage displaying the peptide CPNGVLYVGC or 1 x 10^{11} pfu of phage displaying the peptide CPNGVLYVGC. n= 2 mice per group.

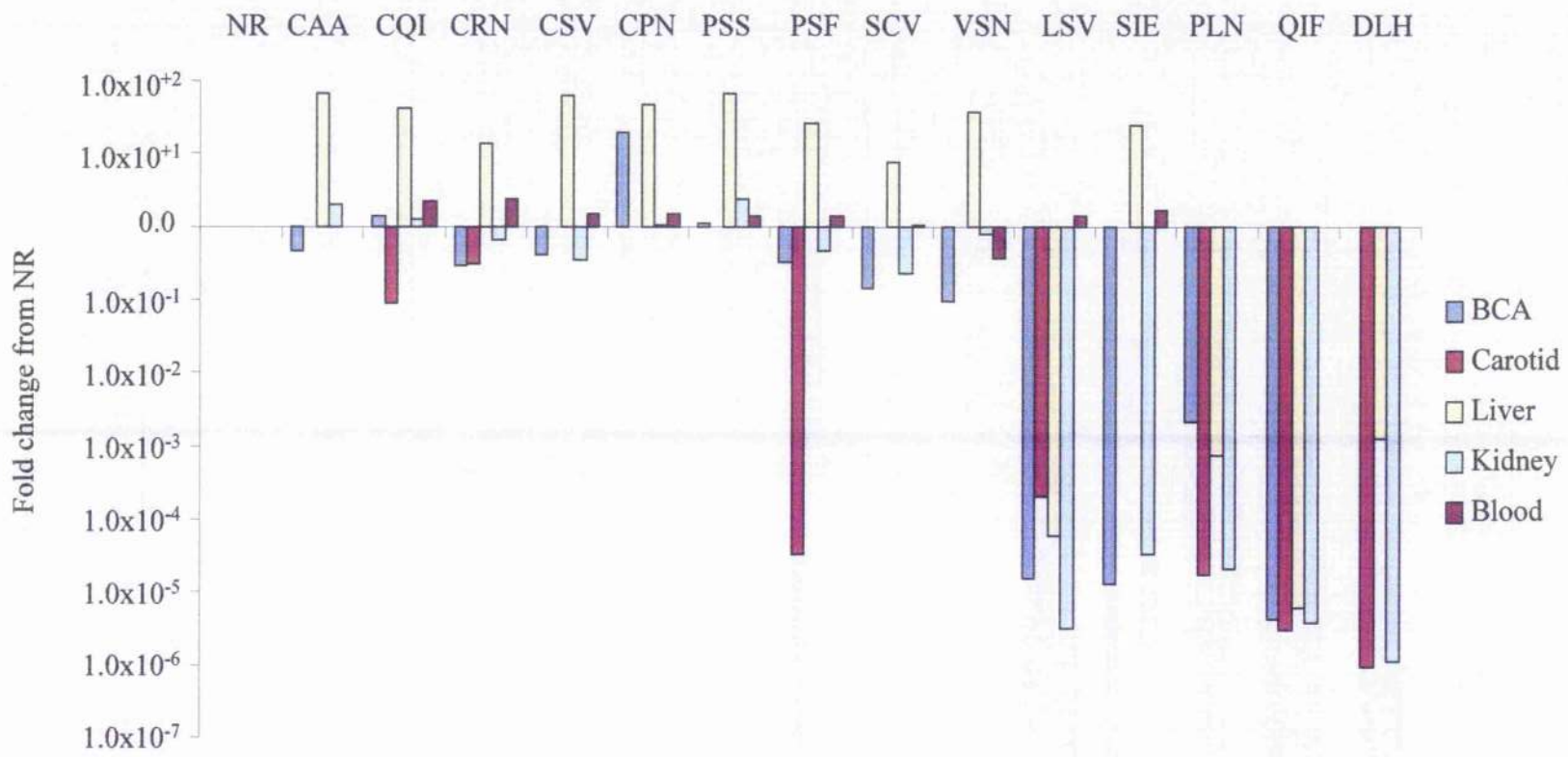


Figure 4.7 Biodistribution profiles of homogeneous phage. Mice (2 per group) received 1 x1 0^{1} ^{1}pfu of phage via tail vein injection and were culled 15 minutes later. Phage were recovered and titred from the tissues shown and compared to phage titres of mice that received NR phage (6 per group). CAA = CAAARVIFRC, CQI = CQIT*DFTSC, CRN = CRNDTHSWSC, CSV = CSVAQVD*GC, CPN = CPNGVLYVGC, PSS = PSSVDNTG, PSF = PSFDSGMN, SCV = SCVTVM*F, VSN= VSNSYYTR, LSV = LSVISS, SIE = SIESSSKR , PLN, PLNDCHSF, QIF = QIF*LFEI, DLH = DLH*LVNH. (* = stop codon)

4.3 Discussion

Biopanning with linear and constrained T7 phage display libraries was performed in an ApoE-/- mouse model of atherosclerotic plaque rupture to identify peptides that selectively target unstable plaques. Five different protocols were used and from each of these a pool of plaque-targeted peptides was identified. BLAST searching of commonly occurring peptide motifs identified some with homology to proteins known to be involved in plaque development whereas for other peptides no homologous protein could be found.

4.3.1 Analysis of original phage libraries

It was decided to perform the biopanning with both linear and constrained T7 based 8-mer libraries. Eight-mer libraries were chosen as they have a large diversity but the peptides are not so long that they significantly impair the rate of phage growth (Clackson and Lowman, 2004). Two libraries were used to increase the likelihood of identifying targeting peptides, as in some cases linear and constrained peptides with the same sequence have been shown to have different affinities for the same receptor (Koivunen *et al.*, 1993). The T7 system was chosen instead of M13 phage as the T7 libraries have been shown to have a wider diversity and less positional amino acid bias than M13 phage libraries (Krumpe *et al.*, 2006), so may be more effective in biopanning experiments. The T7Select10-3b library displays 5-15 copies of the peptide on the surface of the phage capsid at the C-terminal of the 10B capsid protein. Higher and lower copy number libraries are available but this system was used so enough copies of the peptide are displayed on the surface of the phage without inhibiting production of the phage. Libraries with higher copy numbers can cause problems with translation of the modified capsid protein if the *E.coli* producing the phage do not contain sufficient copies of the tRNA molecules required for rarer codons (Krumpe *et al.*, 2006).

In vivo biopanning has commonly been used to identify phage that bind to larger target tissues such as the heart (Zhang *et al.*, 2005). Targeting of unstable atherosclerotic plaques is technically more difficult. Although the mouse model used in this study is one

of the most reliable and accurate models of atherosclerosis, it has been shown that unstable plaques are only found in about 60% of animals and that there is some variation in the degree of atherosclerosis seen in the BCA of the mice (Johnson *et al.*, 2005b). At the time of dissection it was possible to see that the majority of animals had developed atherosclerosis in the BCA, but the extent of this varied between mice and without carrying out histology it is impossible to determine which mice had developed unstable plaques. As the whole of the BCA is required for phage extraction it is impossible to use the tissue for both histology and biopanning. Also, it is not possible to just isolate phage that are binding to the plaques, phage binding to the surrounding healthier vasculature will also have been isolated and amplified. To try and reduce the effect of plaque variability in the model, a minimum of 3 mice was used for each round of biopanning. The biopanning may also have been improved if initially an *ex vivo* negative selection step had been carried out on healthy vasculature to reduce the possibility of recovering phage that bind to healthy areas of the BCA.

Analysis of the amino acid composition of the libraries suggests that some amino acids may be over or under-represented but this could be because only a relative small number of peptides (about 50 for each library) were used in this analysis. Sequencing also showed that both libraries contained many peptides that were not of the expected length. The error could have occurred during construction of the DNA libraries or translation of the capsid protein, but it is unlikely to have had a significant affect on the biopanning results.

Based on the diversities of the libraries (both approximately 1×10^9) it was thought that there was no advantage of using one library over the other.

4.3.2 Biopanning results

For all 5 biopanning experiments, the overall increase in the amount of phage recovered from the BCA in the final round compared to round 1 suggests that a pool of BCA targeted phage have been identified. In the majority of experiments there was a decrease in the amount of phage in the BCA from rounds 1-3 as phage that do not bind

atherosclerotic plaques were sequentially depleted from the library to enable amplification of a pool of targeted phage. When individual phage were picked and amplified (protocol 1) an increase in the amount of phage in the BCA was seen at every round probably because the diversity of the library was reduced more rapidly using this protocol. A similar increase in recoveries was reported by Houston *et al.* (Houston *et al.*, 2001) who performed biopanning to identify plaque-targeting peptides and picked individual phage for amplification at each round.

After four rounds of biopanning with the bulk amplified libraries (protocols 2-5), there was no significant increase in the amount of phage recovered from the kidney and in 3 of the 4 experiments there was no phage recoverable from the fourth round. These results suggest that the phage pools have not become enriched in phage that target the vasculature of tissues other than the BCA, suggesting they may be specific for areas of atherosclerosis. However, only one non-target tissue has been investigated, so to confirm this it would be necessary to analyse the amount of phage accumulating in other non-target tissues. This pattern of results has previously been demonstrated in a number of *in vivo* biopanning experiments (Rajotte *et al.*, 1998, Work *et al.*, 2006), for example, a study to identify brain targeting peptides reported an increase in the amount of phage recovered from the brain and a decrease in the amount recovered from the heart, kidney and lung (Work *et al.*, 2006).

The results also show that the amount of phage found in the BCA is dose dependent as comparing the results from with and without a predose show that where a ten fold higher dose was received there is approximately a 10-fold increase in the amount of phage detected. This suggests that the tissue has not been saturated with phage and that there were still further binding sites available.

4.3.3 Analysis of peptide sequences

Using protocol 1, where individual phages were amplified, by the 3rd round there were several peptides found at a relatively high frequency. The titering results also suggested there was no further enrichment of the phage pool between rounds 2 and 3 so it was

decided that for this experiment 3 rounds of biopanning had produced a pool of peptides with increased selectivity for the atherosclerotic BCA.

After four rounds of biopanning with the bulk amplified libraries (protocols 2-5) there were many repeated peptides found, but the percentage occurrence of these remained relatively low. The BCA titres had not yet reached a plateau, suggesting the library could have been further enriched for targeting peptides. There were few peptides repeated in the 3rd round that were also found repeated in the 4th round, suggesting that analysing results based on these low percentage frequencies (1-2%) is not very reliable. Performing additional rounds of biopanning to further reduce the diversity of the library may have made the identification of targeting peptides easier. However, the identification of the same peptides from the different biopanning reactions is encouraging as it suggests the results are reproducible and therefore it is more likely that these peptides have a high specificity for the BCA. Of particular interest is the peptide LSVISS as it occurred at a relatively high frequency in protocol 1 and was also one of the most common peptides from protocol 2. The selectivity of this peptide requires further analysis as it might not have been selected for just based on its ability to bind the BCA. Some peptides have a negative effect on the growth rate of the phage (Clackson and Lowman, 2004). As LSVISS is a 6-mer peptide in an 8-mer library and only contains small-uncharged amino acids it may have less of an effect on the rate of phage growth, so the amplified peptide pools could have contained more copies of this phage than others. A growth time course experiment is required to determine if this could be the case.

None of the peptides sequenced from the BCA of the fat fed mice were the same as those sequenced from the BCA of mice fed a normal diet (that do not develop atherosclerosis. This suggests the peptides from the fat fed mice are plaque specific and not just BCA specific. However, the sequences from the normal mice were obtained after just one round of biopanning, so their specificity for the BCA might not be reliable at this early stage. This is highlighted by the results in the fat fed mice that showed the phage recovery decreased after the first round, suggesting that some non-specific phage had

been present. It is important that full biopanning is performed in healthy mice so the peptides can be more accurately compared.

The sequencing results showed that the targeted pools contained many phage that encoded for peptides containing stop codons. Other biopanning experiments have also identified peptides with stop codons (Houston *et al.*, 2001, Goldman *et al.*, 2000). It is thought that these phage can actually have a growth advantage over other phage (Houston *et al.*, 2001). Several explanations for how they occur have been proposed. It may be that a truncated protein is produced, or translational read-through might occur (Goldman *et al.*, 2000). Also under some conditions the stop codon UAG is translated as glutamine and UGA as selenocysteine (Bock *et al.*, 1991). It has also been shown that a +1 or -1 frameshift can occur to enable translation to continue past a stop codon (Goldman *et al.*, 2000). One problem with the T7-103b library is that in some cases the phage may not actually display the peptide encoded by the phage genome. The T7 capsid contains a total of 5-15 copies of the 10B and 10A capsid proteins, normally 10B makes up about 10% of the capsid protein but this can vary (Condron *et al.*, 1991). Phage can be assembled with only 10A, so the peptide encoded for by the 10B gene may not always be expressed (Novagen, 2002).

4.3.4 Comparing the different biopanning protocols

Based on the number of peptides identified and their frequency suggests there was no advantage gained by using the predose. However using a predose enabled the use of a lower dose of the phage library, which was advantageous as it was difficult to achieve purified stocks of the required titre. Further testing of the individual peptides inserted into viral vectors is also required to determine whether using the predose increased the specificity of the identified phage by blocking binding to non-specific receptors.

After 4 rounds of biopanning both pools of constrained peptides remained more diverse than the linear pools, possibly because of the slightly higher diversity of the initial CX8C library. The results suggest that there was no advantage in using either the X8 or CX8C library.

Restricting the library by amplifying individual phage at each round has produced fewer peptide sequences that occurred at a higher frequency and it provided a quicker method that required less animals. However, artificially reducing the diversity of the library may potentially remove peptides with a higher specificity, particularly after just one round where the diversity remained high.

In summary, the biopanning profiles and sequencing results suggest that some potential plaque-targeting peptides have been identified. Further testing of the individual peptides incorporated into viral vectors is required to determine whether peptides identified from one of the biopanning protocols have a higher degree of specificity for the plaques.

4.3.5 Analysis of BLAST results

The results of the BLAST searching identified several peptides that have homology to proteins that might have a role in atherosclerosis. Two peptides (QIFLFEIG and SIESSSKR) were found to have homology to ApoB, the main apolipoprotein component of chylomicrons and LDL. ApoB binds to LDLR to mediate uptake of LDL into tissues and is found at high levels within human plaques (Martinet *et al.*, 2003). Of particular interest is the peptide SIESSSKR as this is homologous to amino acids located near the LDLR binding domain (Figure 4.8) and near to a mutation that increases the risk of atherosclerosis development. The R_{3527} to Q_{3527} mutation occurs in familial defective apoB-100 (FDB100) that results in hypercholesterolemia (Innerarity *et al.*, 1987). It causes a conformational change that reduces its affinity to LDL-R to 5-10% of wild type levels as the arginine residue is thought to stabilise 2 clusters of basic amino acids that are required for binding to LDLR (Vrablik *et al.*, 2001).

There were also many peptides with homology to proteins involved in the inflammatory response. For example, 2 peptides (LSTVKTYE and CTS*LNYTYC) with homology to the leukocyte adhesion protein β2 were identified. This protein forms a receptor that binds to many molecules known to be expressed on the surface of plaques. A recent study also found leukocyte adhesion protein β2 expression is upregulated in unstable plaques

A.

3121 anldflnipl tipemrlpyt iittpplkdf slwektglke flkttkqsfd lsvkaqykkn
3181 khrhsitnpl avlcefisqs iksfdrhfek nrnnaldfvt ksynetkikf dkykaekshd
3241 elprtfqipg ytvpvvnvev spftiemsaf gyvfpkavsm psfsilgsdv rvpsytlilp
3301 slelpvlhvp rnlklslpdf kelctishif ipamgnityd fsfkssvitl ntnaelfnqs
3361 divahllsss ssvidalqyk legttrltrk rglklatals lsnkfvegsh nstvslttkn
3421 mevsvakttk aeipilrmnf kqelngntks kptvsssmef kydfnssmly stakgavdhk
3481 lslesltsyf siesstkgdv kgsvlsreys gtiaseanty lnskstrssv klqgtskidd
3541 iwnlevkenf ageatlqriy slwehstknh lqleglfftn gehtskatle lspwqmsalv

B.

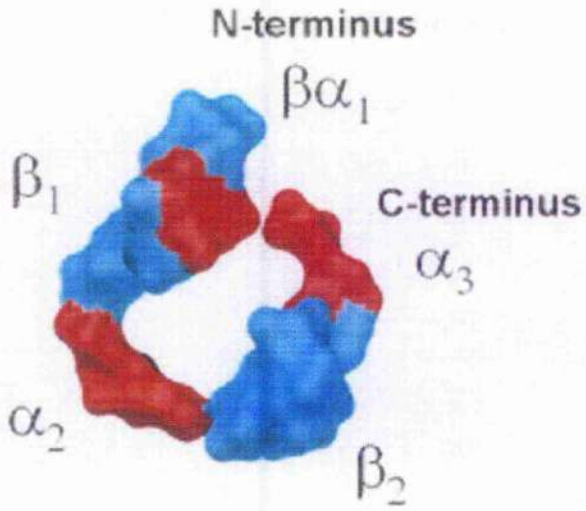


Figure 4.8 ApoB-100. A. Part of the protein sequence of ApoB-100 {Law, 1990 #6150}. The C-terminus of the ApoB LDL receptor binding domain is shown in green and the region to which the peptide SIESSSKR is homologous is shown in red. The R_{3527} .o Q_{3527} mutation (shown in blue) occurs in familial defective apoB-100 (FDB100) (Innerarity *et al.*, 1987). R_{3558} to C_{3558} (shown in blue) also reduces the affinity of ApoB to LDL receptor resulting in subjects developing hypercholesterolemia (Pullinger *et al.*, 1999). **B.** Secondary structure of ApoB-100 imposed onto low resolution model of the protein (from (Johs *et al.*, 2006)). The LDLR binding residues and the region of the peptide homology is located in the β_2 domain.

compared to stable plaques (Papaspyridonos *et al.*, 2006). Integrin β2 is the common subunit of many leukocyte integrins including complement receptor 2 (CR2), CR4 and leukocyte function-associated antigen-1 (LFA-1). It can be expressed on the surface of monocytes, T-cells, macrophages, neutrophils and dendritic cells and is involved in binding to cell adhesion molecules such as intercellular adhesion molecules (ICAMs), extracellular matrix proteins including fibronectin and complement protein 3B. Integrin β2 also binds LDL receptor–related protein (LRP) to enable leukocyte adhesion to the vascular endothelium during the inflammatory response involved in plaque development (Spijkers *et al.*, 2005). The peptide LSTVKYTE has homology to an exposed region of the I domain of the protein (Figure 4.9), which is thought to play a critical role in ligand binding and heterodimer formation (Xiong and Zhang, 2001).

One of the few peptides that produced positive results when tested as an individual phage population (CQ1*DFTSC) was found to have homology to TIMP4. TIMP4 has been associated with the inflammatory response in cardiovascular disease (Koskivirta *et al.*, 2006, Dollery *et al.*, 1999, Li *et al.*, 1999). In healthy arteries it has been found in medial SMC and in advanced plaques it has been found to co-localise with macrophages in areas surrounding the necrotic core (Koskivirta *et al.*, 2006). Its expression is upregulated in inflammatory cardiovascular disorders as it is produced by lymphocytes, mast cells, monocytes and macrophages (Koskivirta *et al.*, 2006). TIMP4 inhibits all known MMPs (Koskivirta *et al.*, 2006) and it may also have a role in apoptosis. It has been shown to inhibit rat aortic SMC migration and induce apoptosis *in vitro* (Guo *et al.*, 2004). *In vivo*, TIMP4 overexpression was shown to reduce neointima formation and increase SMC apoptosis in balloon injured rat carotid arteries (Guo *et al.*, 2004). The TIMP2 homologous peptide CNHRYMQMC (Liu *et al.*, 2003) appears to be a promising atherosclerotic plaque targeting peptide (Chapter 3) and from these preliminary studies this TIMP4 homologous peptide also seems to have potential.

Interestingly a peptide with homology to P-selectin was identified. P-selectin is expressed on the surface of both platelets and endothelial cells and acts as a receptor for leukocyte binding (Vestweber and Blanks, 1999). It is upregulated on the endothelium in

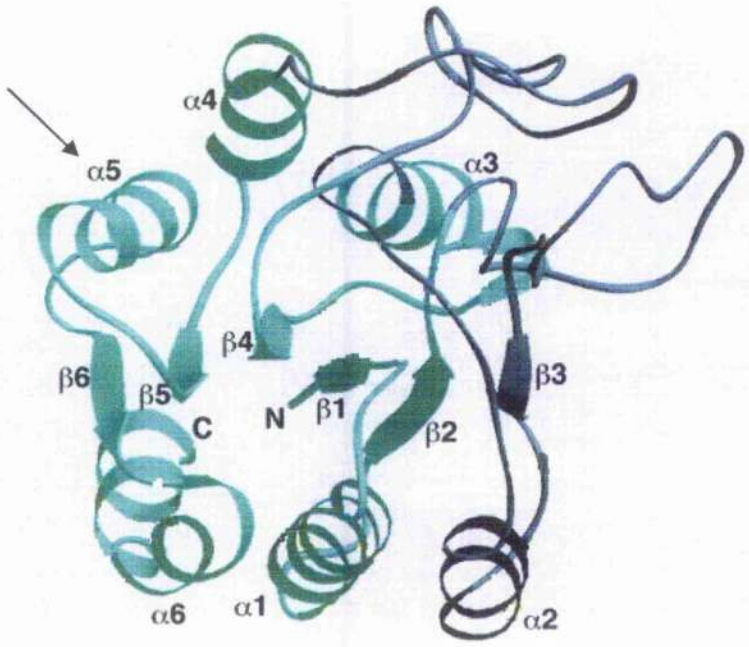


Figure 4.9 Diagram of the β_2 I-like domain model. View of the top of the domain. The peptide LSTVKTYE is homologous to α helix 5. Adapted from (Huang *et al.*, 2000).

atherosclerosis (Johnson Tidey *et al.*, 1994, Molenaar *et al.*, 2003). It has a key role in plaque development and progression as P-selectin-deficient mice develop significantly reduced atherosclerosis (Dong *et al.*, 2000, Johnson *et al.*, 1997). P-selectin is also found at higher levels in the blood as disease progresses (Blann and McCollum, 1998, Ridker *et al.*, 2001). As its expression is increased on the endothelium, it is more likely that this biopanning would have identified a peptide that binds to P-selectin rather than a homologue of P-selectin. However, the ligand for P-selectin, sialyl-Lewis^X carbohydrates has been reported to be expressed on the endothelium, although its role here is unknown (Munro, 1993).

Many of the protein homologues identified have a role in wound healing and blood clotting, so could be expressed at a higher level in unstable plaques that have undergone previous silent ruptures. Therefore these peptides may have a higher specificity for unstable rather than stable plaques. This is of particular interest, as silent ruptures have been shown to be a key feature of the plaques produced in the mouse model used in this study (Johnson *et al.*, 2005b). One of the peptides (YVTMKHHP) has homology to FGF15, which has not been linked to atherosclerosis but has a role in cardiac development (Vincentz JW, 2005). However, other isoforms of FGF are known to be expressed in atherosclerotic plaques (Brogi *et al.*, 1993). They have a role in smooth muscle cell, proliferation, migration and proteoglycan synthesis (Tan *et al.*, 1989). Another peptide (FISVRLS) has homology to fibronectin, which is a known component of atherosclerotic plaques and its expression has been shown to be upregulated in advanced plaques (Hiltunen *et al.*, 2002). It is a chemoattractant for several cell types that play a role in the wound healing process, including fibroblasts, endothelial cells and macrophages. It also generates a scaffold that allows attachment of other extracellular matrix components.

One of the peptides (VSIANNRL) was found to have homology to the extracellular matrix protein laminin. The 37/67 kDa laminin receptor (LamR) has recently been identified as the receptor for cellular attachment of AAV8 and might have a role in the infection pathway of AAV2, 3 and 9 (Akache *et al.*, 2006). LamR is expressed in many

tissues including the heart and liver and is upregulated in many cancers, so any virus or phage that uses it as a receptor is likely to have a broad tropism (Sobel, 1993). Several other viruses including Dengue virus and Sindbis virus also use LamR as a receptor (Thepparit and Smith, 2004, Wang *et al.*, 1992).

Another peptide (C*IEANSLRC) was found to have homology to platelet-activating factor acetylhydrolase (PAF-AH) which has been linked to atherosclerosis. It is produced by macrophages, mast cells and activated platelets (Karabina and Ninio, 2006). Secreted PAF-AH is mainly found associated with LDL (Stafforini *et al.*, 1987). PAF-AH has an anti-atherogenic effect as it degrades PAF (a potent proinflammatory phospholipid molecule generated by leukocytes, myeloid cells, platelets, mast cells, and endothelial cells) and PAF-like molecules produced by LDL oxidation and platelet activation and so reduces the migration of leukocytes into the vessel wall (Lee *et al.*, 1999). But, the products of PAF-AH mediated degradation may have an adverse effect on the vessel wall (Karabina and Ninio, 2006). However, it has been shown to protect against atherosclerosis development in several animal models (Quarack *et al.*, 2001, Turunen *et al.*, 2004). In another study in LDLR-/- mice over-expressing ApoB-100 suggested PAF-AH has a proatherogenic role as the mice developed large lesions and were found to have elevated levels of PAF-AH (Singh *et al.*, 2004). PAF-AH was thought to be upregulated by the high levels of LDL present (Singh *et al.*, 2004). So as yet it is not known whether PAF-AH is pro- or anti- atherogenic or may just be a marker for atherosclerosis (Karabina and Ninio, 2006).

Some of the proteins identified by the BLAST searching have no connection to atherosclerosis and may have just been identified randomly based on the probability of a short peptide sequence existing in a protein. Interestingly the breast cancer susceptibility gene BRCA2 that has not been linked to atherosclerosis was found to have homology to two of the peptides. However BRCA2 is a nuclear protein involved in DNA repair so it is unlikely that it binds to anything expressed on the surface of atherosclerotic plaques. This highlights a limitation of using BLAST searches alone to identify receptors for peptide motifs. However in combination with frequency analysis and screening of

populations of individual phage it can be used to provide additional information about identified peptides and their potential receptors, which can be investigated further.

Some peptides were also found to have homology to hypothetical proteins and proteins with no known function, so these may actually have some relevance.

A major problem with BLAST searching short peptide sequences is that in many cases the binding site of the natural ligand for a receptor is not a linear peptide sequence but is discontinuous (i.e. formed when the protein is folded correctly). As such BLAST searching primary amino acid sequences does not produce definitive results, but can indicate potential homologues that require further investigation to validate them. Due to this and the fact that the BLAST database is incomplete, for many peptides no homology to any known protein is identified.

4.3.6 Analysis of biodistribution profiles of individual phage populations

The initial work with the CPNG peptide suggested that the best way to test the targeting capacity of the individual phage was to administer the phage without a predose as this produced more favorable ratios of phage in the blood: BCA and liver:BCA. This method was also chosen because it would provide a more rigorous testing of the specificity of the phage as any phage without a high specificity for the plaques could still be taken up by the RES.

The results showed that for the majority of the phage tested, compared to NR phage there was no targeting to the BCA, but there was actually an increase in the amount of phage accumulating in the liver. Although phage with the peptides CPN and QIT were found to have higher levels in the BCA, there was also a higher level in the liver, so the peptides may not bind to receptors specific to atherosclerotic plaques. It would be interesting to carry out IHC on the BCA of mice receiving the homogeneous phage to determine more precisely if the phage in the BCA were binding to the plaques or to the surrounding healthier vessel.

One problem with this experiment is that the method used (without a predose) was chosen on the basis of initial results with one of the phage (CPN), which happens to be one of the few phage that did seem to have some degree of targeting to the BCA. For other phage using a predose may have given better results as it would have reduced uptake of phage by the RES so it might have been possible to see targeting to the BCA.

Another problem with this study was that the biopanning was performed in ApoE-/- mice on a C57/Bl6/129SvJ background that have been shown to develop unstable plaques (Johnson *et al.*, 2005b), whereas due to time constraints the individual phage were tested in ApoE-/- mice on a C57/Bl6 which have been shown to develop advanced plaques (See section 3.3.1). If the peptides are highly selective for unstable plaques then they are unlikely to target advanced plaques.

4.3.7 Summary

The biopanning profiles from the BCA and kidney and the sequencing results suggest that the biopanning has successfully reduced the diversity of the original libraries and produced pools of BCA targeted peptides. The BLAST search results have shown several of the peptides have homology to proteins known to be involved in atherosclerosis suggesting some interesting, potential plaque targeting peptides have been identified. However, analysis of the peptides using individual phage populations, in most cases failed to demonstrate targeting to atherosclerotic plaques. Further testing of some of the more promising candidates based on frequency and the BLAST searching results is required. It is necessary to analyse the biodistribution profiles of the individual phage in ApoE-/- mice on a C57/Bl6/129SvJ background and perform detailed IHC to determine whether the phage are binding to atherosclerotic plaques or surrounding vasculature. Alternatively synthetic peptides labeled with fluorescein-5-isothiocyanate (FITC) could be used to analyse the targeting capacity of individual peptides. Ultimately, engineering of the peptides into viral vectors would enable the targeting capacity of the peptides to be fully examined.

Chapter 5:

Development and Characterisation of an AAV2 Based Peptide Library

5.1 Introduction

AAV2 is a promising vector for gene therapy as it causes no known human disease, is minimally immunogenic and can produce sustained transgene expression (Monahan and Samulski, 2000a). It may therefore be particularly good for treating chronic conditions such as atherosclerosis where long-term transgene expression may be beneficial. However due to the natural hepato-tropism of the virus and sequestration in the extracellular matrix of ECs (Pajusola *et al.*, 2002), it provides poor transduction of vascular cells following both local and systemic administration (Richter *et al.*, 2000, White *et al.*, 2004, Maeda *et al.*, 1997, Nicklin *et al.*, 2001a). To use AAV2 as a vector for applications to atherosclerosis it needs to be both detargeted from its native tropism and retargeted to atherosclerotic plaques to produce a more efficient and useful vector. The most effective way of doing this is thought to be via the incorporation of targeting peptides into the HSPG binding site of the AAV2 capsid (commonly after amino acid 587). This can both redirect the virus to an alternative receptor and potentially block it's binding to HSPG (Girod *et al.*, 1999). This has been demonstrated with some success for targeting AAV2 to vascular cells both *in vitro* and *in vivo* (Nicklin *et al.*, 2001a, Work *et al.*, 2006, Work *et al.*, 2004a, White *et al.*, 2004).

The most commonly used method to identify targeting peptides is phage display (see chapter 4). When no prior knowledge of a target receptor is available biopanning with a phage library is an efficient way of identifying peptides that can be used to target viral vectors to a particular tissue or cell type. Although there are many successful examples of this, two main problems have been encountered with this procedure. Not all peptides identified by phage display can be incorporated into viral vectors as peptide insertion can affect the ability of the capsid to assemble (Wickham *et al.*, 1997, Hong and Engler, 1996). For example, when 10 transferrin targeting peptides identified by phage display were inserted into the HI loop of Ad5 only 2 of the peptides had no detrimental effect on Ad production, 4 caused a reduction in the titre of the virus and 4 prevented amplification of the virus (Xia *et al.*, 2000). From our experience approximately 50% of identified peptides can be successfully incorporated into viral vectors. Changing the physical

context in which the peptide is displayed from the phage to the virus may cause alterations in the 3D conformation of the peptide that can alter the affinity of the peptide for its receptor. For example the LSNFHSS peptide identified by phage display as targeting EC, did not improve Ad5 transduction of ECs when it was displayed in the HI loop (Nicklin *et al.*, 2001c). The peptide GETRAPL was identified by phage display for targeting to SMC, but when inserted into both the Ad5 and AAV2 capsid it did not improve transduction of these cells compared to unmodified control virus (Work *et al.*, 2004a).

One recent attempt to overcome the problems encountered when inserting peptides into Ad5 vectors has involved the creation of a context-specific phage library. It has the H and I sheets of the adenovirus knob inserted into the pIII protein of filamentous bacteriophage, with a library of 12-mer peptides inserted into the same position where they would be displayed in the HI loop (Ghosh and Barry, 2005). This library was used to identify a muscle targeting peptide and when the corresponding Ad5 virus was tested it was shown to be a functional and muscle-specific vector (Ghosh and Barry, 2005). Although this overcomes the problem of context specificity and in this case it was successful, it still doesn't overcome the problem that the identified peptide may inhibit virus assembly and function.

An alternative approach developed to produce targeted AAV vectors has completely removed the need to use phage by producing peptide-modified libraries of AAV2 vectors (Perabo *et al.*, 2003, Muller *et al.*, 2003). The libraries were made by inserting random oligonucleotides encoding 7 amino acids into a plasmid encoding the AAV genome to create a plasmid library. Perabo *et al.* (Perabo *et al.*, 2003) then produced the viral library by co-transfecting the plasmid library with an adenovirus helper plasmid. This created a library with random 7-mer peptides inserted after amino acid 587, which had a diversity of 4×10^6 modified virus particles. It was used for *in vitro* biopanning to identify viruses targeted to M-07e (a human megakaryocytic cell line) cells and Mec1 cells (derived from B-cell chronic lymphocytic leukaemia cells) (Perabo *et al.*, 2003). Three of the four viruses identified from the screening showed significantly increased

transduction of the target cells, independently of HSPG binding (Perabo *et al.*, 2003). However, as with all biopanning on cells, peptides that mediate binding to a ubiquitously expressed protein can be identified. Two of the peptides identified contained an RGD motif so they are likely to bind integrins (Perabo *et al.*, 2003, Pasqualini *et al.*, 1995).

Muller *et al.* (Muller *et al.*, 2003) used a two-step system to produce a viral library from the plasmid library. The first step packaged the library genomes into chimeric capsids of both wild type and mutant capsids by transfecting 293T cells with the plasmid library, plasmid encoding wild type capsid and a helper adenovirus plasmid. The resulting shuttle virus library was then infected into 293T cells with a helper Ad virus to produce the virus library (Muller *et al.*, 2003). This 2-step protocol was used to try and avoid producing virions displaying more than one peptide, which could be produced by a cell being transfected by more than one plasmid from the library. However using this method results in the library containing wild type AAV2 (Waterkamp *et al.*, 2006). The resulting library had an estimated diversity of 1×10^7 random 7-mer peptides inserted after residue 587 (Muller *et al.*, 2003). Biopanning performed on HCAEC identified several targeted viruses that were shown to function *in vitro* and targeted the heart *in vivo* (Muller *et al.*, 2003). These studies are proof of concept showing that *in vitro* biopanning with AAV2 based libraries can be used to identify specifically targeted viruses that can efficiently, bind, internalise and translocate to the nucleus in the target cell type. In both cases, *in vitro* the viruses had a high degree of selectivity for target cell types that are normally poorly transduced by AAV2 and their transduction was shown to be mediated by the inserted peptides. Further work is needed to determine their efficacy *in vivo*. It is important to note that the diversity of the AAV libraries is several logs lower than that typically found in phage libraries, so the chance of identifying targeting peptides is therefore reduced. However, these studies have shown that the diversities are still high enough to enable identification of viruses targeted to different cell types.

AAV2 based libraries have also been used to identify mutants with higher resistance to the most commonly occurring neutralising antibodies (Perabo *et al.*, 2006a, Maheshri *et al.*, 2006). Both groups used error prone PCR of the cap gene to create a library of

AAV2 mutants, which was then used in directed evolution experiments to identify antibody resistant virions. Perabo *et al.* biopanned on HeLa cells in the presence of a pool of human sera known to contain AAV2 neutralising antibodies. The most commonly identified point mutations were R459G, R459K and N551D (Perabo *et al.*, 2006a). These residues and 73% of the less frequently occurring mutations are all located in the same region of the capsid, suggesting that an antigenic epitope had been identified (Perabo *et al.*, 2006a). Maheshri *et al.* (Maheshri *et al.*, 2006) performed biopanning in the presence of rabbit anti-AAV2 neutralising sera. This identified mutations N587I and T716A for enabling antibody-evasion. These mutations may be part of several different antibody binding epitopes that may all require mutating to provide a vector, which evades antibody neutralisation in the majority of the human population due to the diversity of antibodies known to exist.

5.1.1 Aims

The main aims of this chapter were to use the AAV2 library created by Perabo *et al.* (Perabo *et al.*, 2003) to:

1. Develop and optimise protocols for biopanning with the AAV library both *in vitro* and *in vivo*.
2. Further characterise the library and the effect of peptide insertions on the infectivity of the virus to study the basic biology of AAV2, to enable better vector development.
3. Identify peptide modified AAV2 vectors that target endothelial cells and atherosclerotic plaques, by performing *in vitro* and *in vivo* biopanning in a mouse model of atherosclerosis.

5.2 Results

5.2.1 Estimation of the diversity of the AAV library

The library contains AAV2 virions with a random 7-mer peptide inserted into the virus capsid at position 587. There is no accurate way of assessing the true diversity of the library, although it has been estimated to contain 4×10^6 different peptides based on the ligation efficiency of the random oligonucleotides into the plasmid used to create the library and the efficiency of transfection during the packaging reaction (Perabo *et al.*, 2003). To try and confirm that the diversity of the plasmid library was maintained in the virus library, the peptide-containing region of the virus library was PCR amplified, cloned and sequenced. Two pools of the library from different synthesis reactions were used. From pool A 84 peptide sequences were obtained and of these 13 sequences occurred more than once (Table 5.1). From pool B, 1 of 34 peptides occurred twice. None of the peptides were found in both pool A and B, suggesting the peptide repetition may be due to sequence bias created by the PCR. Statistical analysis of the peptide sequences based on the frequency of amino acids occurring in different positions within the peptides calculates that the library has a diversity of 5×10^7 different peptides.

The oligonucleotides encoding the peptides were synthesised randomly, so all codons were possible. This means their will be some bias in the frequency of amino acids as some are encoded by 6 different codons whereas others are encoded by only 1 codon. Analysis of the occurrence of each amino acid within the peptides showed that the library contains a higher than expected number of alanine (A), aspartic acid (D), glycine (G) and proline (P) (Figure 5.1). The library had fewer than expected phenylalanine (F), isoleucine (I), lysine (K), asparagine (N) and tryptophan (Y) residues. Several peptides containing stop codons were also identified (Figure 5.1).

Pool A		Pool B
AADLLGP	PWAQRLP	AE-Stop-DARS
AGRDVSL	QATGCHR (2)	AFSGS-Stop-V
ALADSLT	RLTLSCI	AQGYPEG (2)
ALGPRSL	RPFCQLV	CGLSPDL
ALRSTSP	RSRARAG	CSEAPMR
ALVARVN	RWPDQRA	DQDQLSG
APRLSSS	SEARXRA	DRRHTHA
AQELRGH	SEVQRSH	DSHRGHS
CATAANL	SGSDPAD (2)	ERGDPGV
CDSRLRR	SHSRSRA	GAEWAVR
DMAVERA (3)	SLAHSAT	GLPRNLS
DQTYPTE	SMMHRLR	GRSEPDS
DSQPLRW (4)	SPLPPDP (3)	GTQSLAS
DSRSADR	SRGKPIT	GYPRHSE
DTAAYLG	Stop-WLRPHS (2)	HSSRPIS
EAGLTVD (2)	TPSGRSG (2)	IVRRLVR
EGGEPDD	TRL-Stop-QDR	LGAT-Stop-RS
ELGRSDK (2)	TRSGATD	MMTRLGE
EQLSLRE	VPKPGSP	RASCGGA
ERSPPTH	VSSNAVS	RATTPR
FGPGHVV	VSVSAAA	RCAAVPA
FHRAESG	WATADQI	RGRAGSD
GATAGFY	YLDGFHQ	RRRIATA
GNCHETP	YLVGRCQ	RTQLVPC
GRLGVVT	YQDGGTA (3)	SCLQLRA
HLTSPRS (5)		SEAPLGI
IDTLGRA (2)		SELGAFE
MIVPRSC		SGPTMKA
NLGPAMS		SIAPGPS
NRPTPTR (2)		SQHSDGD
PEAARQQ		SR-Stop-PRPV
PGCHLSA		SVSLPQR
PLGPAAA		VLRSPGA
PPPKPTV		
PRAPLHT		
PSALSNM		
PTCRRPP		
PWAHRLA		

Table 5.1 Peptide sequences from the complete AAV library. Positively charged peptides are shown in red and negatively charged peptides are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis.

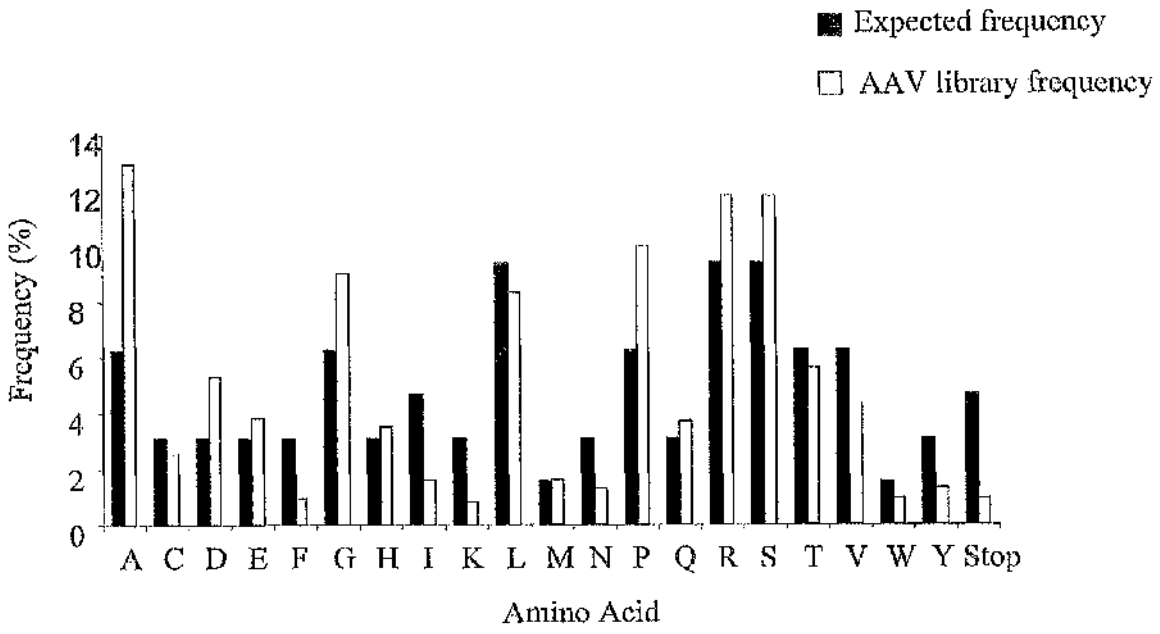


Figure 5.1 Amino acid composition of peptides from the complete AAV library. 118 peptides were sequenced from the complete AAV library and compared to the expected amino acid frequency based on all codons occurring at the same rate. Alanine (A), Cysteine (C), Aspartic acid (D), Glutamic acid (E), Phenylalanine (F), Glycine (G), Histidine (H), Isoleucine (I), Lysine (K), Leucine (L), Methionine (M), Asparagine (N), Proline (P), Glutamine (Q), Arginine (R), Serine, (S), Threonine (T), Valine (V), Tryptophan (W), Tyrosine (Y).

Analysis of the peptides to determine whether any amino acids occur more or less frequently at a specific position within the peptide indicated that for most amino acids there was no positional specificity. The exceptions to this are that acidic amino acids were more commonly found at the N- and C-termini of the peptides (positions 1 and 7) and bulky amino acids were generally found in positions 1 and 2 (Figure 5.2).

5.2.2.1 *In vitro* biopanning with the AAV library

In vitro biopanning was performed on RGE cells to identify endothelial cell-targeted viruses and on HeLa cells as a control, to confirm that different peptide containing viruses can be selected for on different cell types. Taqman analysis of the amount of virus in cell media, pellet and supernatant samples from all six rounds of biopanning shows a similar pattern of results was obtained with all 3 cell fractions (Figure 5.3). The results from the RGE cell samples show an increase in the Ct values over the first three rounds, indicating the amount of virus present has been reduced (Figure 5.3A). Over the last three rounds there was an increase in the amount of virus detected (Figure 5.3A). With HeLa cells a decrease in the amount of virus was also seen over the first three rounds but there was no consistent increase seen in the latter rounds (Figure 5.3B).

HeLa cell samples from rounds 4, 5 and 6 were then used as templates for sequencing of the peptide-containing region of the AAV2 capsid (Figure 5.4). From the mixed sequencing trace in the peptide insertion site, it is clear that even after 6 rounds of biopanning, the cell samples still contained a mixed pool of viruses, although the diversity was greatly reduced from the starting library. PCR analysis of the RGE cell samples from round 6 indicated that the population of viruses contained viruses both with and without a peptide (Figure 5.5A), whereas the HeLa cell samples only contained viruses with an inserted peptide (Figure 5.5B). As a consensus sequence had not been reached after 6 rounds of biopanning, the peptide-encoding region of the virus capsids was amplified by PCR and TA cloned, so individual peptide sequences could be obtained.

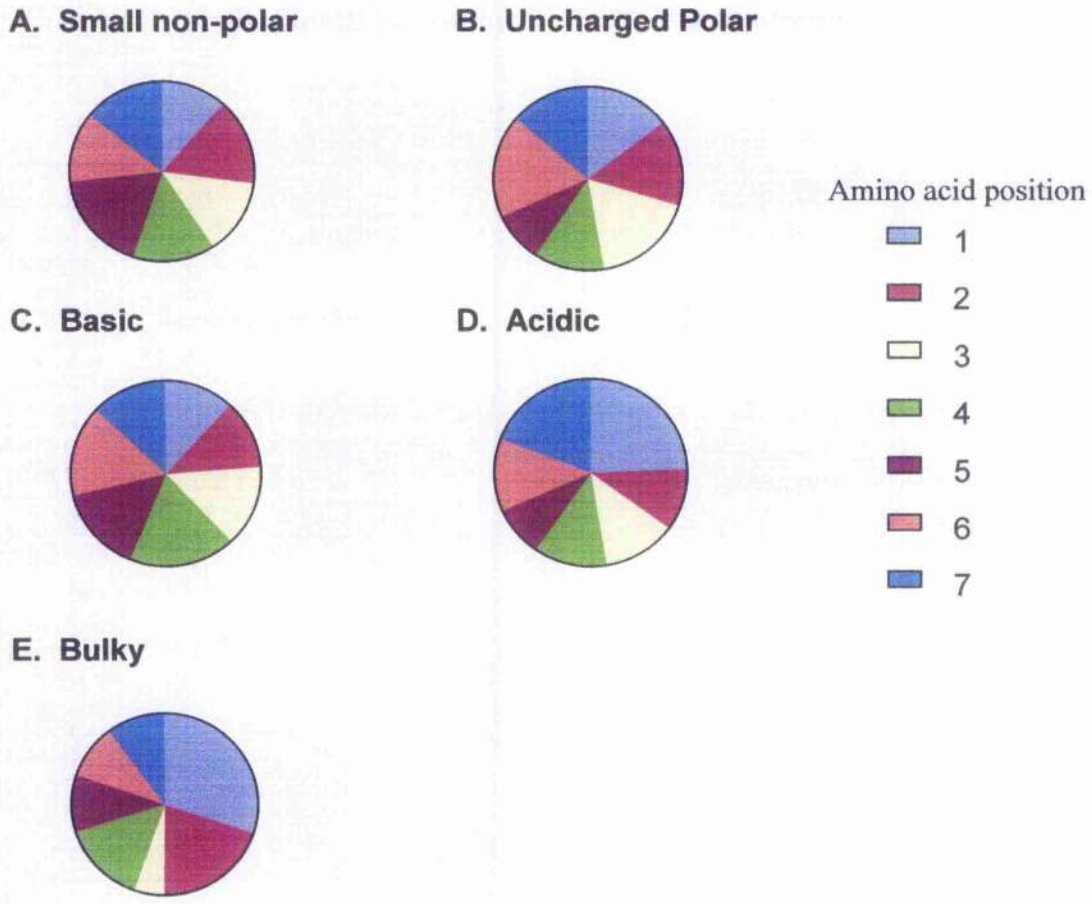


Figure 5.2 Analysis of the amino acid composition of 118 peptides sequenced from the virus library. A. Small non-polar amino acids (A, G, I, L, M, P and V). **B.** Uncharged polar amino acids (S, T, N, Q and C). **C.** Basic amino acids (R, K and H). **D.** Acidic amino acids (D, E). **E.** Bulky amino acids (F, W, Y)

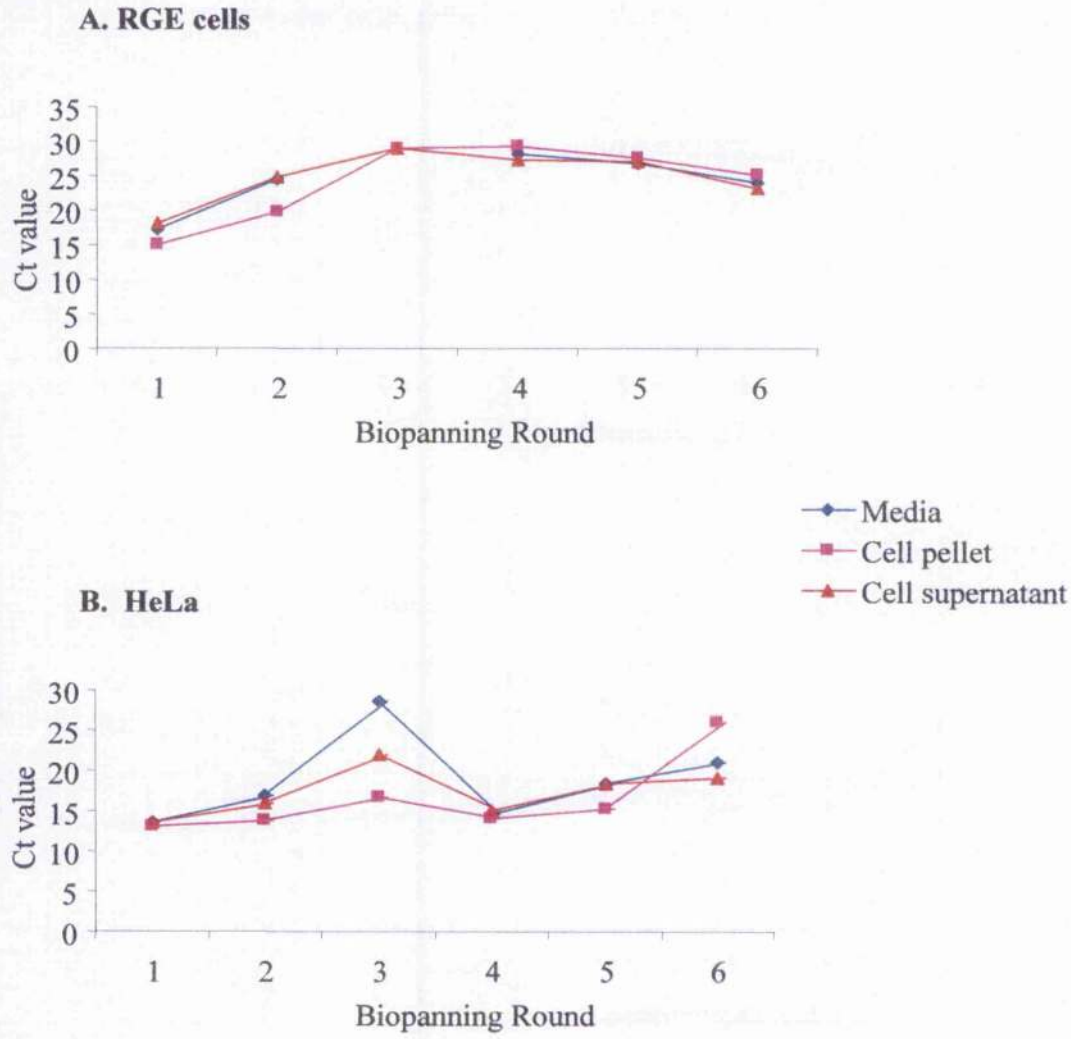


Figure 5.3 Real time PCR results from *in vitro* biopanning with the AAV library. Samples of cell media, pellet and supernatant were isolated from each round of biopanning and used as a template for Taqman real time PCR. **A.** RGE cells. **B.** HeLa cells. To detect the amount of virus in each sample The cycle threshold (Ct) value is the number of PCR cycles required before a significant amount of PCR product above background level has been produced.

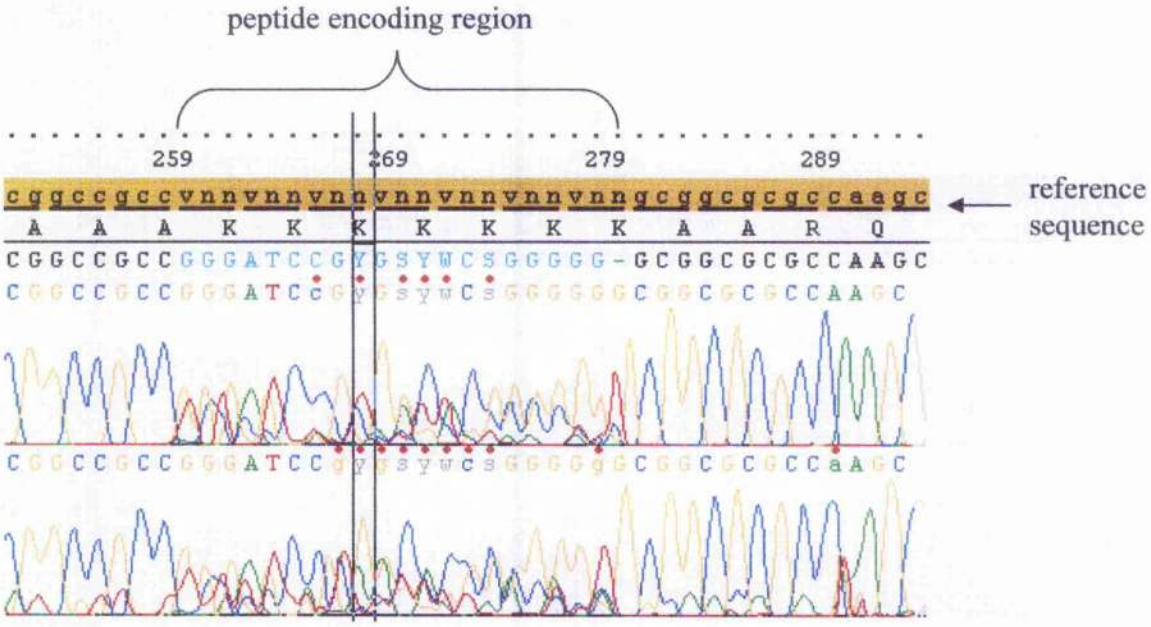


Figure 5.4 Sequence analysis of HeLa biopanning samples. Two sequencing traces of the peptide insertion region of viruses in cell samples from round 6 of biopanning on HeLa cells.

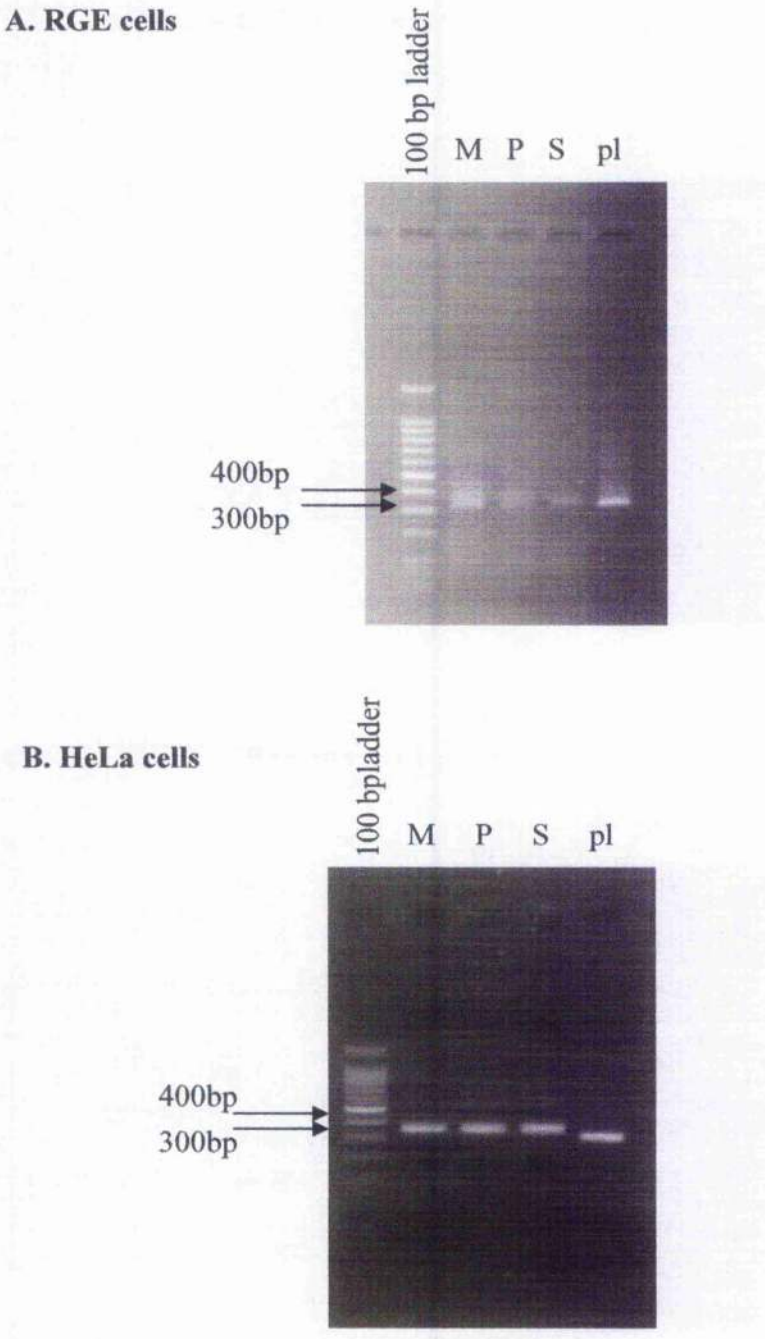


Figure 5.5 PCR analysis of AAV library biopanning samples. Agarose gel electrophoresis of the PCR products of the peptide containing region of the virus library from round 6 of biopanning on **A.** RGE cells and **B.** HeLa cells. The PCR product from AAV library virions containing a peptide is 330 bp where as for wild type AAV without a peptide it is 309 bp. Cell media (M), Cell pellet (P), Cell supernatant (S) and a plasmid positive control (pl) (containing no peptide)

5.2.2.2 Analysis of peptide sequences from biopanning on RGE cells

As the PCR results suggested the majority of viruses from the RGE cell samples contained viruses that had no peptide insertion. From 55 sequences, only 4 were found to contain a peptide (ARSTLSH, SIRGTGG, SQVRATT and SSGTPSR) and these shared no consensus motif.

5.2.2.3 Analysis of peptide sequences from biopanning on HeLa cells

Sequencing of the peptide insertion region of 2 PCR products from round 6 of the biopanning in HeLa cells produced 95 sequences containing 27 different peptides (Table 5.2). Sequencing results identified several consensus motifs that might enable specific targeting of HeLa cells. The most commonly occurring peptide motifs (e.g. ASSPRPS which was in 30% of the peptides) were found in the 2 independent PCRs. BLAST searching the peptide ASSPRPS did not identify homology to any relevant proteins. One commonly occurring motif $SIR^{A}/_{G}T^{G}/_{A}$ was found in 3 peptides from the HeLa biopanning and 1 in the RGE biopanning. This was the only peptide identified from both biopanning experiments suggesting it might bind a receptor found on both cell types.

Interestingly, 84% of the different peptides sequenced from the HeLa cell biopanning had an overall positive charge, 16% were neutral and none were negatively charged (Table 5.2). This is in contrast to the peptides sequenced from the full library that were 46% positive, 32% neutral and 22% negative (Table 5.1). Further analysis of the peptide sequences from round 6 has identified several features of the HeLa selected population of peptides that are different from the composition of peptides in the full library (Figure 5.6). The HeLa targeted peptides contain lower levels of the large amino acids F, W and Y than the original library ($p<0.05$), suggesting that as well as inhibiting capsid formation they may also reduce virus infectivity, however due to the low level seen in both libraries it is difficult to draw any conclusions from this. The percentage occurrence of basic amino acids was not altered from the original library, but within the HeLa targeted pool they were commonly found in positions 5 and 7, where as in the original library they were evenly distributed (Figure 5.6B). There is a significant reduction ($p<0.05$) in the

Pool A

Positive	Neutral
ASSPRPS	ATSTMGG
GASNPAR	DSAVKQT
GRRDSPV (5)	
GSAVKQT	
SIRATGG (9)	

Pool B

Positive	Neutral
AAGSPVR	LDPDSRR
ALTPPRV	TGTAGVA (2)
ANGPLPR	
ASSPRPS (27)	
ATSTMGG	
ATSTRAP	
GNPLSTR (3)	
GRRDSPV (5)	
GSAVKQT (6)	
NRGTTNA (2)	
NTPIQAR	
NTPTQAR (4)	
QHGSAPR	
SAPVPTR (4)	
SIRATAR	
SIRGTGG (8)	
SVAPRGP	
SYSPPVR	
TLIPPGR	
TNPPRTT	
TPAPGSK (2)	
VNPDSRR	

Table 5.2 Peptide sequences from round 5 of biopanning on HeLa cells. The sequences are from 2 PCRs (pool A and B) and are divided on the basis of the overall charge of the peptides (there were no negative peptides). Positively charged amino acids are shown in red and negatively charged amino acids are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis.

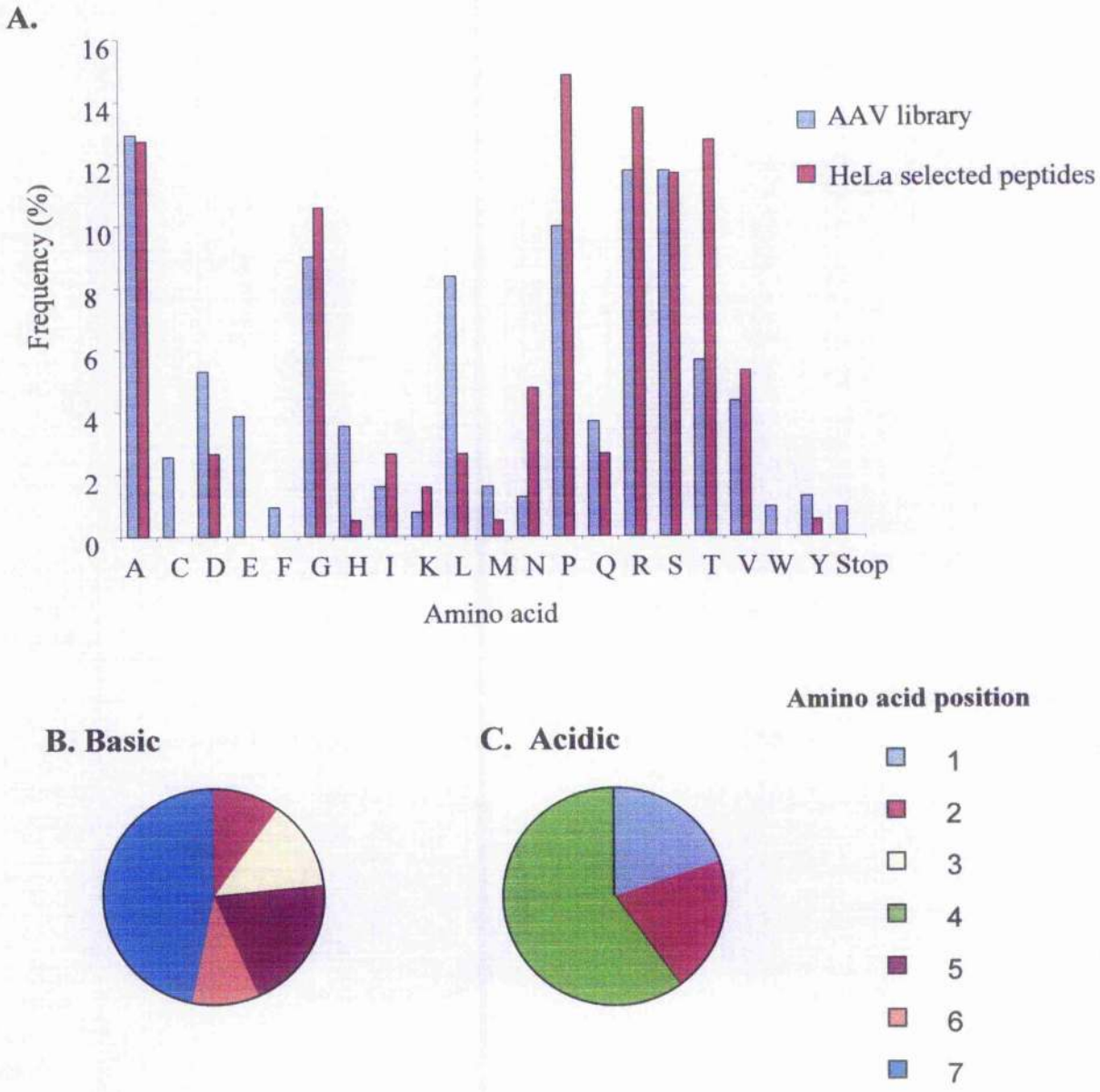


Figure 5.6 Analysis of the amino acid composition of peptides sequenced from round 6 of biopanning on HeLa cells. **A.** Frequency of amino acids within the peptides. **B.** Position of basic amino acids within the peptides. **C.** Position of Acidic amino acids within peptides. Small non-polar amino acids (A, G, I, L, M, P and V). Uncharged polar amino acids (S, T, N, Q and C). Basic amino acids (R, K and H). Acidic amino acids (D, E). Bulky amino acids (F, W, Y)

amount of acidic amino acids (D and E) found in the HeLa targeted peptides and they are only found at residues 1, 2 and 4 (Figure 5.6C). There were no positional effects seen with any of the other amino acid groups (data not shown).

These results and the previously published example (Perabo *et al.*, 2003) suggest that the AAV library can be used for *in vitro* biopanning as several consensus peptides that might target HeLa cells were identified, and the pool of targeted peptides had different features from the original library, indicating that some selection had occurred that required further investigation.

5.2.3 Influence of peptide insertion on heparin binding

One of the main problems encountered when trying to develop peptide-targeted AAV2 vectors is that in some cases HSPG binding can be maintained, despite the insertion of the peptide into a region of the capsid that is known to form the major HSPG binding site. However, the level of this binding varies depending on the peptide (White *et al.*, 2004, Grifman *et al.*, 2001), and it has been noted that in many cases where HSPG binding is maintained, the inserted peptides are positively charged (Work *et al.*, 2006, Work *et al.*, 2004a). Previously biopanning with the AAV library identified 4 targeted viruses, of these 1 was positively charged (RSNAVVP) and still bound heparin whereas the other 3 peptides had no charge and their transduction was independent of heparin (Perabo *et al.*, 2003). So, the library contains both HSPG binding-dependent and independent viruses. For a virus to be targeted to a cell type with a high degree of selectivity it is important that the ability of the virus to bind HSPG is ablated, so the library was separated into pools of HSPG binding and non-binding viruses (based on their affinity for a heparin column) for further biopanning experiments.

The binding library (Table 5.3) and 2 pools of the non-binding library were sequenced (Table 5.4). No peptides were found in both libraries, suggesting the library had been successfully divided. From the binding library 63 different peptide sequences were obtained and of these 4 occurred twice (Table 5.3). More repetition was seen in the non-binding library sequencing, with 10/55 and 12/40 repeated peptides from pools A and B

Positive peptides		**Neutral peptides**
ANSSPGR		ADAQTRI
APASAHV	SSGHQSA	DNAPTTR
APPRSVN (2)	STAQGVR	ESARQAQ
ASRASGA (2)	SVRPSSG	ESLGSGR
ASSGGLK	TATNSSK	GADVSTK
ATRGVPS (2)	TIAPRAT	RRDSNPE
GAHDGVR	TPQPPNR	SGELRGS
GGRPQIV	TPSAKPS	SGSDRDR
GHGLPYV	TQAQLQR	TNDGAVR
GRDMAPR	TRKSPER	VEAARIS
GSKAVGS	TTLNPPR	
GSPKPPG	TTSGNRL	
GTAPRTS	TVRGSAS	
GTSAKGV (2)	VTAHPTR	
GTVSGIR		
GVKREAG		
IGVSTRH		
KLDPTPR		
KLDQMRP		
LPPHHTV		
NATSGPR		
NIRQPTG		
NRGSTVT		
PDPARGK		
PLHNRTS		
PNTNPAR		
PSVPQAR		
PTSGAPK		
QTGTTVR		
RAGSATW		
RASAHPS		
RASAHRS		
RDRPVVS		
RGAQQGA		
RGHGGGE		
RGTTPPV		
RSNSPSR		
SGVHARE		
SQRLPHP		
SRALGTS		

Table 5.3 Peptide sequences from the heparin binding library. The sequences are divided on the basis of the overall charge of the peptides. Positively charged amino acids are shown in red and negatively charged amino acids are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis.

A.

Positive	Neutral	Negative
AAHTAPP	AAVSVSA	APDSAGA
DLRSIRT	ATGPTTL	APTDTDD
GSPKPPG	ATPPQQP	ARADDLE
HTPGGAP	DHGSVAT	ASESPPP
RSSSSEK	DPPPRLQ	DGDDGPA (2)
RTEHLQH	DSHRPDQ	DSSPPGV
SHAAQPT	FGSTTGG	EAGGGAV (3)
SHGEWTR	GAGLGTI (2)	**ERDRGDV (2)**
THSNSQR	GDHLTGM	GNDADER
VMPARLQ	GENQARS	GTDQTPA (2)
	GHDNGPV	PDVTELY
	GNPPTGM (6)	PESEVPM
	GSPAGPN	PNGGDSV (2)
	LPADVLH	PSDPSPT
	LPAGTLQ	RDEPPGM
	NVHSDRD	SAPRDDQ
	PPSATTS (2)	SCQQSDC
	TGGTAST (4)	SGDSLLG
	TPTGNAG	SSLSLES
	TQPGSTG	TAASTEC
	TTTSRES	TRETDSN
		TSQDGVT
		VREHGD
		VRENTGD (2)

B.

Positive	Neutral	Negative
AAHTAPP	APPEQTH	AMSDAPV
APHPGPG	CAARSDL	DLPSDRD (4)
APHPGPS (2)	DHGRAEP	DSHSPDQ
GVKR-stop-AG	DRSPPAT	**ERDRGDV (2)**
IFCGTR-stop	**GNPPTGM (5)**	GDGGSAG
LPTGHVQ	GSPAGPN	ISEEQPV (2)
PPTSVPR	LGAAGPV (2)	PECDVTL
SHGEWTR	NQTAREA	PESEVPM (2)
	PGGSSCS	RDEPPGM
	PHGVSDS (2)	SGDRTSE
	PPSATTS	SGDSLLG (3)
	SAPTTAP	SSLSLES
	SSSGPSG	STTPADG
	TGGTAST (4)	TRADDPP (2)
	TLPPNTS	VADPTFV (2)
		VTETSTS
		VSEVNDV

Table 5.4 Peptide sequences from 2 pools of the non-binding library (A and B). The sequences are divided on the basis of the overall charge of the peptides. Positively charged amino acids are shown in red and negatively charged amino acids are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis. Peptides that were found in both non-binding pools are shown in bold.

(Table 5.4). There were also twelve sequences found in both independent sequencing reactions of the non-binding library (Table 5.4).

The overall charge of the peptides (Table 5.5) suggested that charge does have an effect on the ability of the virus to bind heparin. The majority of peptides from the binding library were positively charged, with the remainder being neutral. All the binding peptides with a net neutral charge contained positively charged residues. The non-binding library was depleted of positively charged peptides and contained a higher percentage of negatively charged peptides. Analysis of the amino acid composition of the libraries (Figure 5.7) shows that the major differences between the non-binding and binding peptides were the levels of R, K, D and E residues.

Investigating the positions of amino acids within the peptides shows that in the heparin binding pool, 41% of the peptides have a positively charged residue at their C-terminal (Figure 5.8). Whereas for the AAV library and non-binding library it is only 15% (Figure 5.2) and 10% respectively (Figure 5.9). In the non-binding library basic amino acids are most commonly found at position 2 whereas in the binding library this is where the fewest are seen. There were no other major differences between the libraries in terms of the position of amino acids within.

5.2.4 *In vitro* characterisation of the non-binding and binding libraries

To characterise the non-binding and binding library pools, experiments were performed on HepG2, and HSVEC. The transduction of the HSPG binding and non-binding pools was compared to that of control viruses AAV2-RC (wild type AAV2 capsid) and A3, a triple mutant (R585A, N587A and R588A) known not to bind HSPG (Wu *et al.*, 2000). In both cell types a similar profile was observed, with AAV2-RC and the binding library producing higher levels of transduction than A3 and the non-binding library (Figure 5.10). Interestingly although heparin binding has been ablated in the triple mutant A3, it is still able to transduce cells, all be it at a much lower level than AAV2-RC. The experiments were repeated (MOI 1000) in the presence of soluble heparin (Figure 5.11). This confirmed that AAV2-RC and the binding library use HSPG as a primary receptor

	Positive	Neutral	Negative
AAV lib	46%	32%	22%
Non-binding library pool A	18%	38%	44%
Non-binding library pool B	19%	38%	43%
Binding library	84%	16%	0

Table 5.5 Summary of the net charges of the peptides sequenced from the complete, non-binding and binding libraries.

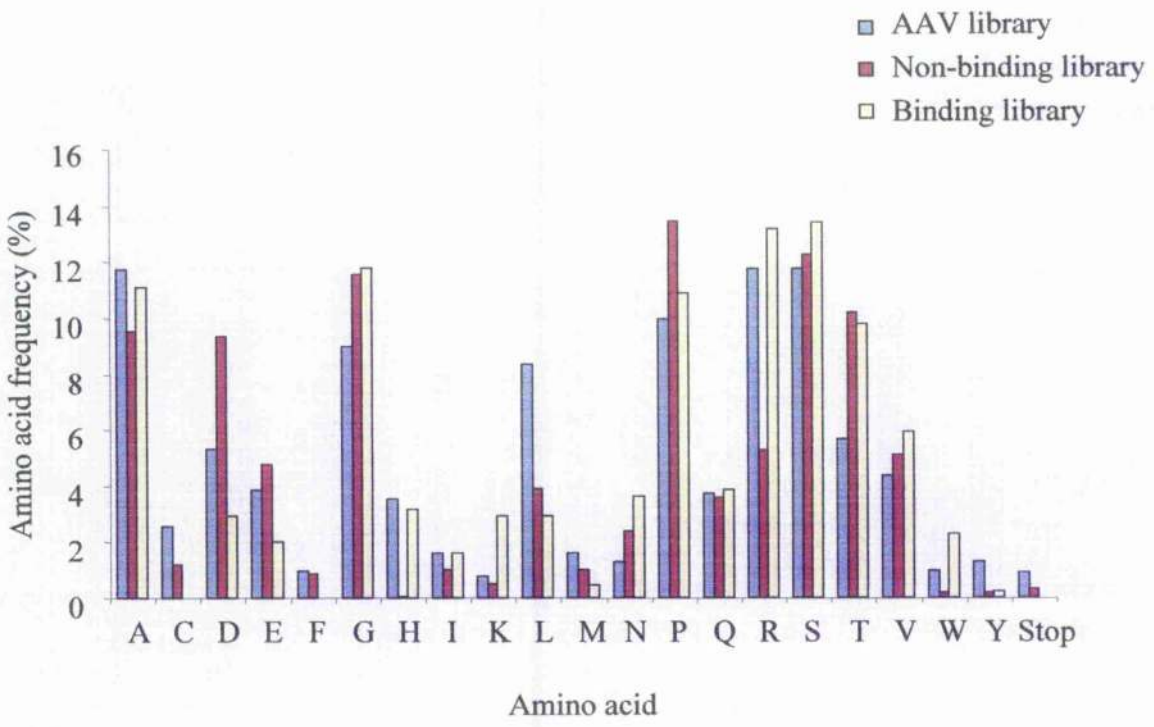


Figure 5.7 Comparison of the amino acid composition of peptides sequenced from the complete, non-binding and binding libraries. Small non-polar amino acids (A, G, I, L, M, P and V). Uncharged polar amino acids (S, T, N, Q and C). Basic amino acids (R, K and H). Acidic amino acids (D, E). Bulky amino acids (F, W, Y)

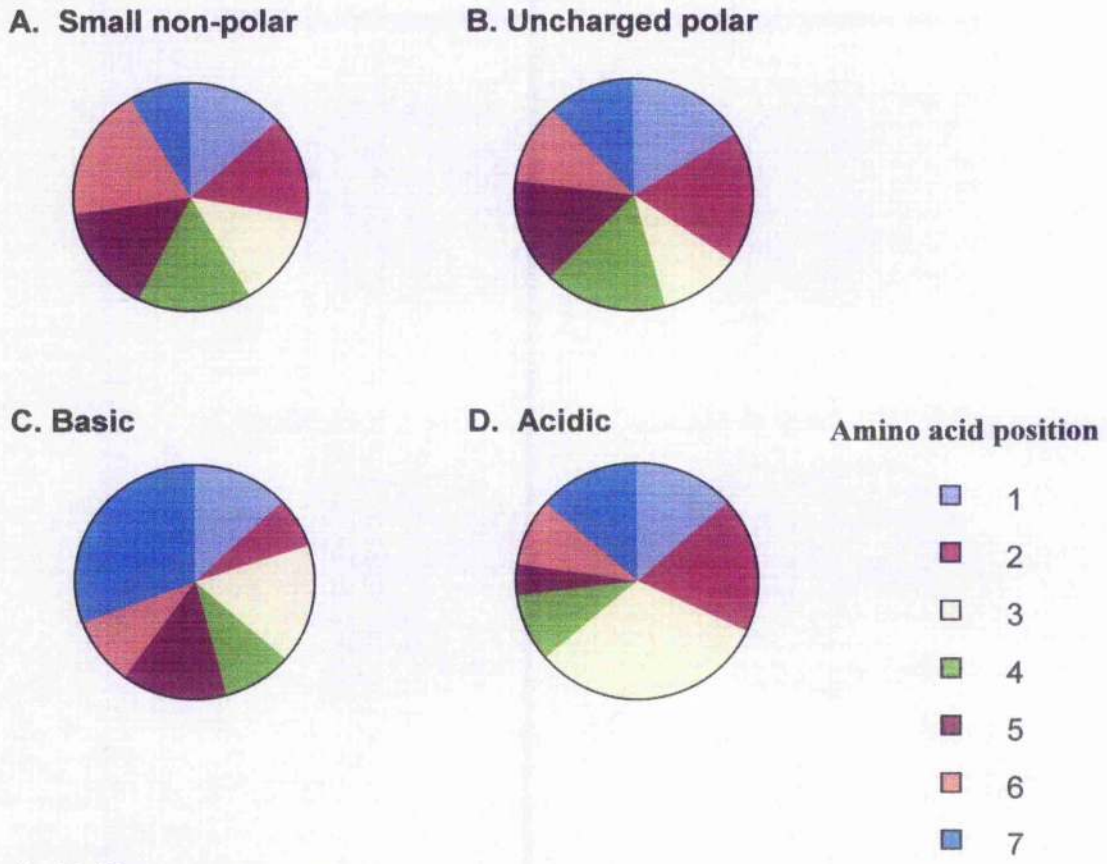


Figure 5.8 Analysis of the peptide composition of peptides sequenced from the binding library. A. Small non-polar amino acids (A, G, I, L, M, P and V). **B.** Uncharged polar amino acids (S, T, N, Q and C). **C.** Basic amino acids (R, K and H). **D.** Acidic amino acids (D, E). **E.** Bulky amino acids (F, W, Y)

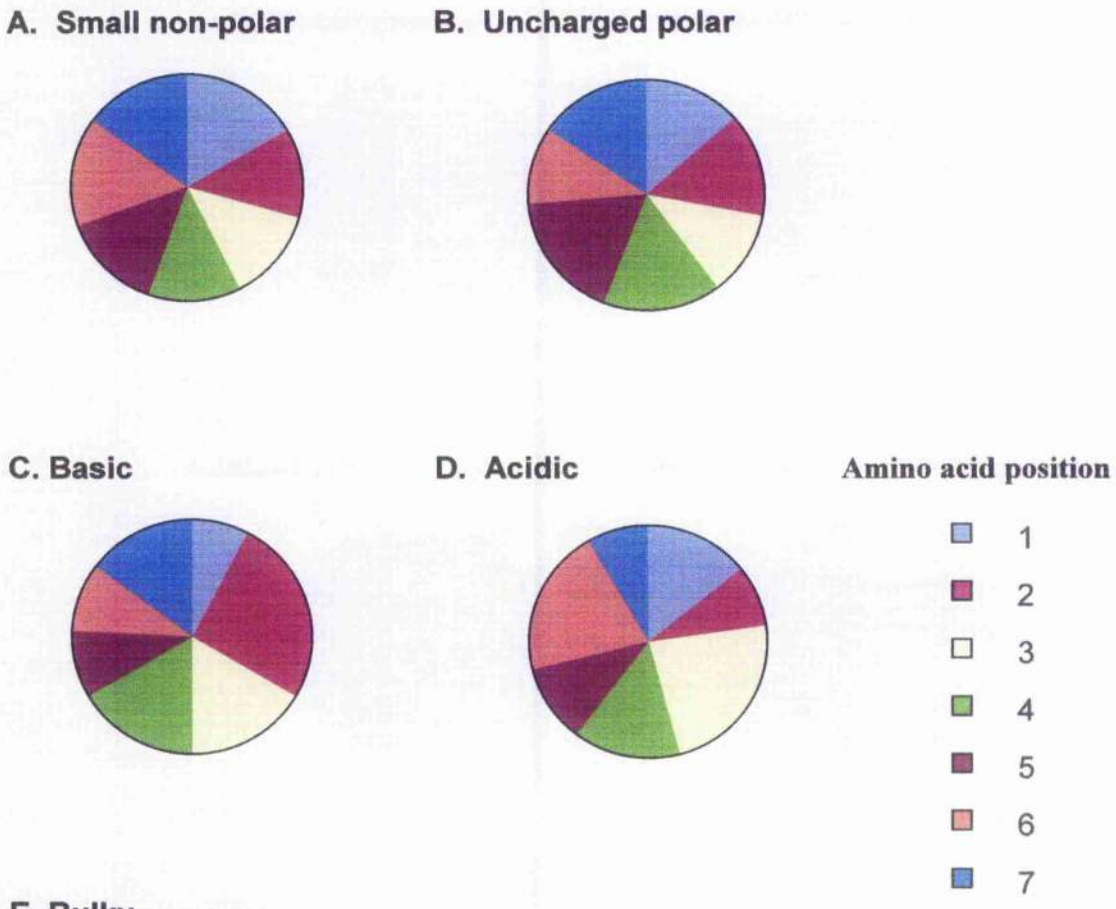


Figure 5.9 Analysis of the peptide composition of peptides sequenced from the non-binding library. A. Small non-polar amino acids (A, G, I, L, M, P and V). **B.** Uncharged polar amino acids (S, T, N, Q and C). **C.** Basic amino acids (R, K and H). **D.** Acidic amino acids (D, E). **E.** Bulky amino acids (F, W, Y)

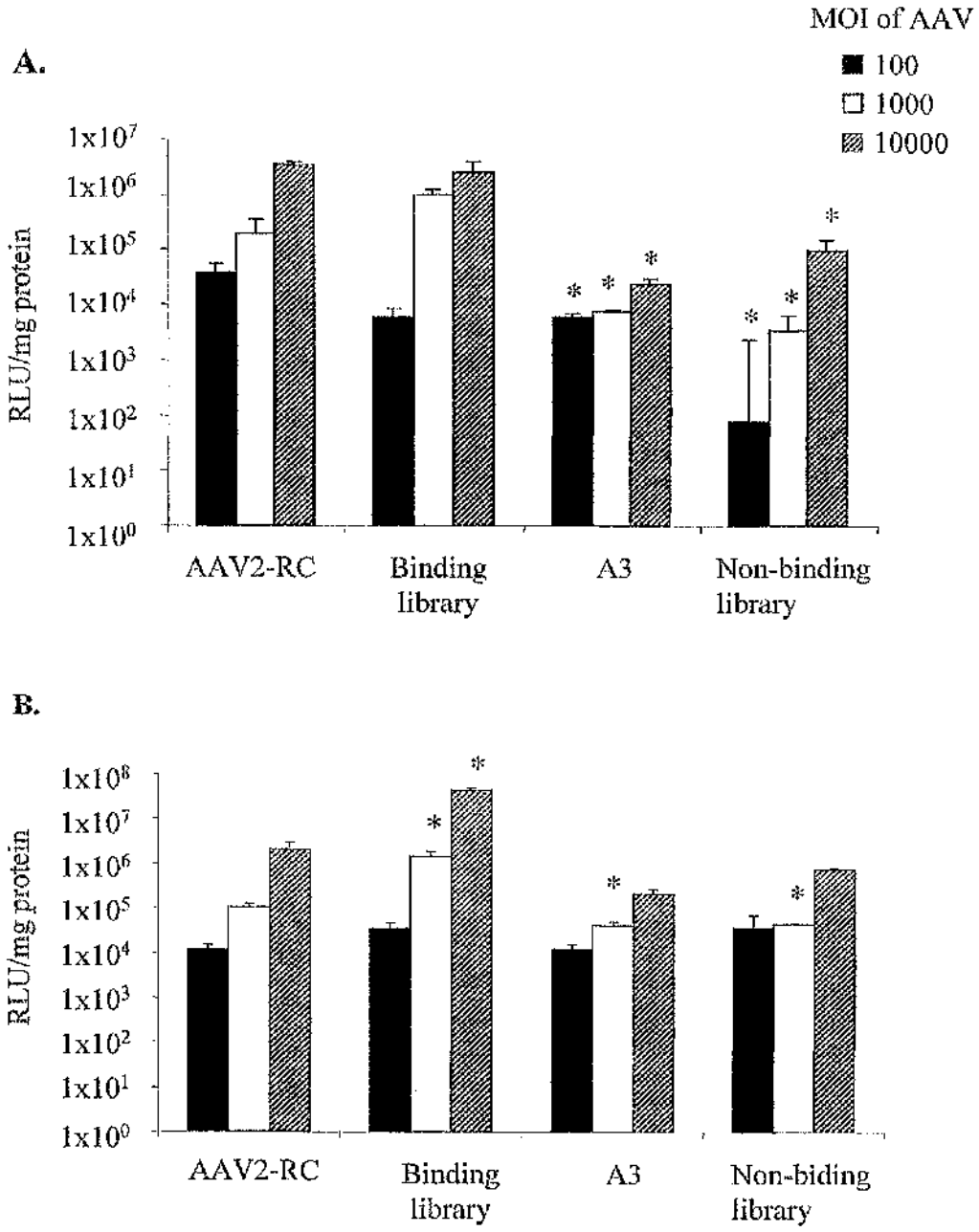


Figure 5.10 *In vitro* analysis of AAV libraries. Transduction of AAV2-RC, binding library, A3 and non-binding library in **A.** HepG2 cells and **B.** HSVECs. Cells were co-infected with AAV MOIs of 100, 1000 and 10000 and wild type Ad MOI 1 and 10 for HepG2 cells and HSVECs respectively. 72 hours post infection a β-gal. assay was performed using cell lysates and results were normalised to the total protein content. *$p<0.05$ vs AAV2-RC

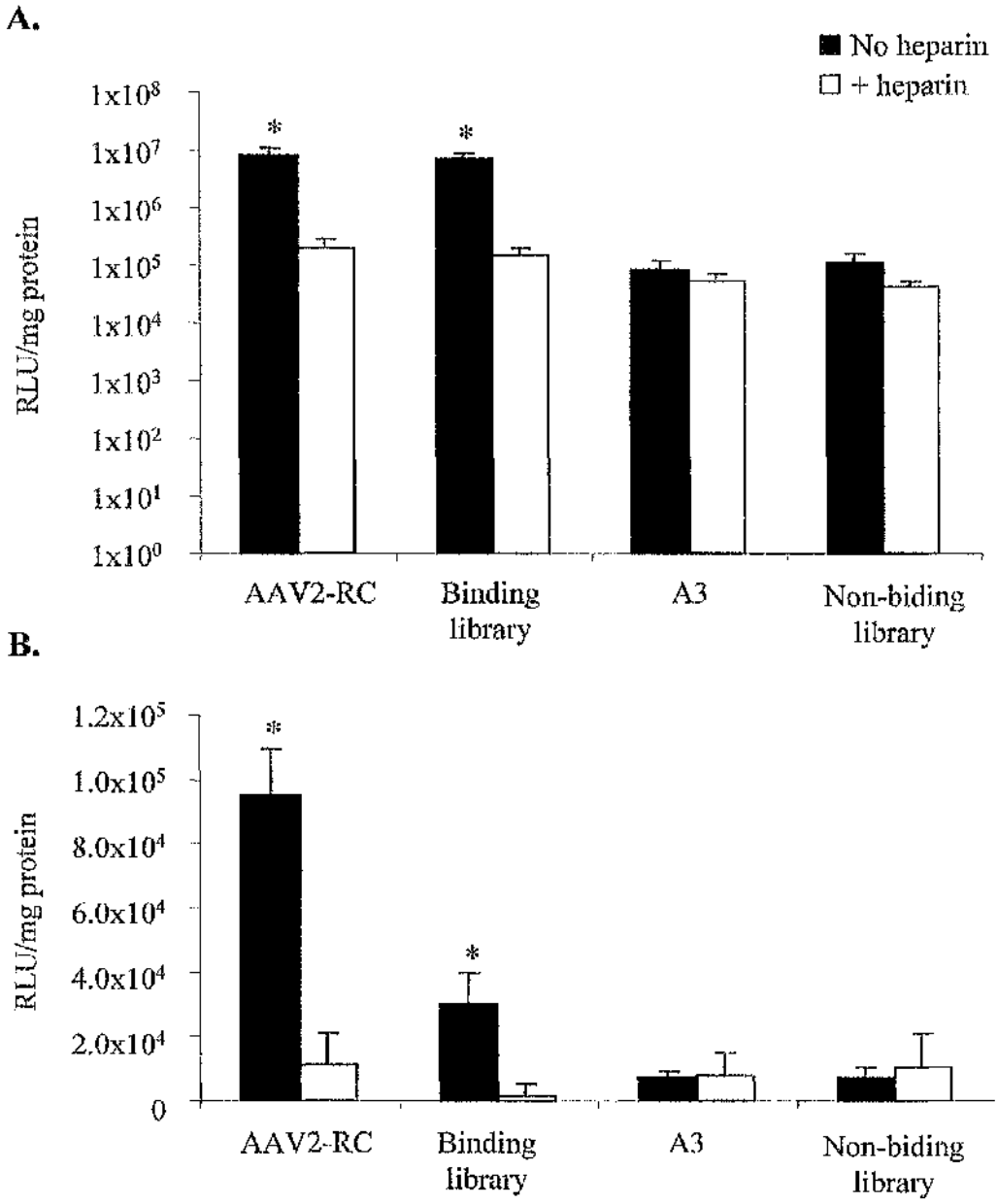


Figure 5.11 Affect of heparin on *in vitro* transduction. Comparing the transduction of AAV2-RC, binding library, A3 and non-binding library in **A.** HepG2 cells and **B.** HSVECs with and without the presence of 10 IU heparin. Cells were co-infected with AAV MOI 1000 and wild type Ad MOI 1 and 10 for HepG2 cells and HSVECs respectively. 72 hours post infection a β-gal. assay was carried out on cell lysates and results were normalised to the total protein content. *$p<0.05$ vs + heparin

as there was a significant decrease in transduction in the presence of heparin, whereas the infection of A3 and the non-binding library was not affected by the presence of heparin, suggesting the use of alternative receptors for cell binding and internalisation.

5.2.5.1 *In vitro* biopanning with the non-binding library

Although the diversity of the non-binding library was reduced from the original library, the initial results suggested that it could provide a useful novel tool for biopanning to identify cell specific viruses that do not interact with HSPG. Biopanning was therefore performed on venous and arterial primary endothelial cells HSVEC and HCAEC to compare viruses that target to different vascular beds and HeLa cells as a control. Five rounds of biopanning were performed, with cell samples taken at each round and used for real time PCR to monitor the course of the biopanning (Figure 5.12). Surprisingly, the profiles show a general trend for a reduction in the amount of AAV present at each round.

5.2.5.2 HeLa targeting peptides from the non-binding library

Sequencing of the PCR amplified peptide-encoding region from round 5 of the biopanning showed only 14/57 (25%) of inserts sequenced contained a peptide. From the 24 hour infection, none of the sequences contained peptides, but only a small sample number was tested. This is in contrast to the results from the biopanning with the full library where they all contained peptides. The 14 peptide sequences consisted of 5 different peptides, none of which were identified by the biopanning with the full library or were found in the sequencing of the non-binding library (Table 5.6). Despite so few sequences being identified, there are some interesting points to note. All the peptides are positively charged, and all have a positively charged residue in the 7^{th} position. This is despite the non-binding library containing a reduced amount of positively charged peptides and only 10% of peptides within the library having a positively charged 7^{th} residue.

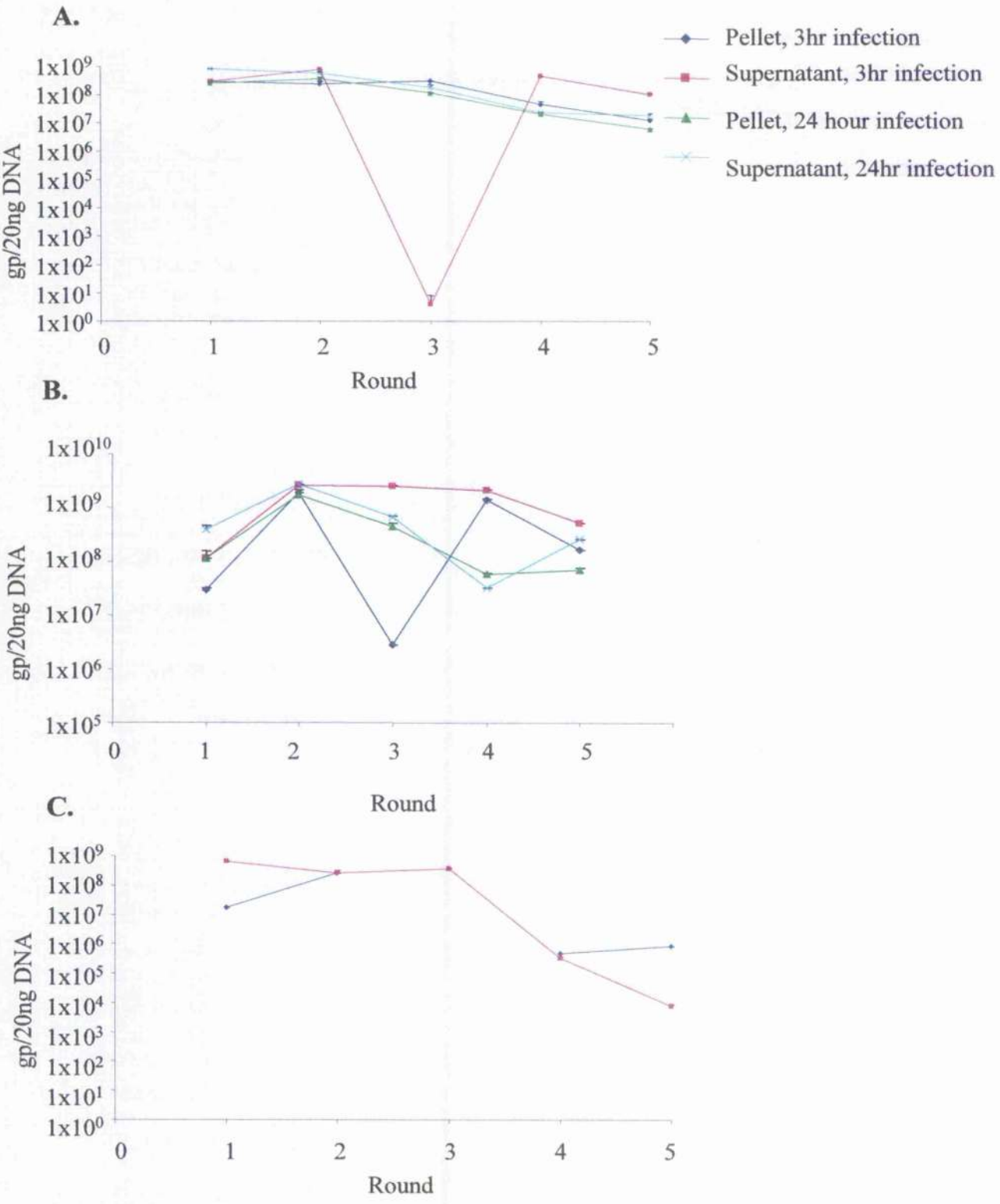


Figure 5.12 Taqman to detect the number of virus particles in cell samples from biopanning with the non-binding library. **A.** HeLa **B.** HSVEC and **C.** HCAEC. The concentration of DNA extracted from the HCAEC cell pellets was not high enough to use in the Taqman assay.

NSAQGPR (5)
TSGVAGR (2)
GRASIQK
TNVPSPR (5)
ASTGLPR

Table 5.6 Peptide sequences from round 5 of biopanning on HeLa cells with the non-binding library using a 3 hour infection time. Positively charged amino acids are shown in red and negatively charged amino acids are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis. There were also 34 sequences that contained no targeting peptide.

5.2.5.3 EC targeting peptides

Biopanning on HSVECs identified 12 and 14 targeting peptides from the 3 hour and 24 hour infections respectively, some of which were found repeatedly (Table 5.7A and 5.7B). Biopanning on HCAEC identified 3 peptides that were also found in the biopanning on HSVEC (Table 5.7). The most commonly identified peptide from the HSVEC biopanning SNSVARP, was identified in both screens and in the HCAEC screen (Table 5.7). The motif SNSV$^{A}/_{V}$ is also found in other peptides. The 2nd most frequent peptide was YNSTQRD, and another commonly occurring motif TAREA were found in both HCAEC and HSVEC biopanning. BLAST searching identified no homology to any proteins of interest. As with the HeLa biopanning, viruses with no peptide were also identified, and they occurred more commonly in the experiment where a 24 hour infection was used. None of the peptides identified in any of the endothelial cell biopanning experiments were also found in any of the biopanning experiments on HeLa cells.

5.2.6 *In vivo* biopanning with the complete AAV library

Whilst the initial *in vitro* work with the non-binding library was being performed, and as the *in vitro* biopanning suggested that the AAV2 library can be used to identify viruses targeted to a particular cell type, methods were developed for *in vivo* biopanning with the complete AAV library. Although the ultimate aim of this work is to identify atherosclerotic plaque targeting viruses, initial *in vivo* experiments were designed to compare the biodistribution profiles of AAV2-RC and the library and to develop protocols for *in vivo* biopanning by focusing on identifying viruses which target the theoretically technically simpler targets, the heart and liver. Comparing the biodistribution of the AAV library with control wild type AAV in ApoE-/- mice shows that the peptide library has altered tropism *in vivo* (Figure 5.13). A lower level of the library was detected in all tissues examined except the heart. The most significant difference seen was in the blood, where 80% of the library was found compared to approximately 30% of the wild type AAV. In other tissues the greatest reduction was seen in the liver and spleen.

A.

Positive	Neutral	Negative
ISAGRTG (3)	GENQARS (4)	YNSVDRD
PIAWARP (3)	GNPPTGM	
SNSNVRD (2)	NQTAREA (4)	
SNSVARP (54)	PTGDVRT	
SNSVVRQ	THTAREA	
	YNSTQRD (47)	

and 13 insertless clones

B.

Positive	Neutral	Negative
APHPGPG	ADNVARP	GNSTPED
ASSGGLK	GNPPTGM (2)	SAPRDDQ
LQTRARP	PAGSSSG	SGDSLLG (3)
RTPNSSP	TNAELPH	SIPDGTV
SNSVARL		
SNSVARP (12)		

and 12 insertless clones

C.

Positive	Neutral	Negative
SNSVARP	NQTAREA (79)	

and 1 insertless clone

D.

Positive	Neutral	Negative
	GENQARS (14)	

and 13 insertless clones

Table 5.7 Peptide sequences from round 5 of biopanning with the non-binding library. A. HSVEC using a 3 hour infection time, **B.** HSVEC using a 24 hour infection time, **C.** HCAEC using a 3 hour infection time and **D.** HCAEC using a 24 hour infection time. The sequences are divided on the basis of the overall charge of the peptides. Positively charged amino acids are shown in red and negatively charged amino acids are in green. For peptides that occurred more than once, the number of copies is indicated in parenthesis.

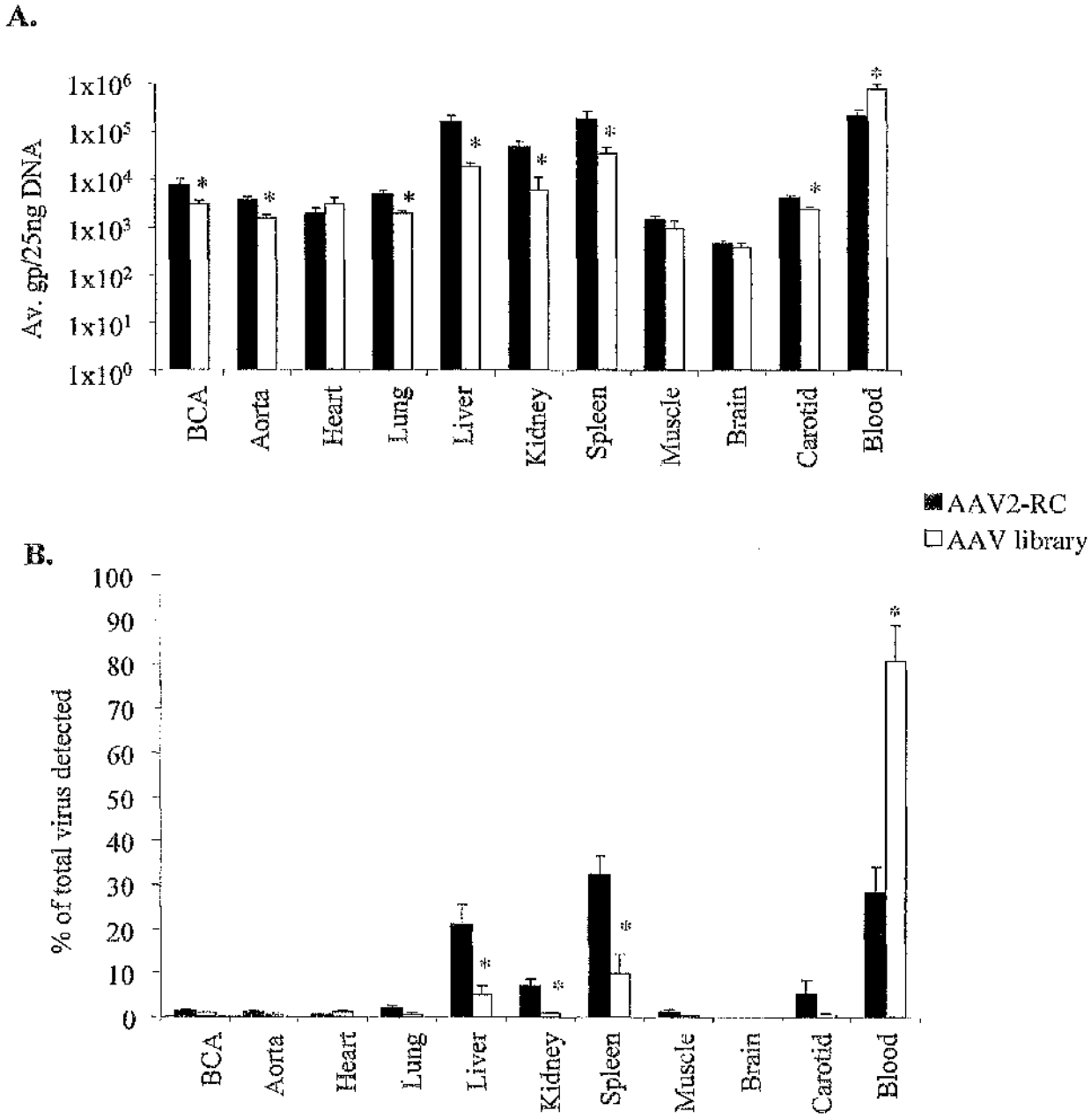


Figure 5.13 *In vivo* biodistribution of AAV2-RC and the complete AAV library. ApoE-/- mice received 4 x 10^{10} gp of virus by tail vein injection. 24 hours post infusion organs were harvested, DNA was extracted and used as a template for Taqman real-time PCR to detect the number of virions in each sample. **A.** Biodistributuion of AAV2-RC and AAV library **B.** Amount of virus detected in each tissue as a percentage of the total amount detected. n=4 per group. *p<0.05 vs AAV2-RC

Sequencing of the peptides isolated from the liver and heart (Table 5.8) showed that as expected, after just one round of biopanning no consensus motifs could be identified. Interestingly the overall charge of the peptides found in the liver has approximately the same percentage of positive and negatively charged peptides as the full library (Table 5.8A), whereas the heart had an increased amount of neutrally charged peptides and a decrease in the amount of positively charged peptides (Table 5.8B). Eight percent of viruses found in the liver had no peptide whereas all viruses sequenced from the heart samples contained peptides.

A second round of biopanning was carried out using pools of viruses synthesised from those isolated in round 1. Comparing the Taqman results of the heart and liver from round 2, show that both biopanning screens had similar biodistribution profiles, although the heart and liver pools did have slightly higher amount of virus in their target organs (Figure 5.14). The only significant differences between the 2 experiments is the liver pool of viruses had about 7-fold more virus detected in the spleen, and with the heart pool of virus there was more than a 300-fold increase in the amount of virus remaining in the blood. To compare the Taqman results from rounds 1 and 2 the amount of virus detected in each tissue has been calculated as a percentage of the total amount of virus detected, as different doses were used at rounds 1 and 2 so they cannot be directly compared. The second round of liver targeted biopanning produced a higher percentage of virus in all tissues except the blood, with the biggest increase seen in the liver and spleen (Figure 5.15A). The results from the heart pool were less encouraging, as there was an increase in the amount of virus found in the blood, and a decrease in that found in every other tissue (Figure 5.15B). Sequencing of peptides isolated from round 2 of the heart biopanning identified 5 peptides that occurred twice (Table 5.9). None of the peptides sequenced in round 2 were also found in round 1.

A. Liver

Positive	Neutral	Negative
GISRTVM	NTVGGDR	RDEPLPD
RHWARRA	DHGRDPN	GEAAG
PTRGVPT	PHGIDPA	DPSALSG
Stop-SCWWMR	AAPTTPT	DCTLGSS
APLHPAS	VAESARP	SEGQLAS
GAQQGHS	RPAQVEW	GAPPDAS
PTALVKL	ADATPRM	PTFNSMC
DRLHEGR	CSEGGHS	DSPDPGD
RRAPHLP	SSPTTPT	VYPALES
GSLSGSH	PSGLPGA	PPGGDVP
RPPASQS	PRTETTP	ALWGFDD
LQRPTRG	GAPDRFV	CDSNPGD
SVPRQTG	HPMPSDP	DYDPVGS
MLAGRT	PPPPGAL	GDGGPGQ
ACQPRTP	PPPSLGN	TATPDDG
SLSHCPA	PTSQLWT	DDSPRPQ
LLRAAAR	PSLLQPP	TQVGEAT
SRATGPC	LCCAPGA	NQLAEAP
VRRSC-Stop-L	VYPSACG	EITGADP
ISPHLER	DLHDRSW	DPDVGSC
PPGFVRA	WPRLDPG	NGTTGDP
HHRRAGS		LSEWPSV
HSAPKSS		ADRTPDV
CATPRCT		
PVCGHAR		
GARLPP		
HWPHALR		
DPSAPRR		
TTSFPHP		
Stop-WAPRTA		
NRASRNC		
SAIRAAS		
APPLSPH		
WACPSHC		
HCCAPGA		
RSRWHSS		
KATQLRV		
KEPRLER		
TWLRDRA		
RSSLRDY		
YNMRTPA		
KATQVRE		
NSAATPR		
RSSLHNY		

Table 5.8 Peptide sequences from round 1 of biopanning with the AAV library in ApoE-/- mice. A. Peptides isolated form the liver, **B.** (shown overleaf) Peptides isolated from the heart. The sequences are divided on the basis of the overall charge of the peptides. Positively charged amino acids are shown in red and negatively charged amino acids are in green. All peptides shown were found once.

B. Heart

Positive	Neutral	Negative
GKSPLVA	ASSHTLD	YCSPGEC
RYPGLTC	ITAPPLA	TDLLPYA
SALRNPC	GHPPGQD	CIAEWIS
HVPRRTN	DLSHATA	CLPDLWA
RPGALLA	PMYDSGR	GSNPDAL
ADTRPTR	PCSSPPT	CAPDPDN
ANGCRYH	VPERPPG	
ISARQPA	GPSPAAV	
	RPVCGLD	
	GSGGSSA	
	GLVALTA	

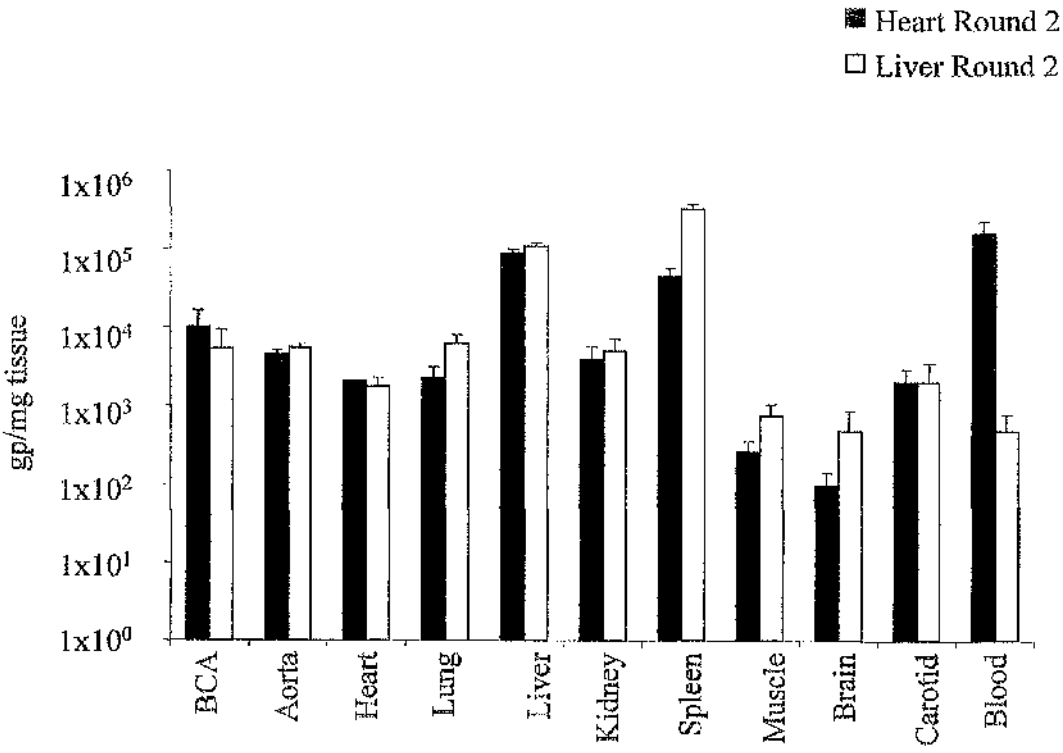


Figure 5.14 Biodistributuion of viruses after 2 rounds of biopanning for heart and liver targeted peptides. ApoE-/- mice received 2 x 10^{10} gp of virus by tail vein injection. 24 hours post infusion organs were harvested, DNA was extracted and used as a template for Taqman real-time PCR to detect the number of virions in each sample. n=3 per group.

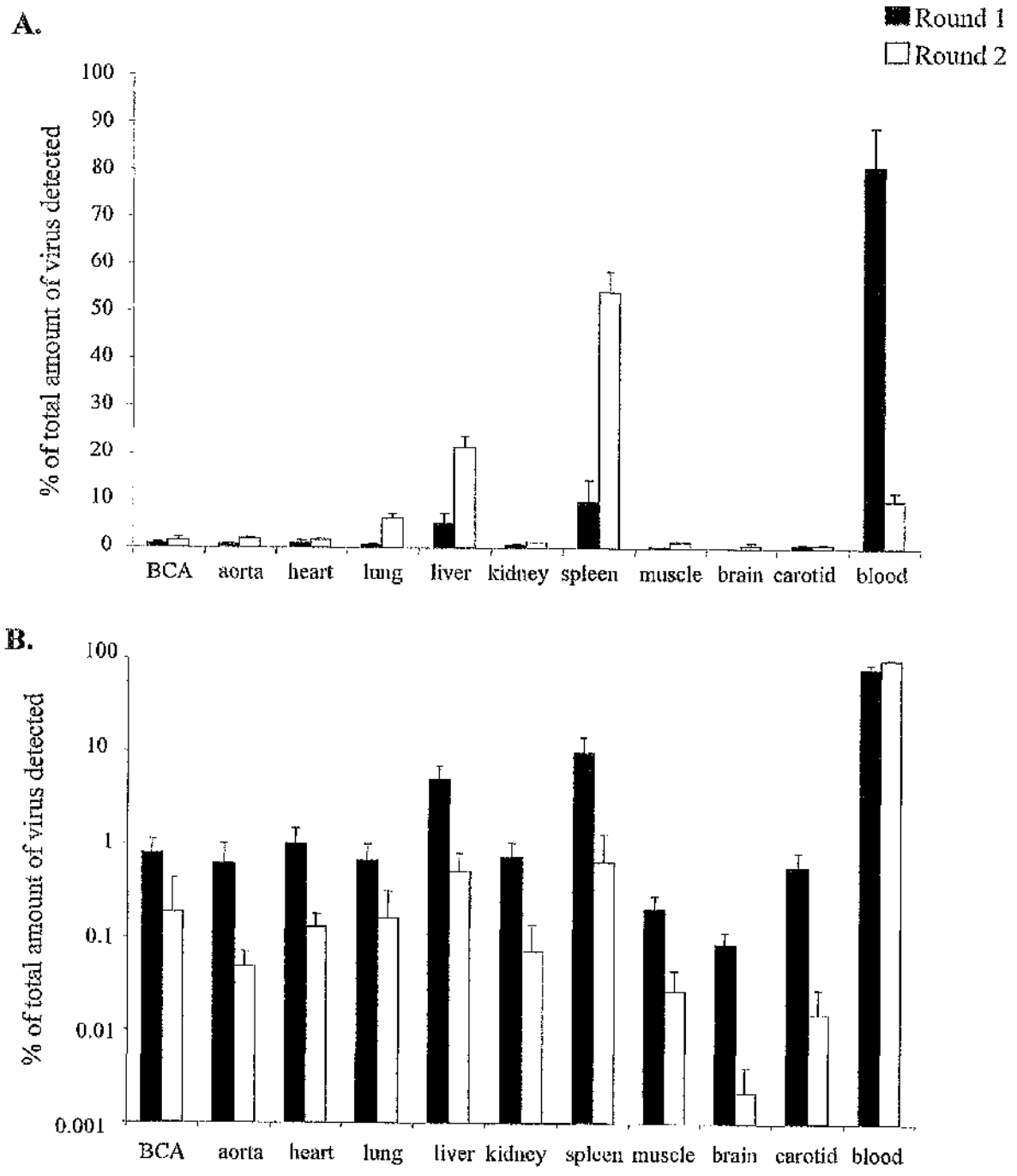


Figure 5.15: Comparison of the biodistribution of viruses from round 1 and 2 of biopanning with the complete AAV library. **A.** Liver targeted biopanning. **B.** Heart targeted biopanning

Positive	*Neutral*	**Negative**
Stop-RLLCRR	Stop-APERGG	DSEDPRA
AAR-Stop-QCR	EGDRRWG	GDSDDQR
AGDAAQR	GAISSSA	QSGADCA (2)
GLLRP-Stop-C	LL-Stop-TVAG	QSGGADA
GPRGRWC (2)	P-Stop-GAPAS	QWDGPGG
GQSAGLR	PGVVDQR	
GRLPP-Stop-G	SLSGPER	
HPGGFCR	SLSQALA	
HRAQERG	SPMAGLA	
LAGRALR (2)		
LGGIRIR		
LHVGRS-Stop		
LL-Stop-TVAR (2)		
PQRAPHG		
PRGLFAR		
PRPELRR		
PWGGPRG		
QHRHLRG (2)		
QQLRESR		
RCDLQQR		
RFGEWPR		
RGCECVR		
RGFRRQR		
RLNRGCR		
RPAHRRA		
RPGHRER		
RPRLVGR		
RRDFRRR		
RRHRPGG		
SARSRFR		
SFCRRGR		
SG-Stop-LAAR		
SGYWRHG		
SLSRPLA		
SPTSPRG		
SRWQV-Stop-R		
SVWRRGR		
VSRR-Stop-QR		
YFSCCWR		
YRSCRLR		

Table 5.9 Peptide sequences from round 2 of the heart biopanning with the AAV library in ApoE-/- mice. The sequences are divided on the basis of the overall charge of the peptides. Positively charged amino acids are shown in red and negatively charged amino acids are in green. The occurrence of repeated peptides is shown in parenthesis.

In round 1 no viruses without peptides were found in the heart, but in round 2, 8% of the sequences had no peptide insertion. Further analysis of the sequences shows that the type of peptides found in the heart at round 2 were very different from R1, as the percentage of positively charged viruses more than doubled (Table 5.10) and of these 69% had an R residue at position 7, which is characteristic of heparin binding. Also, in round 1 no peptides containing stop codons were identified where as in round 2 there were several.

5.2.7 Comparing the non-binding and binding libraries *in vivo*

C57/Bl6 mice received 4 x 10^9 gp of either the binding library, non-binding library, AAV2-RC or A3 viruses (expressing the *lac Z* reporter gene) via tail vein injection. 24 hours post injection mice were sacrificed. DNA was extracted from tissues and used as a template for Taqman real-time PCR. As expected, the AAV2-RC and binding library have a very similar profile with the majority of the viruses being found in the liver and spleen (Figure 5.16). The amount of A3 and non-binding library found in the liver and spleen was reduced compared to both AAV2-RC and the binding library.

	Positive	Neutral	Negative	No peptide
AAV library	46%	32%	22%	0%
Heart R1	32%	42%	24%	0%
Liver R1	46%	22%	24%	8%
Heart R2	69%	14%	9%	8%

Table 5.10 Summary of the net charge of peptides isolated from *in vivo* biopanning with the complete AAV library in ApoE-/- mice.

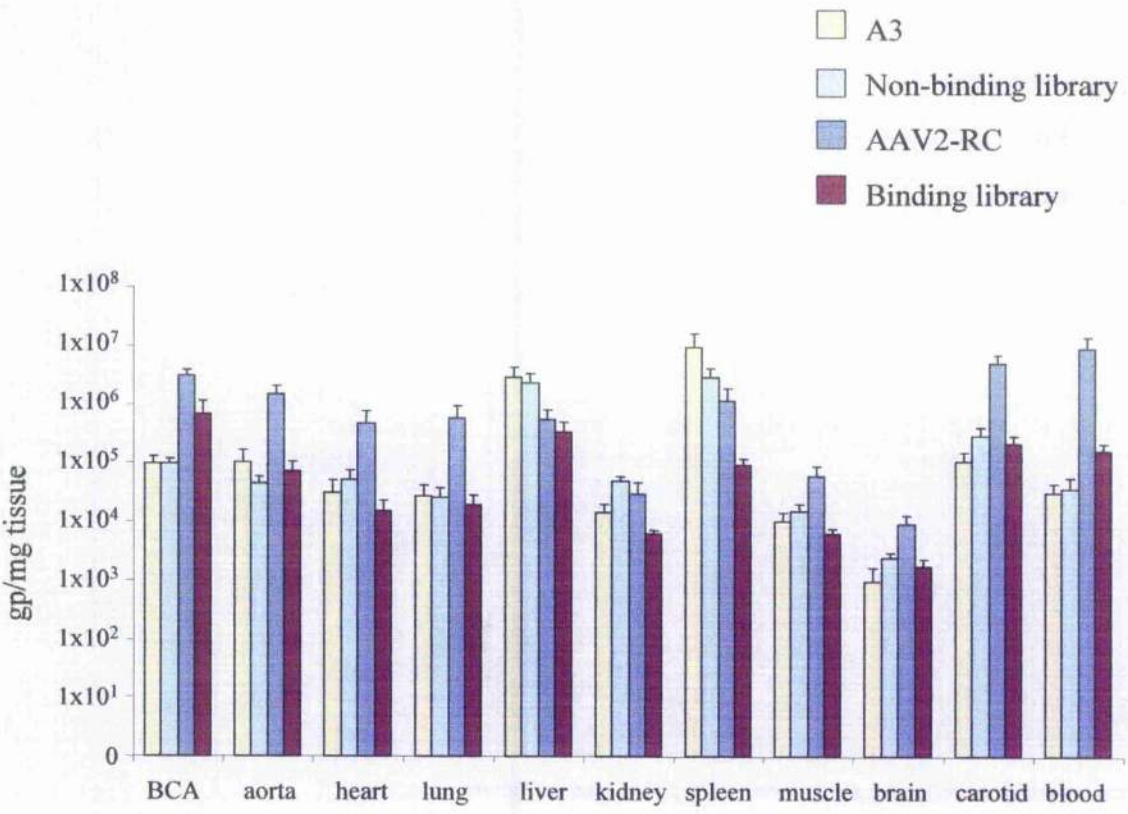


Figure 5.16 ***In vivo*** **biodistribution of AAV libraries.** C57/Bl6 mice received 4 x 10^9 gp of A3, non-binding library, AAV2-RC or binding library. 24 hours after systemic administration DNA was extracted from tissues and used as a template for real-time PCR to detect the number of virions in each sample. n=4 per group.

5.3 Discussion

Not enough is known about AAV2 biology and its cellular interactions to develop specific cell targeted vectors by rational design, although phage display has successfully identified some retargeting peptides without a positive charge that have been shown to alter AAV2 tropism (White *et al.*, 2004, Work *et al.*, 2006). Recent structural (Xie *et al.*, 2002) and mutational studies (Kern *et al.*, 2003, Opie *et al.*, 2003, Girod *et al.*, 1999) have provided some information about the AAV2 process of infection that has enabled the development of an AAV2 based library that can be used to identify cell type specific viruses by biopanning (Perabo *et al.*, 2003). The work in this chapter has used an AAV2 library to try to identify endothelial cell targeting peptides and developed methods for biopanning with the library *in vivo*. Analysis of the library has also provided more information about how peptide insertion affects HSPG binding of the vectors.

5.3.1 Composition of the AAV library

The diversity of peptide libraries is an important determinant in how successful a biopanning experiment can be, as libraries with a lower diversity are less likely to contain peptides with a high affinity for the target. Sequencing of the peptide-containing region of PCR products from the AAV library did identify repeated sequences within just a small number of samples, indicating that the library diversity may be relatively low. However, this may not actually be the case as repeated peptides were only found in sequences from the same PCR product and not from different PCRs. Therefore the repeated peptides may be due to problems with the method used to obtain the sequences. To clone the peptides, the insert region was amplified by 40 cycles of PCR, and this may have introduced some sequence bias. Due to the exponential nature of the reaction any inserts that were amplified in the initial cycles would have been present in significantly higher numbers than other peptides and so are more likely to be repeatedly amplified in subsequent rounds of PCR. Consequently, the cloned peptides may not be a true representation of the library diversity. The bias in the PCR is random, so comparing results from different PCRs should provide a better representation of the diversity. There is no way to directly sequence the peptides from the virus library. Alternatively the plasmid library can be sequenced directly without the requirement for PCR from a pool

of peptides, but this still would not show if the diversity seen in the plasmid library is maintained at the virus level. There is likely to be some decrease in diversity from the plasmid library to the virus library as it is possible that not all peptides present in the plasmid library will be capable of being packaged into the AAV capsid, as some peptide sequences may form a structure that is not compatible with the structure of the surrounding amino acids in the virus capsid.

Statistical analysis based on the sequencing results estimates the library has a diversity of 5×10^7 different peptides, which is about 10 fold higher than the original estimation (Perabo *et al.*, 2003). Both calculations are only estimates so this degree of difference between the two values is to be expected. However, both estimates predict the AAV library has a diversity several logs lower than that produced by standard phage libraries which can display about 1×10^9 different peptides (Hajitou *et al.*, 2006).

Analysis of amino acid composition of the peptides showed large amino acids were possibly under-represented in the library whereas small uncharged peptides were over-represented. This could be because the larger amino acids are more likely to cause a significant alteration in the structure of the AAV capsid and therefore will be less well tolerated and might prevent capsid assembly. Proline was also found to be over-represented in the library, this could be because it can introduce kinks into proteins and this may enable easier incorporation of the peptide into the capsid (Perabo *et al.*, 2006b). Peptides containing stop codons could have occurred due to read through of the stop codons enabling translation of the full AAV capsid, but it is more likely to be due to a problem with the packaging method used to synthesise the library. The library is produced by transfection of cells with a plasmid encoding the capsid gene. If cells become transfected with more than one plasmid then this could result in chimeric virions expressing more than one peptide in their capsid and therefore uncoupling of the genotype from the phenotype. The sequencing results obtained in this study generally agree with an analysis carried out on another pool of the library (Perabo *et al.*, 2006b).

The positional specificity of acidic and bulky amino acids within the peptides may be because in these positions their incorporation may be better tolerated by the capsid structure. Restrictions such as these may reduce the variability seen in peptide sequences, so may reduce the effectiveness of the library for biopanning. However it has been shown that phage libraries also show some bias in amino acid composition and distribution within peptides. For example, a 12-mer M13 library has been shown to have an over-representation of threonine and proline residues, an under-representation of cysteine, arganine, valine and glycine and positional bias with a reduction in arginine residues and an increase in asparagine at the N-terminus and a decrease in acidic residues at the C-terminus (Krumpe *et al.*, 2006). This demonstrates that amino acid bias is not unique to the AAV library so it is not disadvantageous to use the AAV library instead of a phage library for biopanning.

Although the diversity of the library is significantly less than standard phage libraries, it was decided to use the AAV2 library for biopanning as it has several advantages over using phage-based libraries. The AAV library enables identification of the peptide in the correct context, so there are not problems with possible loss of targeting capacity as has been seen when some peptides identified by phage display have been incorporated into viral vectors. Some peptides identified by phage display can inhibit viral capsid assembly when inserted into the virus capsid, for example, in one study producing peptide modified Ad vectors, it was found that 2 out of 6 peptides could not be incorporated into the capsid of an Ad vector (Wickham *et al.*, 1997). Using the AAV library, it is not possible to identify peptides that prevent capsid assembly. Also, unlike phage libraries the AAV library can also be used to select for viruses that most efficiently complete all stages of the infection process and not just binding to the cell surface. Another disadvantage of phage libraries is that different peptides have different effects on the rate of phage growth, so false positive results can occur when biopanning with phage libraries as the screening may just identify a peptide that has no inhibitory effect on the phage replication (Pasqualini and Ruoslahti, 1996).

5.3.2 *In vitro* biopanning with the AAV library

The Taqman profile of the RGE biopanning is typical of a successful biopanning experiment, with a decrease in the amount of virus present over the first three rounds as viruses with a low infectivity for RGE cells are removed, and then an increase in the amount of virus in the later rounds as the more efficiently infective viruses are selected and amplified by replication in the target cells. However, biopanning on HeLa cells did not produce an enrichment in the number of targeted viruses. This could be because HeLa cells are a cancer cell line that us transcriptionally very active, so will express a large range of surface proteins that could potentially act as receptors, so more rounds of biopanning may be required to see further enrichment of targeted viruses. This is supported by the sequencing results of the HeLa targeted peptides that showed there were still a large number of different peptides present.

5.3.2.1 Analysis of peptide sequences from biopanning on RGE cells

The majority of viruses isolated from round 6 of biopanning in RGE cells contained no inserted peptide. The insertless virus could have come from contamination at some stage during the biopanning process or may have been produced during library production. Muller *et al.* (Muller *et al.*, 2003) also detected insertless AAV in the first two rounds of biopanning with an AAV2 based library on HCAEC, but by the 3rd round it was no longer present. They suggest that AAV2 without peptides could have occurred due to recombination events that led to packaging of wild type cap genes into the shuttle capsids during production of the library transfer shuttle vectors, but it was undetectable after 2 rounds as wild type AAV2 poorly infects HCAEC (Muller *et al.*, 2003). If there was a low level of AAV with wild type cap gene in the original library then this would be selected for by biopanning on RGE cells as they are highly permissive to wild type AAV2 (See chapter 3) so will probably replicate more efficiently than the peptide modified viruses. As no viruses without peptides were found when the library was sequenced it seems that the biopanning has selected for them. The four peptides that were present after the six rounds of biopanning may have some specificity for RGE cells, but this would require further work to determine.

5.3.2.2 Analysis of peptide sequences from biopanning on HeLa cells

Several repeated peptides were identified from the biopanning on HeLa cells and as some were found in independent PCRs this suggests their repeated occurrence is not due to bias in the PCR and that they were actually present at a higher level by round 6 of the biopanning. None of the peptides isolated from the 6^{th} round of biopanning were also sequenced in the original library pools. Although some diversity remained in the HeLa targeted pool of peptides after 6 rounds, the biopanning has identified some consensus motifs that may enhance virus transduction of the cell line. The motif SIRGTGG was identified from both the RGE and HeLa cell biopanning suggesting the peptide may have some cross specificity and bind to a receptor expressed on both cell lines. As cell lines and not primary cells were used in this work it is likely they will have a lot of molecular similarities.

5.3.3 Characterisation of heparin binding and non-binding peptides

The sequencing results suggest the non-binding library has a lower diversity than the binding library implying that peptides that enable heparin binding are more common in the library, so might be more compatible with insertion into the AAV capsid than non-binding peptides, as they are likely to cause a smaller alteration in the capsid structure. Therefore to find specifically targeted peptides that do not interact with heparin, the non-binding library may be better to use for biopanning than the full library, as heparin binding viruses seem to be more common than non-binders.

Analysis of the overall charge of peptides isolated from the 2 libraries suggest that positively charged peptides enable the formation of a heparin binding site whereas negatively charged peptides block the ability of the virus to bind heparin. This supports previous findings, but it has not previously been demonstrated with such a large number of peptides. The difference in overall charge of the peptides is due to the over representation of E and D amino acids in the non-binding library and under-representation of K and R. There is no difference in the number of H residues in the non-binding and binding libraries, but H could be considered a neutral amino acid as it has a low pKa value. If H is considered a neutral amino acid then the effect of charge of the

peptide becomes even more pronounced, with less than 10% of the peptides in the non-binding library having a positive charge, while the binding library contains no negatively charged peptides.

This data supports the evidence from structural (Xie *et al.*, 2002) and mutational studies (Kern *et al.*, 2003) that suggest the interaction between HSPG and AAV2 is due to electrostatic interactions of the positively charged amino acids in the virus heparin binding site and negatively charged HSPG. Based on the features of the peptides found in the two libraries a model of how peptide insertion can either maintain or ablate the ability of AAV2 to bind heparin can be proposed (Figure 5.17). Figure 5.17A shows the HSPG binding site found in wild type AAV2. Inserted positively charged peptides may form a heparin binding site in combination with existing capsid R residues (Figure 5.17B) or a new HSPG binding site could be formed independently without any major interactions with existing capsid residues (Figure 5.17C). The peptide sequences suggest a positively charged residue at position 7 is particularly good at enabling reconstruction of the heparin binding site. As the position of the positively charged residue seems to effect HSPG binding this suggests that receptor binding is in part mediated by the amino acids in the virus capsid (Figure 5.17D). A positive charged amino acid at position 7 in this library is separated from R588 by 2 amino acids, which most closely resembles the wild type situation involving R585 and R588. The SMC targeting peptide EYHHYNK that was inserted into AAV2 in the same way as the library peptides also retains a strong heparin dependency, so supports the observations in this study (Work *et al.*, 2004a). In the non-binding library positively charged amino acids were most commonly found in position 2, which is the furthest position form both R585 and R588, so it cannot facilitate the formation of a heparin binding site. Neutral and negatively charged peptides may prevent the formation of an HSPG binding site (Figure 5.17E) by preventing an interaction between R585 and R588 from occurring.

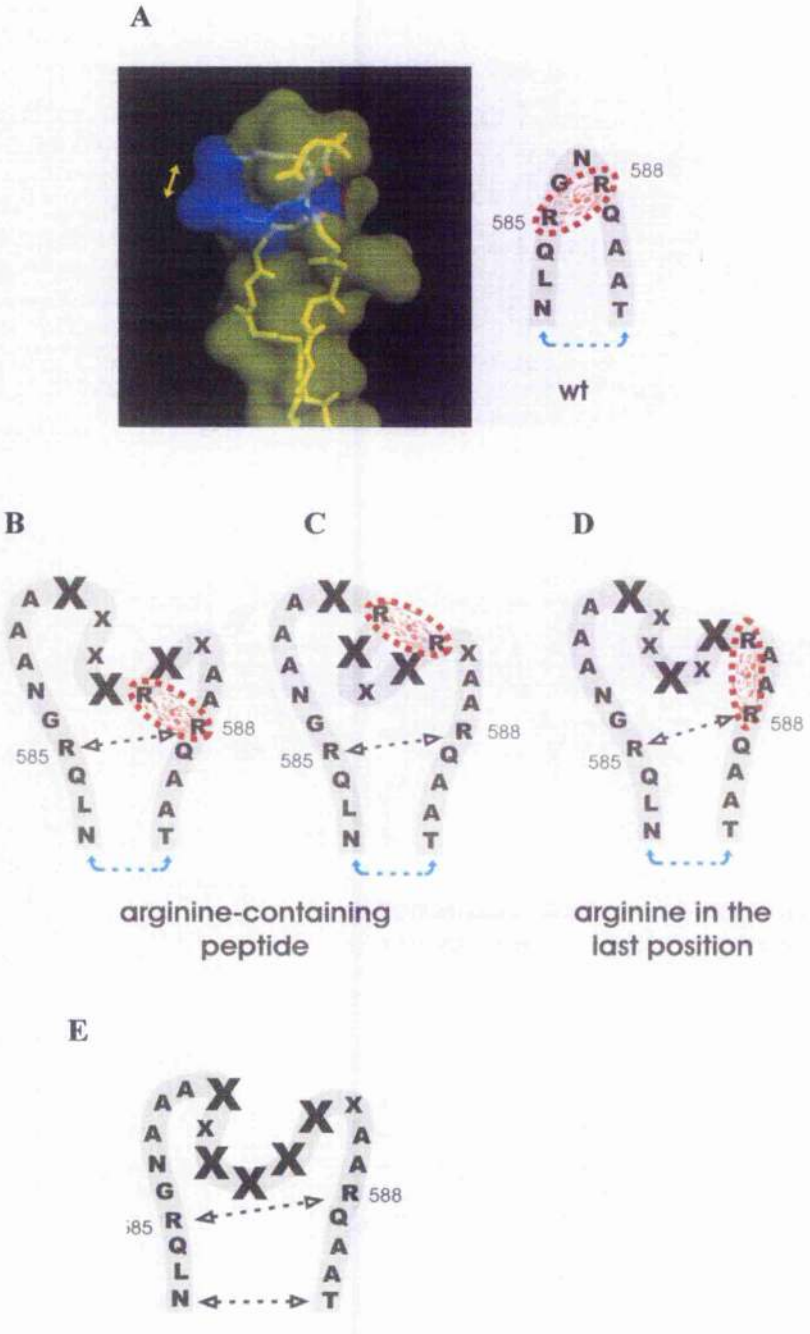


Figure 5.17 Schematic diagrams of the AAV2 HSPG binding site. A. Wild type AAV2, AAV library virions which bind heparin (**B, C and D**) and **E.** AAV library virions which do not bind heparin. (Perabo *et al.*, 2006b)

5.3.4 *In vitro* characterisation of the non-binding and binding libraries

In vitro transduction assays using the 2 library pools on HSVEC and HepG2s showed that the library had been correctly split to produce the non-binding and binding libraries as they produced similar infectivity profiles to the A3 and AAV2-RC viruses respectively. The non-binding library and A3 virus were still able to transduce both cell lines, suggesting that although HSPG is the main receptor for AAV2, other receptors can also be used independently. In both cell types the level of transduction measured from AAV2-RC in the presence of heparin is equivalent to A3, indicating that this is the level of transduction achievable independent of virus:HSPG interaction.

5.3.4.1 Analysis of HeLa targeting peptides identified from the non-binding library

Despite the lack of enrichment seen by the Taqman profiles, the sequencing results show consensus targeting peptides have been identified. Biopanning with both the full library and the non-binding library suggests the infection of HeLa cells seems to be most efficient when the peptides are positively charged and have a positively charged 7^{th} residue, which are also the characteristics of peptides which mediate binding to HSPG. HeLa cells are known to express HSPG (Piñon *et al.*, 2003), so this may be the receptor the viruses are using. Due to the method used to split the AAV library into the non-binding and binding pools, it is possible that there could be a low level of heparin binding viruses (including wild type AAV) in the non-binding pool. Despite this low level, it does seem that particularly in the HeLa cell biopanning, these heparin binding viruses have been selected for. The high level of viruses without peptides identified by this biopanning could be because the non-binding library is depleted in this type of peptide, so the insertless AAV may more efficient at infecting HeLa than the majority of viruses in the non-binding library. These results suggest that AAV2 has evolved to be highly efficient at binding HSPG to infect cells so any alterations we make to this may be reducing its overall transduction efficiency and therefore the selection pressure selects for the few remaining heparin binding viruses, including both wild type virus and peptides which bind to HSPG and most closely mimic the natural AAV capsid structure and infection mechanism. One possible way of overcoming this could be to carry out the first

round of biopanning in the presence of soluble heparin to remove any remaining HSPG binders. Alternatively, the library could be produced on the A3 background, which should reduce the ability of the viruses to bind heparin even if they contain a positively charged peptide.

5.3.4.2 Analysis of EC targeting peptides

EC gene expression varies between different vascular beds as in different tissues the EC are exposed to different environments e.g. varying oxygen concentrations in the blood and are required to carry out different functions depending on their location (Minami and Aird, 2005, Lacorre *et al.*, 2004, Aird, 2004). Despite using arterial and venous endothelial cells, there were some peptides that were identified from biopanning on both cell types (but not HeLa cells) suggesting that they may be EC specific. Identification of the same peptides from different screens shows its occurrence was not due to the random bias in the PCR reaction. Although some peptides were identified from both EC cell types, others were unique to one cell type, demonstrating there are molecular differences between these 2 vascular beds. Comparing the HSVEC targeting peptides from this work with those identified by biopanning on HSVEC using an M13 linear library of 7-mer peptides (L. Work, unpublished) has identified some similarities. From the phage biopanning, 90 different peptides were each found once, and several of these contain motifs found in the non-binding library HSVEC biopanning (Table 5.11). The most commonly occurring similarity has homology to SNSVARP, which suggests the (SV)ARP motif may be important. This motif has also been found in peptides isolated form biopanning with a M13 phage 12-mer library on Human umbilical vein endothelial cells (HUVEC)s. The most commonly occurring peptide from that screen was MSLTTPPAVARP and the motif YARP was also found in another peptide (White *et al.*, 2004). This suggests the motif may target a receptor found on endothelial cells from a range of vascular beds. Comparing the results of the HSVEC biopanning with results from biopanning with an AAV2 library on HCAEC also shows some similarity in motifs identified (Muller *et al.*, 2003). In 2 independent screens the tri-peptide motif NSV was identified (Muller *et al.*, 2003), which was also found in the most commonly identified HSVEC peptide and in the HCAEC screen in this study.

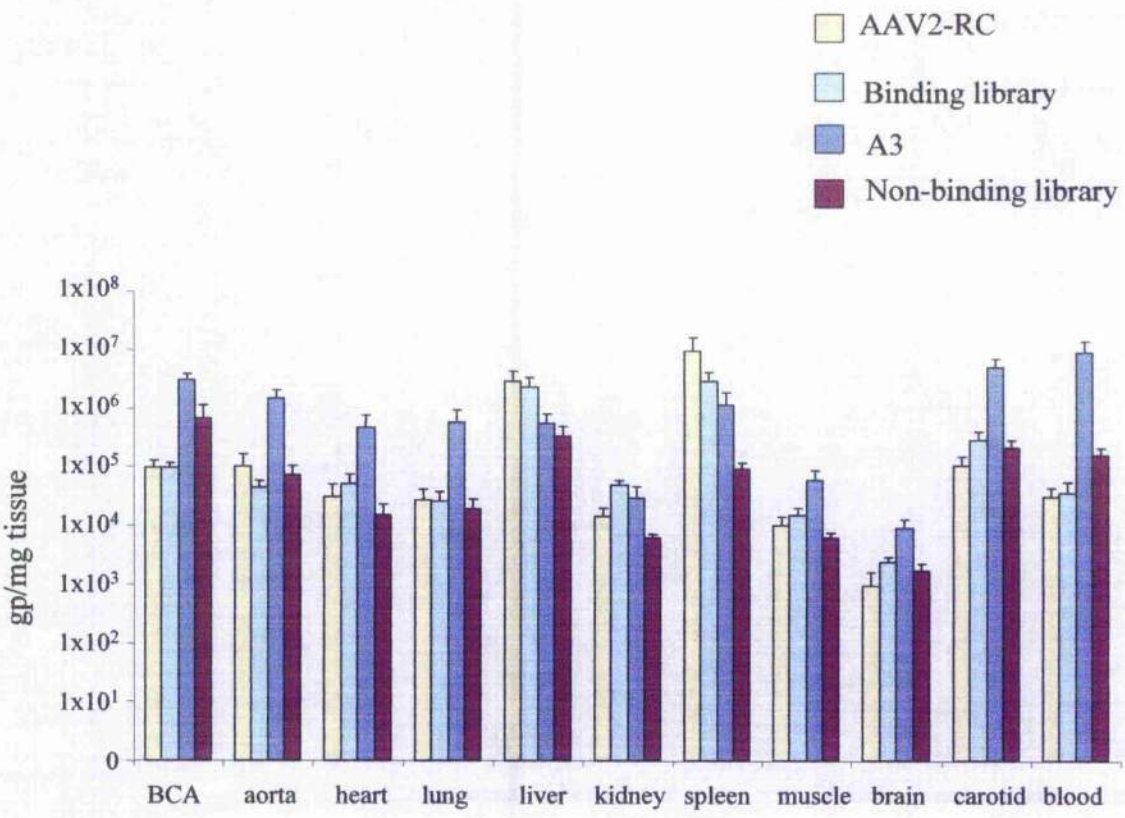


Figure 5.16 ***In vivo*** **biodistribution of AAV libraries.** C57/Bl6 mice received 4 x 10^9 gp of A3, non-binding library, AAV2-RC or binding library. 24 hours after systemic administration DNA was extracted from tissues and used as a template for real-time PCR to detect the number of virions in each sample. n=4 per group.

Interestingly the peptide GENQARS that was found in both the HSVEC and HCAEC biopanning has also been identified from biopanning with the complete AAV2 library on Mec1 cells (a human cell line derived from B-cell chronic lymphocytic leukemia (B-CLL) (Perabo *et al.*, 2003). Further worked showed the virus infected primary leukemia cells from B-CLL patients significantly better than wild type AAV2 and that the transduction is heparin independent and can be competed by soluble peptide (Perabo *et al.*, 2003). It also transduces HeLa and CO-115 (human colon carcinoma) cells at an equivalent level to wild type AAV2 (Perabo *et al.*, 2003). Therefore these cell lines may have a common receptor to which GENQARS binds. As GENQARS has been identified from biopanning on 3 different cell types it may be a particularly efficient virus in the later stages of transduction. Identification of a peptide known to mediate infection independently of heparin acts as proof of concept as it shows a heparin independent virus has been isolated from the non-binding library.

From all 3 cell types, most information was gained from the 3 hour infection time rather than 24 hours. For HeLa and HCAEC more viruses without peptides were identified in the 24 hour screen and for HSVEC more consensus motifs could be identified from the 3 hour screen. This could be because a shorter infection time will only select for the most efficient viruses for binding and internalisation, so the less specific viruses which bind to a receptor which is expressed at a lower level or bind with a lower affinity are more likely to be removed.

The presence of wild type AAV2 in the library does seem to have reduced the number of targeting peptides identified from some of the screens. However it seems to be due to the wild type capsid infecting the cells more efficiently than the majority of the peptide modified viruses, so in these cases the identification of more, but less efficient viruses may not actually be advantageous. Waterkamp *et al.* (Waterkamp *et al.*, 2006) have altered the way they produce their AAV library to eliminate any wild type AAV2 contamination. To avoid homologous recombination between wild type and library genomes a synthetic cap gene containing alternative codons was used to produce the

library (Waterkamp *et al.*, 2006). *In vitro* biopanning experiments comparing the contaminated and wild type free library showed targeting peptides could be identified from both libraries, although some of the selected peptides identified using the new library seemed to have a better degree of cell specificity than those identified with the original library (Waterkamp *et al.*, 2006).

5.3.5 *In vivo* biopanning with the complete AAV library

Comparing the biodistribution of the AAV library with AAV2-RC showed that insertion of peptides into the capsid of AAV does alter the tropism of the virus as the majority of the AAV library was found to still be circulating in the blood 24 hours after vector administration indicating that the majority of the library virions did not have a strong affinity for any accessible receptor, so they remained in the blood. Peptide insertion seems to prolong the amount of time the virus can remain in the blood before being cleared from the body or infect a tissue such as the liver. Increased levels of virus in the blood have been found with other peptide modified AAV2 vectors (White *et al.*, 2004). Higher levels of the library were detected in the heart, and interestingly an HSPG detargeted virus with the mutations R585E and R484E has also been found predominantly in the heart suggesting that a secondary receptor for AAV2 may be expressed at high levels in the heart (Kern *et al.*, 2003, Müller *et al.*, 2006). Also, several wild type viruses were found in the liver, but not in the heart. This supports previous studies that have shown that wild type AAV does not have an efficient tropism for the heart after systemic administration (Müller *et al.*, 2006, White *et al.*, 2004, Work *et al.*, 2006).

After 2 rounds of biopanning the biodistribution of the pool of viruses from the liver biopanning suggest that the biopanning may have selected viruses that are efficiently taken up by the reticulo-endothelial system as higher levels of the virus were found in the liver, spleen and lungs. The biodistribution profile of the heart pool of viruses suggests the pool mainly contains detargeted virus as their was a large increase in the amount of virus found in the blood, suggesting viruses that target other tissues have been removed in the first round of biopanning. Some repeated peptides were isolated from the heart

suggesting that there was some reduction in the diversity of the library and a degree of enrichment for heart targeting peptides had occurred. However, the majority of peptides isolated in round 2 had features typical of peptides that bind HSPG (a net positive charge and a positively charged 7^{th} residue). The Taqman profile did not show any enrichment for viruses targeted to the heart, the amount of virus without a peptide had increased and the majority of peptides identified contained features typical of peptides that bind heparin, so it was decided not to continue with this biopanning. Instead, the possibility of using the non-binding library for *in vivo* biopanning was investigated.

5.3.6 Comparing the non-binding and binding libraries *in vivo*

Comparing the libraries with AAV2-RC and A3 *in vivo* showed similar results to the *in vitro* comparison, as similar biodistribution profiles were achieved with AAV2-RC and the binding library and the A3 virus and non-binding library. This suggests that the majority of virions in the binding library use HSPG as a receptor. Reduced amounts of the non-binding library were found in the liver and spleen suggesting that HSPG binding ablation does detarget the virus from its native tropism, as has previously been seen with other HSPG mutants (Kern *et al.*, 2003) and targeted viruses modified with HSPG independent peptides (White *et al.*, 2004, Work *et al.*, 2006). Ablation of HSPG binding seems to reduce the overall infectivity of the virus as more A3 and non-binding viruses were found in the blood than the other 2 viruses. The increase with the non-binding library was not as great as that seen with A3 as the inserted peptides may be enabling virus to use alternative receptors and so restores some of the virus infectivity.

These results suggest that *in vivo* biopanning with the non-binding library may provide a way of identifying viruses which are detargeted from the natural tropism of AAV2 and retargeted to specific tissues of interest.

5.3.7 Summary

The work in this chapter has focused on characterising the AAV library and developing methods for effectively using the library for both *in vitro* and *in vivo* biopanning. It has demonstrated that using the complete AAV library it is difficult to identify peptides that

ablate HSPG binding, as these seem to be both more compatible with the AAV structure and more efficient at mediating infection, so the biopanning process actually selects for these viruses. Biopanning with the HSPG non-binding library seems to have been more successful and has identified 4 potential endothelial cell targeted AAV2 vectors containing the peptides GENQARS, SNSVARP, YNSTQRD and NQTAREA which may infect cells independently of an interaction with HSPG. These vectors are currently being synthesised so their selectivity for endothelial cells both *in vitro* and *in vivo* and their ability to bind heparin can be further investigated.

The complete AAV library was used to develop methods for *in vivo* biopanning. After 2 rounds of biopanning, sequencing results suggest that it may be possible to identify consensus sequences after 3-4 rounds, but to avoid the identification of heparin binding peptides it is likely that viruses with better selectivity will be identified using the non-binding library.

Work carried out with the full AAV library and the non-binding and binding library supports previous studies that have indicated that insertion of positively charged peptides into the heparin binding site in the AAV2 capsid is able to maintain some degree of heparin binding. In the case of the library used for this work, a positively charged residue at position 7 within the peptide seems to be particularly efficient at restoring heparin binding ability. This positional affect highlights one of the advantages of using the AAV2 library instead of phage, as it shows the context specificity of the peptide is important as the heparin binding site must be formed by the peptide and existing capsid residues for the position of the charged residue to be important. As well as providing information about peptides which enable targeting of particular cell types/tissues, the information gathered in this chapter will enable a more accurate prediction of which peptides identified by phage display can be successfully incorporated into the AAV capsid to produce functional viruses which do not interact with heparin

6.1
General Discussion

The initial optimism surrounding the potential to use gene therapy to treat a wide range of diseases receeded following a number of negative clinical trials and treatments that resulted in serious adverse effects. However, improvements in the understanding of the mechanisms involved in successful gene delivery and vector optimisation have regenerated interest in this field. Cardiovascular gene therapy is an advancing area as the knowledge of both the disease and gene therapy vectors is improving. Following many successful studies in animal disease models over 100 clinical trials have now been undertaken (http://www.wiley.co.uk/wileychi/genmed/clinical/) for diseases such as ischemia and vein graft failure (Alexander *et al.*, 2005, Losordo *et al.*, 2002). However, further advancements in vectors are still required for clinical gene therapy for CVD to be realised.

Gene therapy vectors are ideally required to be non-toxic, non-immunogenic, stable in blood and producible on a large scale. Ideally a vector to treat atherosclerosis would provide a high level of transgene expression specifically in the atherosclerotic plaque following systemic administration of the vector. Highly localised gene expression reduces the likelihood of vector toxicity, reduces the possibility of adverse affects due to gene expression in non-target tissues and reduces the dose of the vector required. As atherosclerosis is a chronic condition long-term transgene expression may be required for a treatment to be effective.

The aim of this study was to develop atherosclerotic plaque targeted viral vectors using three different approaches:

1. Previously identified plaque targeting peptides were inserted into Ad and AAV vectors to test their targeting capacity and compare the efficiency of different vector platforms.
2. *In vivo* phage display was performed to identify peptides that target unstable atherosclerotic plaques.
3. Biopanning with the AAV library was used to identify EC targeting peptides and further develop the use of this technology.

6.1 Atherosclerotic plaque targeted viral vectors

The previously identified plaque targeting peptides CAP, CNH and CQE were inserted into the capsid of Ad and AAV based vectors to compare the targeting capacity of the vectors both *in vitro* and *in vivo* in a mouse model of atherosclerosis. Initial testing of AdKO1 vectors suggested they had a tropism for vascular cells *in vitro*. Incorporation of the peptides into the CAR and HSPG detargeted vector AdKO1S* produced vectors that inefficiently infected all cell types tested. These results, in combination with results from other studies that have failed to demonstrate retargeting of AdKO1S* vectors (Bayo-Puxan *et al.*, 2006) (A. Kritz, in press) suggest that the platform vector is defective. Although it remains to be proven exactly why the KO1S* mutation produces an inefficient vector, based on this evidence it is unlikely that the KO1S* platform can be efficiently targeted to any cell type.

Insertion of the CAP, CQE and CNH peptides into the fiber of the Ad5/19p vector significantly increased the transduction of all cell types tested *in vitro*, suggesting that peptide insertion caused non-specific cellular uptake of the vectors. *In vivo* results showed the peptide-targeted vectors were inefficient and not selective for the vasculature. The lack of specificity of the Ad5/19p vectors may be due to the position into which the targeting peptides were inserted. The peptides may not be sufficiently exposed on the capsid surface or the conformation that the peptides form in the Ad19p fiber might not enable receptor binding.

The most promising results were achieved with the vectors AAV2-CNH and AAV2-CAP, which were found to have an increased tropism for the BCA and aorta of atherosclerotic mice. The lack of retargeting of AAV2-CAP in healthy mice suggests this vector is selectively targeted to plaques and not just vascular targeted. Immunohistochemistry to identify what cell types are infected by the viruses and a biodistribution study of AAV2-CNH in healthy mice will be performed to provide more detailed information about the selectivity of the vectors and their likely effectiveness as plaque targeted gene therapy vectors. Ultimately the most important test of the vectors

will be to determine whether the vectors expressing a therapeutic gene can produce levels of transgene expression that result in a therapeutic effect in the ApoE-/- mouse model. There are a number of genes that could potentially have a therapeutic effect if they were delivered to atherosclerotic plaques. For example, TIMP2 is thought to inhibit the migration of macrophages and reduce smooth muscle cell apoptosis in plaques (Johnson *et al.*, 2006), therefore it is a promising candidate gene for a treatment aiming to stabilise vulnerable plaques. Also, targeted delivery of anti-inflammatory cytokines such as IL-10 or TGFβ could reduce the inflammatory component of plaque progression and thereby stabalise plaques. Systemic over-expression of these cytokines has previously been shown to slow the progression of atherosclerosis in mouse models of the disease (Liu *et al.*, 2005, Yoshioka *et al.*, 2004, Li *et al.*, 2006).

Although the biodistribution studies showed the peptide-modified AAV vectors target the vasculature compared to untargeted AAV2, there was still a significant amount of the vector found in non-target organs such as the liver and spleen. To improve this, further modifications could be made to the capsid. *In vitro* experiments showed that both AAV2-CAP and AAV2-CNH are still able to bind heparin, which may be why the detargeting was not more efficient. If the vectors were produced on the A3 background, which has additional mutations that reduce heparin binding (Wu *et al.*, 2000), then the specificity of the vectors may be increased. Ultimately, these transductionally targeted vectors could also be combined with transcriptional targeting to increase their specificity. The expression cassette used in the vectors in this study contained a CMV promoter, which is active in a wide range of cell types. To increase the specificity of these vectors EC or SMC specific promoters such as flt-1 (Nicklin *et al.*, 2001b) or SM-22α (Moessler *et al.*, 1996) could be used. If the vectors are found to be vascular-specific rather than specific for atherosclerotic plaques then hypoxic response elements (Modlich *et al.*, 2000, Houston *et al.*, 1999) could be used to increase the specificity of the vectors.

It is advantageous that the most promising results were achieved with the AAV based vectors as they can produce long term transgene expression so are more likely to provide an effective form of treatment for atherosclerosis. For Ad to be a useful vector in this

situation, targeting peptides could be engineered into HdAds, which have been shown to provide effective gene therapy of atherosclerosis for over 2.5 years in ApoE-/- mice (Kim *et al.*, 2001).

As it has been proposed that the CAP peptide binds Grp78 (Liu *et al.*, 2003), which is thought to be upregulated in a number of diseases (Shin *et al.*, 2003, Asplin *et al.*, 2000), it would be interesting to test the targeting capacity of the CAP modified vectors in models of cancer as other putative Grp78 binding peptides have been characterised in melanoma, prostate and breast cancer xenograft models (Arap *et al.*, 2004). It would also be interesting to compare the plaque targeting efficiency of these other Grp78 binding peptides (Kim *et al.*, 2006, Blond-Elguindi *et al.*, 1993) in ApoE-/- mice.

6.2 Identification of peptides targeted to unstable plaques

Although biopanning has previously been performed in mouse models of atherosclerosis (Houston *et al.*, 2001, Liu *et al.*, 2003, Kelly *et al.*, 2006) no studies have focused on targeting of advanced, unstable plaques, which are responsible for the majority of deaths due to atherosclerosis (Falk *et al.*, 1995, Davies, 1996). To identify peptides that target unstable plaques, biopanning was performed in ApoE-/- mice on a C57/Bl6/129SvJ background fed a high fat Western diet. This diet has previously been shown to lead to the development of rupture-prone plaques in approximately 60% of mice (Johnson *et al.*, 2005b). The amount of phage recovered from the BCA and kidney at each round of biopanning suggests that pools of peptides specific for the BCA of atherosclerotic mice were produced. BLAST searching the peptides that occurred at the highest frequency identified peptides with homology to many proteins known to be involved in atherosclerosis. Particularly encouraging was the identification of peptides with homology to proteins involved in thrombus formation, which suggests they may have a higher degree of specificity for unstable plaques that have undergone previously silent rupture.

To further examine the targeting capacity of the identified peptides they could be incorporated into viral vectors. Based on the comparison of the vectors with the CAP,

CNH and CQE peptides it seems that AAV2 vectors would provide the best platform for this. Candidate peptides should be chosen based on the frequency with which they were identified from the biopanning and the BLAST search results. The work with the AAV library also suggests that positively charged peptides would produce a less effectively detargeted vector, so should be avoided. Based on these criteria some of the peptides which should be investigated further include LSVISS, CSNSLL*PNMC, CTS*LNYTYC, CQIT*DFTSC and QIFLFEIG.

In addition to providing a tool for targeting gene therapy vectors, peptides that target unstable plaques could also be used for the development of molecular imaging probes for non-invasive imaging techniques to aid the detection of vulnerable plaques. Although there have been some advances in identifying unstable plaques, there is still a requirement for the development of a non-invasive diagnostic system which can accurately predict the presence of unstable plaques in patients as 30-50% of people of people with severe atherosclerosis have no symptoms until they suffer a heart attack, which in many cases can result in death (Morteza *et al.*, 2006).

One example of this used probes that target VCAM-1, which is expressed on the endothelium from the early stages of atherosclerosis (Iiyama *et al.*, 1999, Davies *et al.*, 1993). Radiolabelled VCAM-1 antibodies have been used for imaging VCAM-1 expression but the target to background ratio remained low, limiting their efficiency for *in vivo* cardiovascular imaging (Kelly *et al.*, 2005). A VCAM-1 binding peptide identified by phage display was combined with a magnetofluorescent nanoparticle to produce an imaging agent (Kelly *et al.*, 2005). Using fluorescence microscopy this was found to colocalise with VCAM-1 expressing cells in atherosclerotic lesions of ApoE-/- mice (Kelly *et al.*, 2005). For this procedure linear peptides were found to be more effective than constrained peptides as a larger number of linear peptides can be attached to each nanoparticle and so they provide more efficient targeting (Kelly *et al.*, 2006).

Targeting peptides have also been used to enhance magnetic resonance imaging (MRI). To enhance the quality of MRI scans, contrast agents containing the paramagnetic

material gadolinium combined with a targeting moiety can be used. For example, Dirksen *et al.* (Dirksen *et al.*, 2005) developed a contrast agent by combining an atom of gadolinium, avidin and the cyclic peptide cNGR, which binds CD13 (an enzyme overexpressed in growing blood vessels) enabling angiogenic blood vessels involved in cancer development to be viewed. The peptides identified in this study have the potential to be utilised in similar systems to specifically diagnose unstable plaques.

6.3 Development of the AAV library

The work with the AAV library has focused on developing the technology so that the library can be efficiently utilised for both *in vivo* and *in vitro* biopanning. In the process, several EC targeting viruses have been identified from the *in vitro* biopanning. Populations of the individual viruses require further testing. Virus binding to heparin columns will be analysed to confirm they are unable to bind heparin and *in vitro* and *in vivo* studies will be performed to determine the tropism of these viruses.

Biopanning with the AAV2 library proved to be more of a challenge than originally anticipated as the biopanning process seems to select for the low level of wild type AAV present in the library and peptide-modified viruses that bind heparin. This suggests AAV2 is highly evolved to very efficiently use HSPG as a receptor and any changes made to the virus reduce its efficiency. Biopanning with the heparin non-binding library helped to overcome this problem but further improvements could still be made. If the library was produced on the detargeted A3 background this would reduce the likelihood of the peptide insertion being able to maintain the HSPG binding site. *In vitro* biopanning could also be performed in the presence of heparin to block the transduction of heparin binding viruses. Although the procedures involved are technically challenging, the methods developed for *in vivo* biopanning can now be utilised with the heparin non-binding library in ApoE-/- mice to identify plaque specific vectors that do not bind HSPG.

This work has also provided more information about which targeting peptides produce more efficient detargeting of AAV. This is important to further the development of vectors targeted via peptides identified by phage display.

6.4 Summary

The work presented in this thesis has made progress towards the development of atherosclerotic plaque targeted vectors and has provided further characterisation of Ad and AAV platform vectors which can be applied to the development of vectors targeted to other cell types. The plaque targeted vectors may also be used to provide more information about the molecular mechanisms of the disease by using the vectors to over-express genes specifically in plaques. Further work aimed at characterising the most promising vectors, first at the level of reporter gene expression but ultimately by testing therapeutic transgene expression is required to prove whether significant gains can be made in treatment.

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