Novel strategies to inhibit smooth muscle cell hyperplasia and intimal thickening

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Novel Strategies to Inhibit Smooth Muscle Cell Hyperplasia and Intimal Thickening

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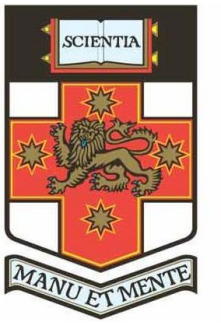

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Coronary artery disease (CAD), underpinned by atherosclerosis, remains a leading cause of morbidity and mortality, particularly in the Western World. Although the advent of percutaneous transluminal coronary angioplasty (PTCA) has provided a fundamental change in the treatment of CAD and drug-eluting stents (DES) have brought about marked improvement, there still remain significant challenges such as restenosis and late stent thrombosis. Coronary artery bypass grafting (CABG) has been acknowledged as the most effective way to treat CAD. However saphenous vein graft failures still present a problem due to stenosis. Vascular smooth muscle cell (VSMC) proliferation and migration is the primary driver of restenosis after percutaneous coronary interventions (PCI) and vein graft failure after CABG. Endothelial dysfunction also plays an important role in both restenosis and late thrombosis following PCI. Therefore, key to the prevention of restenosis and late stent thrombosis is to suppress SMC proliferation and migration, and to enhance re-endothelialisation. The broad aim of work in this thesis is to seek more effective strategies to inhibit SMC hyperplasia and intimal thickening while promoting re-endothelialisation.

More specifically, the effects of three kinds of bio-molecules on prevention of restenosis are investigated in this thesis. Firstly, a novel "cocktail" consisting of a combination of VEGF-A, VEGF-D and cRGD, which has not been studied on SMC and EC in response to injury, is tested in a rat carotid balloon injury model. Secondly, the efficacy of the DNAzyme, Dz13, targeting c-Jun is tested in the rabbit autologous vein bypass graft model using the lipid-based transfection agent 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) / 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Lastly, the anti-restenotic potential of miR-191, a natural microRNA inhibitor of the immediate early gene Egr-1, is examined in the balloon-injured rat carotid artery model.

The data presented in this thesis demonstrates that localised delivery of Dz13, miR191 and a novel cocktail of VEGF-A, VEGF-D and cRGD can inhibit neointima formation in animal models. According to these studies, the cocktail, Dz13 and miR191 may be useful approaches for reducing in-stent restenosis and late thrombosis. The ability of these biomolecules to be delivered at a local level makes them ideal for inclusion in stent-based strategies.

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Abstract

Coronary artery disease (CAD), underpinned by atherosclerosis, remains a leading cause of morbidity and mortality, particularly in the Western World. Although the advent of percutaneous transluminal coronary angioplasty (PTCA) has provided a fundamental change in the treatment of CAD and drug-eluting stents (DES) have brought about marked improvement, there still remain significant challenges such as restenosis and late stent thrombosis. Coronary artery bypass grafting (CABG) has been acknowledged as the most effective way to treat CAD. However saphenous vein graft failures still present a problem due to stenosis. Vascular smooth muscle cell (VSMC) proliferation and migration is the primary driver of restenosis after percutaneous coronary interventions (PCI) and vein graft failure after CABG. Endothelial dysfunction also plays an important role in both restenosis and late thrombosis following PCI. Therefore, key to the prevention of restenosis and late stent thrombosis is to suppress SMC proliferation and migration, and to enhance re-endothelialisation. The broad aim of work in this thesis is to seek more effective strategies to inhibit SMC hyperplasia and intimal thickening while promoting re-endothelialisation.

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Publications arising from this thesis

Published articles

Yue Li, Ravinay Bhindi, and Levon M. Khachigian, Recent developments in drug-eluting stents. ***Journal of Molecular Medicine*** 2011. 89(6): p. 545-53.

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Under review

Yue Li, Lucinda S. McRobb, Roland Stocker, Levon M. Khachigian, NOS-dependent inhibition of intimal thickening and accelerated re-endothelialization after vascular injury with a novel VEGF/cRGD cocktail. (Under review)

Margaret Patrikakis, **Yue Li**, Lucinda S. McRobb and Levon M. Khachigian, Inhibition of intimal thickening by microRNA miR-191 targeting the zinc finger transcription factor Egr-1 in vascular smooth muscle cells**.** (Under review)

Conference Presentations

1. Yue Li, Kristine Malabanan, Ravinay Bhindi, Levon M. Khachigian "A 'cocktail' for inhibition of smooth muscle cell growth and promotion of endothelialisation". Poster at the Australian Vascular Biology Society, 19th National Scientific Conference, Sydney September 2011.
2. Yue Li, Kristine Malabanan, Ravinay Bhindi, Levon M. Khachigian "Novel cocktail inhibits intimal thickening after balloon injury and stimulates re-endothelialisation". Poster at the Australian Vascular Biology Society, 20th National Scientific Conference, Queensland, September 2012.

Originality Statement

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Signed..... Date.....

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Abbreviations

AGE	advanced glycation end-product
Ala	alanine
AP1	activating protein-1
ApoE	apolipoprotein E
Arg	arginine
Asp	aspartic acid
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BMS	bare-metal stents
BP	biodegradable polymer
CABG	coronary Artery Bypass Grafting
CAD	coronary artery disease
CDK	Cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
cRGD	cyclicArg-Gly-Asp peptide
CVD	cardiovascular disease
Cys	cysteine
DES	drug eluting stent
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC	endothelial cell
ECM	extracellular matrix
EES	everolimus-eluting stents
Egr-1	early growth response-1
EPC	endothelial progenitor cell
eNOS	endothelial nitric oxide synthase
EDTA	ethylenediaminetetraacetic acid
FGF-2	fibroblast growth factor 2
Gly	glycine

h	hour
HMEC	humanmicrovascular endothelial cell
IAM	iodoacetamide
IEL	internal elastic lamina
IMA	internal mammary artery
IOD	Integrated Optical Density
L	litre
LAD	left anterior descending artery
LbL	layer-by-layer
LLL	late luminal loss
L-NAME	nitro-L-arginine methyl ester
MACE	major adverse cardiac events
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
min	minutes
mL	milliliter
MMPs	metalloproteinases
MR	moderate-release
mRNA	messenger RNA
ng	nanogram
nM	nanomolar
NO	nitric oxide
NOS3	nitric oxide synthase 3 (endothelial nitric oxide synthase)
OSS	oscillatory shear stress
PAC	plasma-activated coating
PBS	phosphate buffered saline
PCI	percutaneous Coronary Intervention
PCNA	proliferating Cell Nuclear Antigen
PDCD4	programmed cell death protein 4
PDGF	platelet derived growth factor
PDK1	phosphoinositide-dependent kinase-1
Pen	penicillamine
PES	paclitaxel-eluting stent
PF	polymer-free
PI3K	phosphoinositide 3-kinase
PlGF	placenta growth factor
PKC	protein kinase C

POSS-PCL	polyhedraloligomericsilsesquioxane poly caprolactone
POSS-PCU	polyhedraloligomericsilsesquioxane poly (carbonate-urea) urethane
Pro	proline
PSS	pulsatile shear stress
PTCA	percutaneous transluminal coronary angioplasty
PTEN	phosphatase and tensin homology
PTK	protein-tyrosine kinase
RA	rheumatoid arthritis
RASMC	rat aortic smooth muscle cell
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
s	seconds
SDS	sodium dodecyl sulphate
SES	sirolimus eluting stents
Ser	serine
siRNA	small interfering RNA
SMC	smooth muscle cell
SR	slow-release
TdT	terminal deoxynucleotidyl transferase
TGF-ß	transforming growth factor-ß
TLR	target lesion revascularization
TVR	target vessel revascularization
µg	microgram
µL	microliter
µM	micromolar
V	volts
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells
YY1	yin yang-1
ZES	zotarolimus-eluting stents

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Chapter 1

1. Chapter I

Introduction

1.1 Coronary Artery Disease

1.1.1 Overview of coronary artery disease

Coronary artery disease (CAD) is a leading cause of death in the world. It is caused by atherosclerosis, a build up of fatty materials in the arterial wall, which reduces coronary blood flow and results in ischemia of the heart muscle.

The treatment of CAD involves pharmacologic therapy, risk factor management and revascularisation. Revascularisation, adopted for patients who have severe heart ischemia, includes two strategies - percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) surgery. During PCI, a deflated balloon and coronary artery stent on a catheter is inserted from the inguinal femoral artery or radial artery into the blocked area inside the coronary artery to clear the blockage of coronary artery. CABG is a surgical revascularisation procedure to bypass the blocked artery and improve blood supply to the heart muscle. Comparing with CABG which opens the thoracic cavity under general anesthesia and using ventilator, PCI is minimally invasive, and enables the patients to have faster postoperative recovery and fewer postoperative complications. Both these two strategies have provided a fundamental change in the treatment of CAD.

However, there are limitations impeding the longer term success of PCI and CABG. The main problems are restenosis and thrombosis. Restenosis is the recurrence of stenosis in coronary arteries treated with PCI or in vein bypass grafts after CABG. Thrombosis is one of the main reasons for CABG failure in early stages (less than 30 days after surgery). In PCI therapy, late stent thrombosis occurs 30 days following the implantation of a drug eluting stent (DES). This problem, partly caused by delayed stent endothelialisation, has become significant.

For these ongoing issues, the work in this thesis has set out to investigate new targets involved in disease progression and to test novel treatment modalities that inhibit smooth muscle cell (SMC) hyperplasia and /or promote re-endothelialisation.

1.1.2 Global burden of coronary artery disease

Coronary artery disease (CAD) is a leading cause of death worldwide and is projected to remain so for the next 20 years [1]. The Global Burden of Disease Study 2004 update (WHO) showed that every year there were 3.8 million men and 3.4 million women dying from CAD globally [2].

1.1.2.1 Coronary artery disease in developed countries

There are about 940 million people living in developed countries such as the United States, Canada, Australia, New Zealand and countries of the European Union. Of this 15% of the world's population, CAD remains a key cause of morbidity and mortality, and is underpinned by atherosclerosis [3].

American Heart Association (AHA) Heart Disease and Stroke statistics displayed a striking mortality rate. According to 2008 mortality data in the United States, 1 in every 6 deaths was caused by CAD [4]. Coronary events will occur approximately once every 25 seconds and result in one death nearly every minute [4].

In Europe, CAD is the most common cause of mortality with 1.92 million deaths every year. According to EU death statistics, CAD ranks first as the single cause of death [5]. CAD resulted death rates are above 22% in women and over 21% in men.

In Australia, the self-reported 2007–2008 National Health Survey (NHS) estimated that about 685,000 people had CAD, equating to 3% of the Australian population. Among these people, 353,000 had suffered from angina and 449,000 had experienced ischemic heart disease (IHD) [6]. In 2007, CAD caused 22,727 deaths in Australia, which accounted for 50% of deaths caused by cardiovascular disease [7]. CAD causes more deaths in Australia compared to any other disease, contributing 17% of all deaths [6].

1.1.2.2 Coronary artery disease in developing countries

The WHO Global Burden of Disease (GBD) investigation in 1990 indicated that among the 6.2 million deaths from CAD, 3.5 million occurred in developing countries [8]. The projection also forecasts that in these countries there would be 7.8 million CAD deaths out of the total number of 11.1 million deaths in 2020 [8]. In the East Asia and Pacific (EAP) region alone, the WHO Global Burden of Disease (GBD) reported that approximately 1.2 million deaths were caused by CAD in 2004 and 8.2 million people suffered from angina indicating that CAD has become a significant and growing burden in East Asia [9].

1.1.3 The pathophysiology of coronary artery disease

The coronary arteries are the arteries that supply blood to the myocardium which makes up the muscular wall of the heart. The main cause of coronary artery disease is atherosclerosis, a build up of fatty materials in the coronary arterial wall, which reduces coronary blood flow and results in ischemia of the heart muscle.

1.1.3.1 Normal lumen diameter of the coronary artery has great significance for the heart

The coronary circulation is the blood circulation that feeds the vessels of the myocardium. These blood vessels include the coronary artery and cardiac veins which service blood supply to the heart. Different from most other vascular beds, the myocardium extracts approximately 75% of arterial oxygen at rest time, near the maximum of myocardial oxygen extraction [10]. Due to the high oxygen extraction at rest time, more coronary blood flow is needed to meet the increase of myocardial oxygen consumption. Therefore, it is of greater significance for the heart than the other organs to maintain normal arterial lumen diameter and blood flow.

1.1.3.2 The layers of the normal artery wall

The normal artery wall consists of three layers: the tunica intima, tunica media and tunica adventitia. Structurally, the intima refers to a continuous monolayer of endothelial cells (EC) that is built on a basement membrane. The media is made up of vascular smooth muscle cells (VSMC), which are inlayed in a matrix, and in this matrix, elastic lamellae sheets are interwoven. The adventitia is composed of fibroblasts, loose connective tissue capillaries, and fat cells [11]. These layers are separated by elastic lamina **(Figure 1.1).**

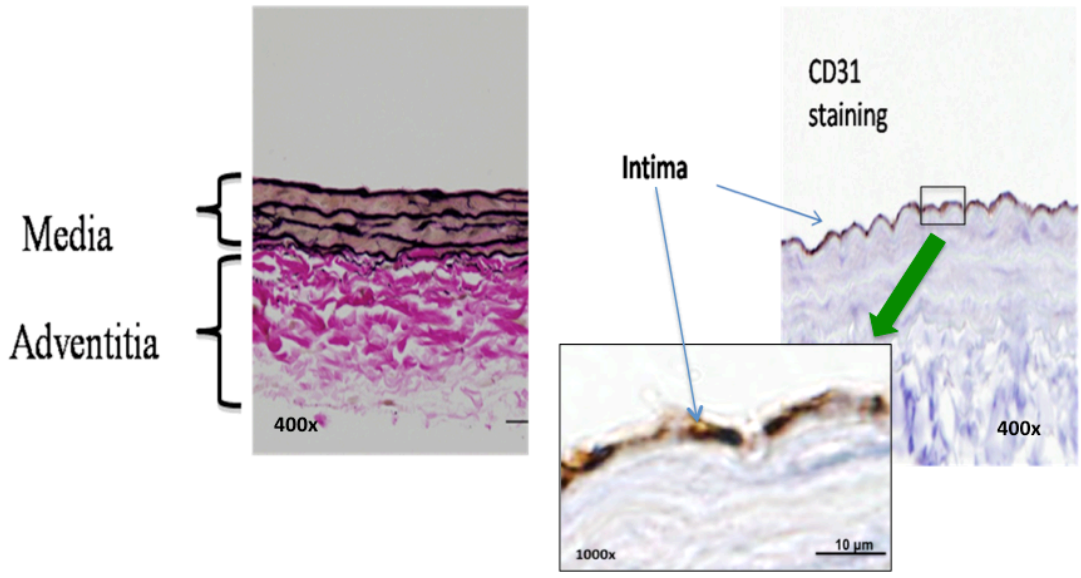


Figure 1.1 Normal rat carotid artery structure

Normal artery wall comprises intima, media and adventitia, and these three layers are separated by elastic lamina. CD31 staining shows that the intima is composed of a continuous monolayer of endothelial cells.

1.1.3.3 Atherogenesis

Atherogenesis, one kind of arteriosclerosis, is the formation and development of atheromatous plaques in the walls of medium to large arteries. Accumulation of fatty materials result in the thickening of the artery wall. Inflammatory processes play a major role in all the developmental stages of atherogenesis [12].

Inflammation is caused by endothelial dysfunction, which is associated with many high risk factors such as hypercholesterolemia, smoking, hypertension, diabetes mellitus, infection and elevated plasma levels of homocysteine, etc. [13]. Endothelial dysfunction results in the increased attraction of circulating monocytes to the inflamed area and their differentiation to macrophages and unregulated uptake of cholesterol in the arterial wall. This increases local content of inflammatory cytokines and matrix metalloproteases (MMPs) that disrupt the extracellular matrix, the elastic lamina and stimulate the activation of medial SMC. Activation of differentiated SMC in the medial compartment involves their dedifferentiation to a more synthetic phenotype capable of proliferation, migration, production of MMPs and extracellular matrix proteins. It is the hyperplasia of these cells, their movement into the intimal space and their production of extracellular matrix that further contributes to the thickening of the arterial wall that can lead to blockage and clinical consequences [14-16].

During atheroma development, changes in the artery wall can be compensated by arterial remodeling. However, as the inflammation is sustained, more and more lipids, macrophages and SMC accumulate, and focal necrosis occurs due to the death of lipid-laden macrophages. The collection of lipids and macrophages result in the formation of vulnerable plaque which consists of a thin fibrous cap, a core

that includes leukocytes, lipids and debris. T-cells produced factors such as interleukin-1 (IL-1), Fas ligand (Fas-L) and interferon gamma (IFN-γ) which are potent inducers of EC and SMC apoptosis, causing erosion and rupture of the vulnerable plaques, leading to thrombus formation [11].

1.1.3.4 Atherogenesis and coronary artery disease

Atherogenesis is a chronic, slowly progressive process and usually asymptomatic. However, when an atheroma ruptures and ulcerates, tissue fragments are released into the circulating blood, promoting an immediate blood clotting cascade at the ulcer site. This triggers a series of events causing enlargement of the clot which may obstruct blood flow acutely. If a complete blockage takes place, myocardial ischemia and damage result, leading to myocardial infarction (MI).

Associated with a non-fatal MI is the fibrous organisation of the blood clot. Fibrous tissue grows around the rupture, developing to stenosis or even closure of the artery lumen. A single plaque rupture may not cause total closure of the lumen. However, repeated ruptures and clot patches can produce more stenosis over time, developing into a persistent localised narrowing or blockage of the arterial lumen. Although the development of stenosis can be slow, atheroma ulceration is a sudden event due to instability and this is the major cause of MI.

The consequence of stenosis is ischemia. The narrowing of the arterial lumen decreases blood flow and causes failure of adequate oxygen delivery. The oxygen demand of the heart cannot be met and the tissue is in oxygen debt. Transient and even mild ischemia can cause anginal pain with electrocardiogram changes [17]. Chronic moderate ischemia weakens the contraction of the heart and develops into

myocardial hibernation [18, 19].

1.1.4 Treatment of coronary artery disease

Current treatment of CAD involves both primary prevention (before any major cardiovascular event in high-risk individuals) and secondary prevention (to prevent occurrence or recurrence of acute myocardial infarction in CAD patients). Treatment can involve risk factor management, CAD pharmacologic therapy, and revascularisation. In primary prevention, the main treatment is risk factor management which may involve management of hypertension, hyperlipidaemia and diabetes mellitus. Risk factor management can stabilize the formation of atheromatous plaque and prevent or slowly progress.

Risk factor management remains an important component of therapy in secondary prevention in combination with further CAD pharmacologic therapy, which may involve administration of anti-platelet agents such as Aspirin and Clopidogrel (Plavix) which are used to inhibit the aggregation of platelets and hence prevent thrombogenesis in the coronary artery or anti-anginalagents, such as beta blockers, calcium channel blockers and nitrates, which are administered to help reduce cardiac oxygen consumption, prevent coronary spasm, expand the coronary artery and thus relieve angina [20]. For severe CAD patients however, neither pharmacologic therapy nor risk factor management can effectively relieve symptoms and in this situation revascularization is necessary to improve blood supply to the heart, decrease patient symptoms, decrease adverse cardiac events and improve the quality of patients lives [21, 22]. At present, two strategies are adopted for revascularisation: percutaneous coronary intervention (PCI); and

coronary artery bypass grafting (CABG). Both are applied to patients who have severe heart ischemia where the symptoms cannot be relieved by therapeutic management or agents.

1.2 Percutaneous coronary intervention (PCI) therapy and coronary artery bypass grafting (CABG)

Percutaneous coronary intervention (PCI) and CABG are important treatment approaches in the management of symptomatic CAD. In Australia during 2007-2008, the rate of PCI procedures was 155 per 100,000 population while the rate of CABG procedures was 61 per 100,000 population [7].

1.2.1 Percutaneous coronary interventions

PCIs are applied to blocked coronary arteries so that adequate blood flow can be restored. Two types of procedures are currently performed. One is percutaneous transluminal coronary angioplasty (PTCA) without stent. Briefly speaking, a small balloon is inserted into the blocked area inside the coronary artery, and with the inflation of the balloon, the blockage is cleared, and then the balloon is removed. The other procedure is coronary stenting, in which the PTCA procedure is carried out first, followed by stent implantation at the narrowed or blocked site(s) in the artery. Different from PTCA, in coronary stenting, the stents are expanded and left at the once blocked area to prevent arterial recoil.

1.2.1.1 The improvement in coronary artery disease treatment brought by drug-eluting stents

The advent of percutaneous transluminal coronary angioplasty (PTCA) provided a

fundamental change in the treatment of CAD. Pioneered by Andreas Gruentzig in 1977, PTCA helped provide immediate symptomatic relief, but presented the problem of restenosis, which arose from recoil and intimal hyperplasia at the site of balloon injury. Prior to the availability of stents, restenosis occurred at a rate of 30-40% of patients at 1-2 years [23-26], and was reported to be even higher at 5 years in the Bypass Angioplasty Revascularisation Investigation [27].

Intracoronary bare-metal stents (BMS) were introduced in 1986, and served as mechanical scaffolds to prevent elastic recoil, and negative remodeling. BMS brought important advantages over balloon angioplasty alone with reduced major adverse cardiac events (MACE), such as repeated revascularisation, angiographic restenosis, and re-occlusion. However, these events were still not infrequent [25, 26, 28]. The SIRIUS trial showed 36% restenosis in the BMS group, and this was a major issue particularly in patients with diabetes, small vessels, long lesions, multiple lesions, and bifurcations.

In 2003, a further development was the introduction of the drug-eluting stents (DES) [29]. DES reduced rates of restenosis and repeated revascularisation compared with BMS [30, 31]. The safety and efficacy of DES for the treatment of CAD and the reduction of restenosis have been demonstrated in a number of trials, which include:

(1) Early trials in simple lesions, such as RAVEL (the 5-year rate of TLR, 10.3% in sirolimus-eluting stents (SES), 26% in BMS) [32], TAXUS-II (at 5 years, rates of TLR were 18.4%, 10.3%, and 4.5% for the BMS, TAXUS SR, and TAXUS MR group, respectively) [33];

(2) “Pivotal” DES approval trials in moderate complex disease, such as SIRIUS (at 5 years, in SES versus BMS patients, TLR was 9.4% versus 24.2%) [34] and TAXUS IV (at 5 years, TLR rate of 14.6% in TAXUS and 21.4% in BMS) [35];

(3) Trials with broader inclusion criteria, such as SIRTAX (compared SES and paclitaxel-eluting stents (PES), presumed SES superiority, not a multicenter study) [36] and SORT OUT II (clinical outcomes did not show significant differences between SES and PES) [37];

(4) Registry data, such as RESEARCH and T-SEARCH (compared with BMS, the use of SES and PES brought a sustained benefit in decreasing TVR at 6 years. 14% in SES and PES, 18% in BMS) [38] and SCAAR (DES vs BMS, restenosis decreased by almost 50%) [31];

(5) Key meta-analyses [39-42], which suggest that DES are safe and efficacious, and from the perspective of mortality and MI risks associated with stents, DES and BMS are similar. DES result in a marked reduction in TLR [39-42], see Table 1.1.

Table 1.1 Clinical trials relating to safety and efficacy of DES

Clinical trial	No. of patients	Follow-up period	Conclusion
(i)			
RAVEL	238 patients	5 years	SES had significantly lower TLR rate at 5 years than BMS
TAXUS-II	536 patients	5 years	The sustained efficacy of SR and MR stents was indicated by lowered rates of target-vessel and target-lesion revascularisation The long-term safety of the TAXUS stent system was supported by low rates of death, myocardial infarction, and stent thrombosis
(ii)			
SIRIUS	1,058 patients	5 years	SES significantly reduced repeat revascularisation, and had similar safety (death and myocardial infarction) to BMS in patients with non-complex CAD
TAXUS-IV	446 patients	5 years	After five years, TAXUS MR stent had similar MACE and TVR, and reduced TLR rates compared with BMS in treatment of complex coronary lesions
(iii)			
SIRTAX	1,012 patients not a multicenter study	9 months	SES was superior to PES in reducing Restenosis in Segment, LLL in Stents, TVR, MACE
SORT OUT II	2,098 patients	5 years	The randomised trial outcomes showed no significant differences between SES and PES.
(iv)			
RESEARCH And T-SEACH	1,537 patients	6 years	SES and PES had similar safety and efficacy in the treatment of coronary lesions Both DES had a sustained advantage in decreasing TVR and MACE compared with BMS at 6 years
SCAAR 2009	47,967 patients	1 to 5 years	DES had a similar long-term rate of death or MI and reduced the rate of restenosis among high-risk patients compared with BMS
(v)			
Stettler *et al*. meta-analyses **[39]**	18,023 patients	4 years	DES and BMS had similar mortality risk. SES seemed to have clinical superiority over BMS and PES
Stone *et al*. meta-analyses **[40]**	5,261 patients	4 years	SES and PES had higher rate of Stent thrombosis after 1 year than BMS Both DES significantly reduced TLR Both DES had no significant differences in the rates of death or MI at 4 years
Kastrati *et al*. meta-analyses **[41]**	4,958 patients	12.1 to 58.9 months	SES had no significant effects on overall long-term survival and survival free of MI compared with BMS SES sustainably reduced reintervention SES had no difference from BMS in the risk of stent thrombosis
Kirtane *et al*. meta-analyses **[42]**	192,371 patients	>=1 year	DES significantly reduced TVR compared with BMS in both RCTs and observational studies The safety and efficacy of DES were suggested in both on-label and off-label use In comparing DES and BMS on safety and efficacy, RCT data were different from observational data

1.2.1.2 Problems of restenosis and late thrombosis in percutaneous coronary intervention

Restenosis is the recurrence of stenosis in coronary arteries treated with PCI and primarily involves the injury-induced stimulation of medial SMC to de-differentiate and proliferate leading to intimal hyperplasia [43]. Late stent thrombosis is thrombogenesis occurring after 30 days following the implantation of DES.

Despite marked improvements in the treatment of CAD brought about by DES, significant challenges still remain. Firstly, restenosis may still occur, especially in long lesions, small coronary arteries, bifurcation lesions, multi-vessel disease, and in the setting of diabetes. Although SIRIUS showed a beneficial effect of sirolimus-eluting stents (SES) over bare-metal stents (BMS), 8-month angiographic follow-up showed an in-stent restenosis rate of 3% (versus 35% in BMS group) and in-segment restenosis rate of 9% (versus 36% in BMS group). In the overlapping stent (for long lesions) subgroup, in-stent restenosis rate was 7.1% (versus 42.7% in BMS group), while in-segment restenosis rate was 8.8% (versus 42.7% in BMS group). In the diabetes patient sub-group, in-stent restenosis rate was 8.3% (versus 48.5% in BMS group), while in-segment restenosis rate was 17.6% (versus 50.5%in BMS group). In the bifurcation lesion sub-group, the restenosis rate in the main vessel was 6–6.2%, while the branch vessel restenosis rate was 18.7-24%. In small coronary arteries (diameter < 2.75 mm) group, the restenosis rate was 14.9% (versus 39.9% in BMS group [22].

The main reason for the occurrence of restenosis after stenting is intimal hyperplasia, which primarily results from the proliferation and migration of VSMC [44]. In the early stages after stent implantation, VSMC proliferation is induced

partly in response to the acute injury, and at the late stage, VSMC proliferate around the stent struts, the foreign matter. The excessive VSMC proliferation causes immoderate intimal hyperplasia, which leads to the narrowing of the arterial lumen [45]. It has been identified that internal elastic lamina (IEL) rupture plays an important role in the initial step of VSMC proliferation from the tunica media to the intima in a rat carotid artery balloon injury model [46, 47].

The second challenge to DES remains the problem of late stent thrombosis. There is some evidence suggesting that a small but incremental risk of potential late stent thrombosis is associated with DES use [48] and is caused in part by delayed stent endothelialisation. It has been proved that the endothelium is crucial to maintain vascular health due to the anti-inflammatory and anticoagulant properties of EC [49, 50]. Late stent thrombosis correlates with altered vascular healing, which includes incomplete vessel wall stent re-endothelialisation and regional positive remodeling [48]. Consequently, continuation of dual-anti-platelet therapy is required for one or more years after DES placement [51, 52]. According to post-mortem analysis of human coronary artery stents, impaired endothelialisation is an important factor of stent thrombosis following DES implantation, and it may increase the risk beyond 30 days [53-55]. Bern-Rotterdam registry (8,146 patients) showed that after DES (SES or PES) implantation, late stent thrombosis rate was 0.4–0.6% annually up to 4 years. Incidence of late stent thrombosis was higher in PES group (0.63% annual rate) than SES group (0.44%; p=0.047) [56]. In a trial involving 12,395 patients, very late definite stent thrombosis (between 12 and 15 months after implantation) was more frequent in DES group than BMS group [57]. In a trial involving 2,557 patients, follow-up results of 2.3 years revealed that stent thrombosis occurred in 2.2% of patients with diabetes versus 1.0% of patients without diabetes [58].

Allergic reactions to the polymers adopted for DES have also been considered a possible cause of very late stent thrombosis. Cook *et al.* found increased numbers of eosinophils in coronary aspirates from patients suffering from very late stent thrombosis, particularly in SES [59]. Recent studies also suggest that in-stent neo-atherosclerosis is an important substrate for extended phase in-stent restenosis as well as late stent thrombosis [60]. Other evidence suggests that chronic inflammation and incompetent endothelial function can trigger or cause late de novo neo-atherosclerosis in implanted BMS and DES, and that may be a main mechanical cause for the late phase in-stent restenosis and thrombosis [60-62].

1.2.2 Development of drug-eluting stents

In efforts to address the clinical problems of restenosis and late thrombosis, DES have undergone improvements in the following three aspects: the stent platform, drug and polymer. Each of these aspects will be discussed in detail below.

Stent platform

The mechanical properties of DES are critical to clinical performance [63]. Strut configuration, thickness, and materials have been improved to enhance delivery while reducing damage to the vessel [64]. For example, stents designed with thinner struts are likely to endothelialise more rapidly and cause less injury to the vessel wall [65]. The ISAR-STEREO study showed a thinner-strut stent could significantly reduce angiographic and clinical restenosis after coronary artery stenting [66]. The ISAR-STEREO-2 study compared stents with thin strut and thick strut and the outcomes showed that the stent with thinner struts decreased angiographic and clinical restenosis [67]. Another example is the cobalt chromium

BMS with thinner struts. Compared with stainless steel BMS, this stent has better deliverability and causes less target lesion revascularisation [68].

Drugs

Currently, sirolimus (rapamycin) eluting stents (SES), paclitaxel-eluting stents (PES), zotarolimus-eluting stents (ZES), and everolimus-eluting stents (EES) have been approved in Europe and North America [69]. An intriguing future prospect is the possibility of new types of drugs, such as catalytic DNA molecules [70] or gene therapeutic approaches involving key transcription factors that regulate cell growth and migration, being locally delivered in a DES setting for the prevention of intimal thickening. Examples of such promising agents include DNAzymes targeting the bZIP protein c-Jun [71, 72], the zinc finger immediate-early gene product, early growth response-1 (Egr-1) [73], and the GLI-Kruppel class transcription factor yin yang-1 (YY1) [74].

Polymer

The nature of the polymer has attracted considerable interest in recent years. It provides an important platform for drug-loading and release kinetics. Several pharmacological agents and different types of polymers, permanent or biodegradable, have been employed. The most studied DES, SES (Cypher), and PES (Taxus) both use permanent polymers. Although mid-term efficacy has been proven, there still exists potential for late stent thrombosis [56, 75-78].

Polymers in DES have potential for pro-inflammatory, thrombosis, and hypersensitivity reaction [55, 59, 78-84]. To avoid these negative influences, two strategies have been utilised. One is using biodegradable polymers to store and

control the release of incorporated drugs. Via biodegradable polymer (BP), sirolimus is released gradually, and the polymer will be absent from the vessel wall, whereby the putative nidus for persistent inflammation and thrombosis is removed. The other strategy is using polymer-free (PF) DES platforms. On a PF sirolimus stent, polymer is absent, and sirolimus is coated on the stent directly [78]. Both strategies can reduce incidence of late acute stent thrombosis and the need for prolonged thienopyridine therapy (and bleeding risks) [78, 85].

1.2.3 Coronary artery bypass grafting

Coronary artery bypass grafting (CABG), commonly acknowledged as the most effective way to treat CAD, is a surgical revascularisation procedure which takes a saphenous vein graft or an internal mammary artery graft from the patient and attaches it to the coronary artery on the outside of the heart to bypass the blocked artery and improve blood supply to the heart muscle and hence ameliorate its ischemic condition.

CABG, which was first introduced in the 1960s [86-88], is presently among the most commonly performed surgical procedure in the treatment of CAD. Every year, there are approximately 1 million CABG performed in the world [89]. Even now when PCI has become popular, it is still the standard to treat patients with three-vessel or left main coronary artery disease [90, 91]. Saphenous vein grafts remain the most common type of conduits that are used for CABG. In the current standard, usually saphenous vein grafts are used to bypass the blockage in the left circumflex coronary artery and right coronary artery, while left internal mammary artery grafts are adopted for left anterior descending artery bypass. Sabik *et al.*

reported that of the 10,881 CABG surgeries performed between 1972 and 1999, 8,733 applied saphenous vein grafts and 2,121 used internal mammary arteries [92].

However, saphenous vein graft failure due to stenosis continues, which impedes the longer term success of CABG. Since CABG was introduced over 50 years ago, there remains an unacceptably high rate of vein graft failure. Sabik *et al.* reported the 78%, 65% and 57% patency of saphenous veins at 1, 5 and 10 years after surgery respectively [92]. Campeau *et al.* published angiographic data demonstrating yearly occlusion rates of saphenous vein grafts of 2.1% per year between years 1 and 5, and 5.2% between years 5 and12 [93, 94]. Fitzgibbon *et al.* showed that, after CABG, the cumulative occlusion rate was 8% at the early stage, 13% at one year, 20% at five years, 41% at 7.5 years, 41% at 10 years and 45% at more than 11.5 years [95].

The reasons for vein graft failure have been investigated. After implantation, the resident cells in the graft are exposed to a new microenvironment, with a static-to-shear insult, increased shear stress, pulsatile stress, circumferential stress and radial deformation [96]. This can lead to the hyperplasia of SMC in the intima and accelerated atherosclerosis after 1 month of CABG. Firstly, from 1 to 12 months after CABG, the graft has to adapt to higher arterial pressures, and the endothelial layer becomes damaged, SMC from the medial layer eventually accumulate in the intima and proliferate causing intimal hyperplasia [97]. Secondly, after 12 months of surgery, accelerated atherosclerosis can cause graft stenosis and occlusion [97]. Vein graft atheromas, in contrast to native coronary atheromas, are diffuse, concentric, with little calcification and poorly developed or absent fibrous caps [98]

and can result in frequent thrombosis which can block the grafts [99].

1.2.4 Cellular and molecular mechanisms of neointimal formation after PCI and CABG

As mentioned previously, the artery wall is composed of the tunica intima, tunica media and tunica adventitia. The intima is made of a continuous monolayer of endothelial cells (EC) resting on a basement membrane. This EC layer constructs a permeable barrier between blood and media, and also plays an important role in vascular functions [100]. The medial layer comprises several layers of smooth muscle cells (SMC) which are inlayed in extracellular matrix (ECM). Under normal conditions, SMC can regulate the vessel diameter by contraction and relaxation, but in some physiological cases, factors such as post-vascular injury, pregnancy and exercise, trigger SMC to de-differentiate from a contractile to a synthetic phenotype affecting vascular remodeling via enhancing proliferation and migration as well as synthesising growth factors, proteases and ECM components [101].

1.2.4.1 Mechanisms of VSMC proliferation and migration

Pathologically, restenosis after PCI and vein graft failure after CABG are primarily characterized by the proliferation and migration of these activated medial SMC [44, 97]. The responses of SMC to therapeutic vascular injury and hemodynamic stress include phenotypic switching, migration and proliferation. SMC transform from a contractile phenotype to synthetic phenotype which has a high protein synthesis, proliferation and migratory activity, and this phenotypic switching is induced by basic fibroblast growth factor (bFGF) and platelet derived growth factor-BB (PDGF-BB) [102]. SMC proliferation and migration in the vascular wall are stimulated by growth factors such as bFGF and PDGF which stimulate SMC signal transduction

cascades such as the Ras mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)- Akt pathways to enhance SMC proliferation, and the Rho kinase family to promote SMC migration [103]. The growth factors activate protein-tyrosine kinase (PTK) receptors and cause receptor phosphorylation and subsequent binding with adaptor proteins which results in a series of activations of Ras, Raf MAPK kinase (MAPKK)/MEK and p44 MAPK/p42 MAPK (ERK1/ERK2) [104]. Activated MAPK enters the nucleus to induce transcription of growth-promoting genes to regulate SMC proliferation, migration and phenotypic switching. The phosphorylated PTK receptors also activate PI3K which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 binds with Akt to anchor Akt to the cell membrane where Akt is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK1). Phosphorylated Akt then activates rapamycin (mTOR)-raptor which can induce transcription of growth-promoting genes and lead to the survival, proliferation and cycling of the cell [104].

In EC, vascular endothelial growth factor (VEGF) binds to its tyrosine kinase receptor leading to activation of PI3K-Akt. The activated Akt phosphorylates eNOS, enhancing its activity and increasing the production of nitric oxide (NO) [105]. NO inhibits vascular SMC proliferation and literature indicates this effect is related to NO causing cell cycle arrest [106]. NO results in an immediate cGMP-independent S-phase block in the cell cycle, and leads to a shift back from the G1-S boundary to a quiescent G0-like state [106].

Injury to the vessel wall typically causes endothelial denudation or dysfunction [47, 96, 107], which results in decreased NO production. The injured endothelia release

fibroblast growth factor 2 (FGF-2) that binds to its tyrosine kinase receptor on the cell surface of vascular SMC and activates the ERK and JNK kinase signaling cascades [108]. These kinases activate transcription factors such as early growth response protein 1 (Egr-1) and activating protein-1 (AP-1) which promote the release of growth factors such as PDGF [108]. These then act in a paracrine and autocrine fashion to further stimulate the dedifferentiation of vascular SMC and their proliferation and migration. Simultaneously, injury and the loss of endothelium lead to the deposition of platelets which also release PDGF and other mitogenic factors. These growth factors also penetrate the vascular wall, bind to their receptors and trigger the proliferation and migration of medial VSMC [107].

c-Jun plays an important role in this progress as it is an important subunit of the transcription factor AP-1 and is necessary for G1 phase progression of the cell cycle [107]. c-jun regulates cyclin D1 transcription and is important for the maintenance of cyclin D1 kinase activity [109]. It has been identified that overexpression of c-Jun can reduce p53 and p21 level. As p53 is a cell cycle arrest inducer and p21 is a cyclin-dependent kinase (CDK) inhibitor, c-Jun overexpression in cells consequently enhances cell proliferation and migration [110].

The immediate-early gene Egr-1 plays a crucial role in the proliferation and migration of VSMC induced by mechanical injury. The Egr-1 protein works as a master regulator in this process via the control of an array of downstream genes by initiating transcription at promoters containing specific recognition elements. It can regulate a cascade of downstream genes involved in proliferation and migration [108, 111]. It has been definitively proven that there is a causal association between Egr-1 and PDGF expression, i.e. PDGF and FGF can stimulate

expression of Egr-1 and the increased Egr-1 can activate PDGF or FGF transcription [108].

The activation of vascular SMC signal transduction pathways also induces cytoskeletal remodeling which causes changes in VSMC adhesiveness to the matrix, activates motor proteins, and consequently results in the migration of SMC [107]. Previous studies have shown that a variety of integrins are present on the cell surface of SMC and that they are responsible for adhesion to the ECM as well as cell movement regulation [112-114]. It has been shown that cyclic RGD peptides can greatly suppress the PDGF-directed VSMC migration via binding to integrins αvβ3 and αvβ5 [112]. The migration of VSMC is also associated with the cell cycle and proliferation. To be specific, VSMC migration only occurs in G1 phase of the cell cycle. As negative regulators of the cell cycle, CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$can also significantly inhibit VSMC migration. It has been indicated that $p27^{Kip1}$ can decrease lamellipodia formation and actin filament reorganisation, as well as focal adhesions. These cellular changes are related to the migration of VSMC [107].

1.2.4.2 VSMC proliferation and migration and diabetes

Many randomised clinical trials have shown that DES markedly reduced rates of restenosis compared with BMS [115]. Nevertheless, despite the use of DES, diabetic patients have a higher rate of restenosis after PCI treatment compared with patients without diabetes [116, 117]. Further, clinical trials also indicate more frequent vein graft failure in diabetics after CABG [117]. It is becoming clear that restenosis after PCI and vein graft failure after CABG associated with diabetes is multifactorial. Diabetes increases VSMC migration and proliferation after PCI, to

which there are several factors associated, such as high glucose, advanced glycation end-products (AGEs), non-esterified fatty acids, and leptin [118].

Although there is no direct evidence showing hyperglycemia directly stimulates VSMC proliferation and migration in diabetes, some studies have indicated that high glucose enhances the expression of growth factors and their receptors in VSMC, such as vascular endothelial growth factor (VEGF), transforming growth factor-ß (TGF-ß), FGF-2 and PDGF-β receptor, which can simulate VSMC proliferation and migration [118-121]. Similarly, it has been shown that hyperglycemia increases the formation of AGEs, which can increase expression of VSMC growth factors, such as PDGF and Insulin-like growth factor (IGF-I), in monocytes [118, 122, 123].

Diabetes is associated with elevation of circulating non-esterified fatty acids, which can enter the arterial wall and are crucial in inducing and stimulating the proliferation and migration of VSMC [118]. This stimulation is mediated by protein kinase C (PKC), mitogen-activated protein kinase (MAPK/ERK), increased reactive oxygen species (ROS) formation, and lipoxygenase-bioactivated metabolites [124, 125]. Diabetes often coexists with increased plasma leptin levels. It has been shown that leptin promotes VSMC proliferation and migration and hence increases neointimal hyperplasia in a PI3K- dependent fashion via activating the mammalian target of rapamycin (mTOR) signaling pathway [126].

1.2.5 Animal models for investigating therapeutic treatments for CAD

1.2.5.1 Rat carotid artery balloon injury model

The rat carotid artery balloon injury model, first described by Clowes in 1983 [127,

128], is commonly used for investigating the arterial response to acute experimental injury. In the model, a balloon catheter is used to make mechanical damage to the vessel lining, which completely removes the intimal endothelial layer and hence induces reproducible remodeling. This balloon injury in the rat model imitates the injury to the coronary artery by balloon in human PCI. The response to the injury mainly involves endothelial denudation, VSMC migration, proliferation and apoptosis, promotion of matrix synthesis, and formation of an invasive neointima. Therefore, this rat model is valuable for the investigation of restenosis and re-endothelialisation in PCI.

Fully-grown male Sprague-Dawley rats are the most commonly used in this model as fluctuating hormone levels in female animals can influence cellular function(s). Briefly, the operation respectively isolates a segment of carotid artery and femoral artery in an anaesthetised rat, makes an arteriotomy incision in the femoral artery so that a 2F balloon catheter can be inserted and advanced through to the left common carotid artery. The balloon is then repeatedly inflated and withdrawn through the left carotid to remove the EC lining and elicit mural distension, which is followed by the removal of the catheter and tying off of the femoral artery. For the measurement of cellular and molecular changes as well as neointima formation, relevant histological studies can be carried out.

It should be noted that neointima formation is proportional to the degree of artery injury in this model. Indolfi *et al.* evaluated the artery injury at 0, 0.5, 1.0, 1.5 and 2.0 atm and demonstrated that VSMC proliferation is related to balloon inflation pressure [129].

Although the rat carotid artery balloon injury models itself on the human PTCA injury, there are limitations of using this model. The arteries used in the rat model are normal without pre-existing atherosclerosis lesions. Yet in the clinical practice, balloon angioplasty is applied to diseased arteries in humans. This difference is the main limitation of the animal model as it influences the different arterial responses to balloon injury. In the rat model, the response to injury involves primarily the migration and proliferation of VSMC. In diseased human arteries, however, the situation is more complex, involving interactions between VSMC, EC, macrophages, and T-cells [130]. Drawn from our group practice, wound infection and pneumonia are the main post-operation complications in this model. These inflammations may affect the neointima formation. Further, from the perspective of anatomy, compared with human arteries, the arteries chosen in the rat model have less medial wall elastin, with a condensed subintimal layer, but without the presence of vasa vasorum [131]. Besides, this rat model may be unsuitable to evaluate the effect of novel stents due to the small size of arteries [132].

In spite of these limitations, the rat carotid artery balloon injury model has been, and remains, a valuable model approach to investigate the mechanisms involved in the injury response and the potential of novel therapeutics to attenuate disease processes.

1.2.5.2 Venous bypass graft model

Vein grafts have been widely used to bypass the blocked arteries, but they themselves may fail due to thrombosis and intimal hyperplasia after surgery. When autogenous veins are used as grafts to bypass the blockages in the arteries, they are constantly exposed to arterial pressure and flow, undergo increased static and

pulsatile deformations and stresses, as well as altered shear stress at the interface of blood and intima, and hence subject themselves to intimal hyperplasia and medial thickening [96].

Animal venous bypass graft models are important tools to investigate the potential of novel therapeutics to prevent graft failure. These experiments have been implemented in various animals such as mice [133], rats [134], rabbits [135], pigs [136], sheep [137], dogs [96] and monkeys [138].

In such investigations, large animals are preferred over small animals as they have similar vascular anatomy and haemodynamics to that found in humans [139], and sufficient tissue can be collected for analyses and used for evaluation of surgical and anaesthetic techniques for further improvement. Cost is a main disadvantage of using large animals, as dedicated surgical areas and instruments are required and the whole surgical process needs more than one person to collaborate and perform. Another disadvantage is the greater amount of test drugs required if administered systemically. To solve this issue, local delivery techniques can be used to reduce the drug doses required, as the vein grafts can be incubated with drugs before grafting to the artery [140].

The rabbit autologous vein bypass graft model was introduced by Andrew Murday in 1983 [141]. The model usually removes the external jugular veins and implants them into the exposed carotid arteries as grafts [142-145]. After halting blood flow, the jugular vein orientation is reversed and anastomosed into the artery end-to-end or end-to-side utilising running sutures.

After the autologous transplantation in the rabbit vein bypass graft model, increasing thickening occurs to the vein graft commencing around 7 days and this continues for at least 6 months and can lead to graft stenosis.[144, 145] The formation of this neointima is the result of SMC migration and proliferation as well as the synthesis of quantities of collagen produced by these SMC [143]. Accompanying this process is the expansive remodelling [145, 146]. The changes in the vein grafts of this model are similar to those in human vessels after CABG, therefore, the rabbit model has been used to study the risk factors for vein graft failure, including hypertension, dyslipidemia, and diabetes [139, 145].

The rabbit vein bypass graft model has been found to have significant advantages in studying the process of intimal hyperplasia. Compared with some other large animal models, the rabbit model is cheaper, with low mortality and high graft patency rate. Moreover, rabbit autologous vein grafts can be used to compare with human coronary vein grafts from the perspective of histological changes [141, 147-149].

1.3 Investigating novel biomolecules as therapeutic approaches to understand and inhibit SMC hyperplasia and intimal thickening

1.3.1 Overview

Drug-eluting stents have been widely used for CAD treatment, restenosis and late stent thrombosis still present challenges. The key to solving these problems is to inhibit SMC proliferation and migration whilst enhancing re-endothelialisation.

Coronary artery bypass grafting (CABG) is one of the most commonly used heart surgical procedures for the treatment of ischemic heart disease. Saphenous vein

grafts have been the most common type of conduit for CABG. However, the failure of saphenous vein grafts caused by stenosis impedes the longer-term success of CABG. A key cellular event in the process of vein graft stenosis is SMC hyperplasia.

In this study, three approaches have been investigated to inhibit VSMC hyperplasia and intimal thickening. Firstly, a novel "cocktail", which consists of a combination of VEGF-A, VEGF-D and cRGD, was tested in the rat carotid balloon injury model. Secondly, the efficacy of the DNAzyme, Dz13, targeting c-Jun was examined in the rabbit vein graft model. Thirdly, the anti-restenotic potential of miR-191, a natural microRNA inhibitor of the immediate early gene Egr-1, was examined in the balloon-injured rat carotid artery model. The following provides some background in relation to these molecules and their targets.

1.3.2 A novel "cocktail" of VEGF-A, VEGF-D and cRGD.

The novel "cocktail" tested in this study with respect to anti-restenotic potential consists of a combination of VEGF-A, VEGF-D and cRGD. The literature suggests advantageous properties of each component that together may provide greater anti-restenotic benefit than when administered alone.

1.3.2.1 Two isoforms of vascular endothelial growth factor (VEGF)- VEGF-A and VEGF-D

VEGF-A and VEGF-D are members of a family of angiogenic growth factors, which includes VEGF-A, -B, -C, -D, -E, -F and PlGF (placenta growth factor) [150, 151]. They stimulate endothelial cell proliferation, accelerate repair and have been shown to promote mobilisation and differentiation of endothelial progenitor cells

(EPC) [152-154]. VEGF-A binds two tyrosine kinase receptors (VEGFR-1 and VEGFR-2). VEGF-D binds to VEGFR-2 and VEGFR-3. These receptors are expressed almost exclusively on endothelial cells [155].

VEGF-A

VEGF-A is also called vascular permeability factor (VPF). It is a basic mediator of angiogenesis, such as embryogenesis, corpus luteum formation, tumor growth and wound healing as well as cardiac compensatory angiogenesis [156, 157]. It is a crucial molecule eliciting EC proliferation, migration and tube formation [157]. VEGF-A has five known isoforms: VEGF-A-121, VEGF-A-145, VEGF-A-165, VEGF-A-189, and VEGF-A-206. Only VEGF-A-121, VEGF-A-145, and VEGF-A-165 are easily diffusible and their biological effects on EC have been identified [150].

VEGF-A has been shown to promote endothelial cell (EC) proliferation and migration [158, 159]. It is necessary for EC survival and to enhance EC growth through inducing the expression of anti-apoptotic proteins Bcl-2 and A1 in EC, typically associated with the activation of the phosphatidylinositol-3 kinase (PI-3-K) signaling and Akt pathways [160]. In EC, VEGF-A can also induce the expression of endothelial nitric oxide synthase (eNOS) and promote the production of NO, a key regulator of vasodilatation and vascular homeostasis [161]. VEGF-A has effects not only on EC but also on EPC. Iwaguro *et al.* indicated that VEGF-A enhanced EPC proliferation *in vitro* and *in vivo* [162]. VEGF-A has also been used as a stent coating. Recently, Yang *et al.* reported that VEGF-A plasmid-coated stents enhanced early re-endothelialisation through release of the VEGF-A gene [163]. Wang *et al*. demonstrated that VEGF-A-coated stents promoted EC

proliferation and re-endothelialisation [164].

VEGF-D

VEGF-D is a secreted glycoprotein [165]. It is also known as c-fos-induced growth factor (FIGF) and binds to VEGFR-2 and VEGFR-3 in human cells but activates only VEGFR-3 in mice [166].

VEGF-D is evident in developing human atherosclerotic lesions, however, in complicated lesions, VEGF-D levels are decreased in an immature form [167]. VEGF-D has also been identified in promoting angiogenesis *in vitro* and *in vivo*, enhancing EC mitogenesis *in vitro* and lymph-angiogenesis in tumors [168-170]. The latter study, which delivered VEGF-D gene via adenovirus into the rabbit hind limb skeletal muscles, indicated that VEGF-D had the strongest angiogenic and lymph-angiogenic effects in the VEGF family [171]. As with VEGF-A, VEGF-D can also induce NO production from EC [171, 172]. Local VEGF-D gene transfer was evaluated in a rabbit balloon denudation model in which adenoviral-delivered VEGF-D decreased neointima formation via NO-dependent mechanisms [173]. VEGF-D gene transfer has also been previously used to prevent graft-vein stenosis in dialysis patients [174]. VEGF-D gene was transfected via an adenoviral vector, and the delivery was achieved by locally placing it around the adventitial surface of a graft-vein anastomosis.

1.3.2.2 Cyclic Arg-Gly-Asp peptide (cRGD)

The RGD sequence (Arg-Gly-Asp) is a basic unit of the cellular recognition system in the extracellular matrix (ECM). The ECM is constituted by an insoluble network of proteins and carbohydrates. The proteins containing an Arg-Gly-Asp (RGD)

sequence, originally found in fibronectin in 1984 [175], along with their receptors (integrins), construct the cell adhesion recognition system. The RGD sequence is recognised by integrins including αvß3, α5ß1, αvß1, αvß5 and αIIßIII, a family of cell-surface transmembrane proteins which function as receptors to ECM proteins [176]. Integrin-based cell adhesions are integral to transmission of mechanical stimuli between cells and the ECM and affect proliferation, differentiation and migration [177-180]. The integrins mediate cell attachment to the ECM and are also involved in specialised interactions between cells [181]. In the interaction between the ECM proteins and integrins, the RGD sequence plays a very important role, such as providing cells with signals for polarity, position, migration, differentiation and growth [175, 182].

Structurally, all integrins consist of two subunits, α and ß [183, 184]. There are sixteen distinct α subunits and eight ß subunits in the integrin family. The α-subunits are each non-covalently combined with a ß-subunit. It has been identified that there are 24 different types of integrins in humans via different combinations of subunits [180, 181, 185]. An individual integrin can recognise several distinct ECM matrix proteins and each ECM protein can be recognised by more than one integrin. Only a subset of integrins (8 out of 24) that have an additional structural domain are capable of recognising the sequence of RGD in the ligands involving an acidic amino acid in the interaction sites [181].

RGD adhesive peptides have different effects when being applied in different ways. When RGD is attached to a surface, it can enhance cell attachment, however, when it is used in solution, it will prevent the cell attachment [186]. RGD-based peptides have been used to coat various artificial surfaces so that tissue

compatibility can be improved. Meanwhile, soluble RGD peptides are promising drugs to treat many diseases through binding to individual integrins [187, 188]. Insoluble RGD application, RGD peptides, or compounds mimicking RGD, have acted as anti-thrombotics via suppressing the function of αIIbß3 integrin [175]. The use of RGD peptides and anti-integrin antibodies can inhibit tumor growth through suppression of angiogenesis [175].

RGD plays an important role in re-endothelialisation after interventional therapy. To inhibit neointima formation after PCI, a complete and functional endothelium is required [189] and it has been proved that endothelial progenitor cells (EPC) contribute to re-endothelialisation after vascular injury [190, 191]. The studies on hematopoietic stem cells have indicated that integrins have a critical role in the re-endothelialisation process [192] and it has also been identified that integrins and integrin-binding cRGD are expressed on EPC [190, 192]. cRGD is cyclic Arg-Gly-Asp peptide that can enhance endothelialisation via attracting EPC and stimulating EPC differentiation [193]. cRGD selectively binds to avß3 which is expressed on growth factor-activated EC [194] and promotes endothelial migration to VEGF [195]. The cRGD peptide, with its hydrophilic characteristics, has higher affinity to the αvß3-integrin receptor. Use of a cRGD-coated stent has previously demonstrated an ability to decrease intimal hyperplasia through promoting the clustering of circulating EPC and endothelialisation [193].

1.3.3 c-Jun-targeting DNAzyme (Dz13)

DNAzymes are synthetic, single-stranded DNA-based catalysts that are designed to cleave the target messenger RNA (mRNA) sequence. They follow the Watson-

Crick base-pairing rules, binding to the complementary sequence in the target mRNA and cleave the mRNA as predetermined [196, 197] **(Figure 1.2).**

The potential for the therapeutic application of DNAzymes is limited by its ability to be delivered to the correct tissue [198]. Thus delivery modality is very important to obtain high efficiency with low non-specific toxicity. The function of the delivery methods is to carry DNAzymes into the organs, tissue and cells to bind and cleave the target mRNA. Appropriate delivery methods for DNAzyme can improve the pharmacokinetics and pharmacodynamics of DNAzyme. DNAzymes have previously been delivered by electroporation and vectors [199, 200]. There are two classifications in vector-based delivery, one is viral, e.g. retrovirus, adenovirus, the other is non-viral, e.g. cationic liposomal formulations. Our group clinical trials have utilised cationic liposomal formulations to deliver DNAzymes. As a versatile DNA delivery means, liposomes are non-immunogenic in nature, low cost and easy to produce [201].

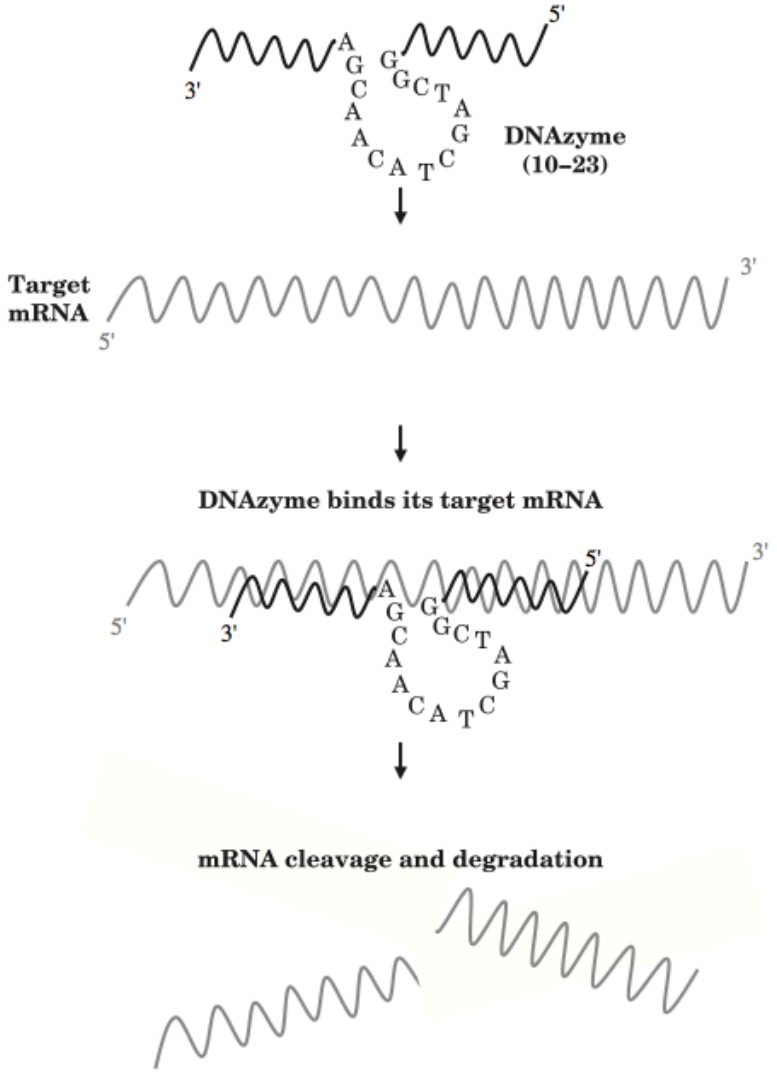


Figure 1.2 Simple schematic of DNAzyme binding to target mRNA and cleaving its sequence. (The figure extracted from **Internal Medicine Journal,** ***2009. 39(4): p. 249-51)***

DNAzymes, single- stranded DNA molecules, bind to their complementary sequence in the target mRNA and cleave the link between an unpaired purine (A or G) and a paired pyrimidine (C or U) in the RNA by the cation-dependent domain of the DNAzyme [197]

Pathogenically, neo-atherosclerosis and restenosis after PCI are initiated by the mechanical injury to cells in the wall of the artery, which induces the signaling and transcriptional pathways in VSMC and chronic inflammation in the artery wall, leading to VSMC migration and proliferation as well as *de novo* atherosclerosis, and resulting in intimal hyperplasia and thrombosis [60]. Animal models have indicated that arterial injury also induces c-Jun expression transiently [71]. Our group has previously proven that c-Jun plays an important role in initiating the injury-induced transcription which controls SMC proliferation and migration and promotes intimal hyperplasia [148]. Dz13 is a DNAzyme targeting c-Jun a 39-kDa basic-region leucine zipper protein belonging to the activating protein-1 (AP1) family of transcription factors [202]. Dz13 was shown to suppress c-Jun expression in cells *in vitro* and *in vivo*, thereby blocking the growth of VSMC, abrogating VSMC repair after scraping injury, and inhibiting intimal thickening in injured carotid arteries [71]. Previous study of our group has demonstrated using the rat balloon injury model that Dz13 targeting c-Jun suppresses intimal thickening in injured carotid arteries [72]. It was also demonstrated by our group that Dz13 (transfected with FuGENE6, a proprietary multi-component lipid-based transfection agent that cannot be used in humans) suppressed intimal hyperplasia in human saphenous veins and vein grafts after autologous end-to-side transplantation in rabbits [148]. Moreover, it has been shown that Dz13 inhibits inflammation by targeting c-Jun [203]. As discussed before, inflammation plays an important role in atherosclerosis progression. Thus Dz13 has the potential to prevent neo-atherosclerosis after revascularisation therapy. In this thesis the efficacy of Dz13 was investigated in the rabbit vein graft bypass model using a delivery approach involving the transfection agent 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and this delivery vehicle has been approved for use in humans.

1.3.4 MicroRNAs (miRs)

Another approach investigated in this work was the use of an early growth response-1 (Egr-1)-targeting miR-191. The rat carotid artery balloon injury model was used to examine the effects of miR-191 on intimal thickening after balloon injury on rat arteries. The reason of using the rat balloon injury model in the miR-191 study is that this model represents a fairly simple form of restenosis encompassing the basic arterial response to injury which primarily involves the migration and proliferation of VSMC.

MicroRNAs (miRs) are endogenous, noncoding, single-stranded RNAs which consist of 18–22 nucleotides and act as gene regulators. miRs can directly degrade or suppress the translation of messenger RNA (mRNA) by adhering to their target genes in 3'-untranslated regions (3'-UTRs) [204]. miRs are involved and play a crucial role in a variety of biological processes [205]. Recent studies have shown that many pathological progresses are related to the expression of miRs, which includes inflammatory pathologies and a range of cardiovascular diseases [206]. It has recently been determined that miRs can be transferred through gap junctions or secretion between cells [207-209].

1.3.4.1 The biogenesis of microRNA

Most of miR genes are mapped in intronic regions. The biogenesis of microRNA involves three stages: primary miR, precursor miR and mature miR, as shown in **Figure 1.3**. In the nucleus, miR genes are transcribed by RNA polymerase II (pol II) or RNA polymerase III (pol III) [210, 211] into primary miR (pri-miRs) which are

structured with caps and polyadenylated (poly A) tails [212]. The pri-miR is then endonucleolytically cleaved by the RNase III enzyme Drosha (RNASEN) and DiGeorge critical region 8 (DGCR8) - formed nuclear microprocessor complex and generates precursor-miR (pre-miR) which has a hairpin loop structure [213-215]. Following transcription, the complex formed by Exportin-5 (XPO5) and Ran-GTP cofactor exports pre-miR out of the nucleus into the cytoplasm [216], where the pre-miR hairpin loop is cleaved off by the RISC complex formed by RNase III Dicer, Argonaute 2 (Ago2) and Tar RNA binding protein (TRBP). This modulation process transforms the pre-miR into a miR duplex, which contains a guide strand (mature miR) and a passenger strand (miR*) [217-219]. The guide strand is then incorporated into miRISC, a miR-induced silencing complex containing Dicer and related proteins [220], meanwhile, the passenger strand miR* is released, followed by rapid degradation. The miR, included in the miRISC, binds to the sequence of the target mRNA by base-pairing rules causing translational repression or direct mRNA degradation [221, 222].

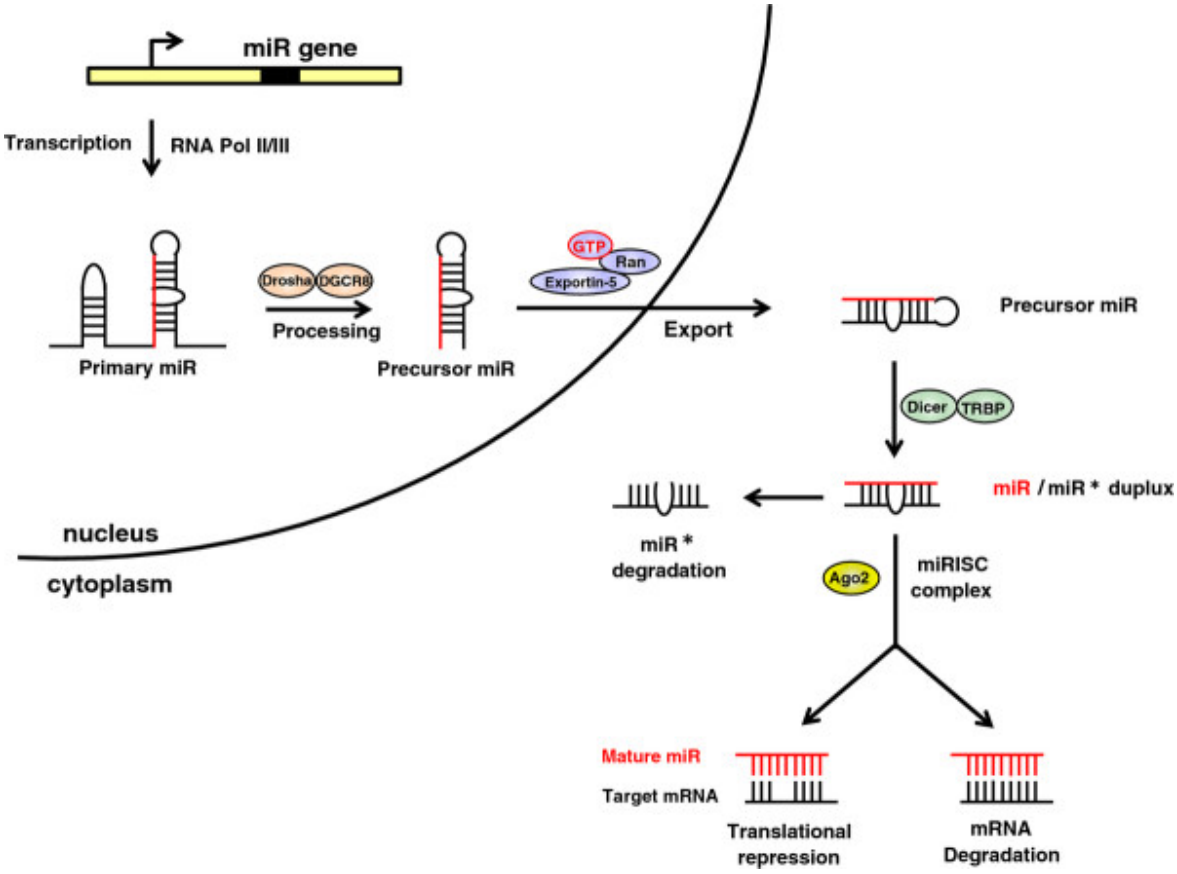


Figure 1.3 The canonical pathway of miR processing. (The figure extracted from* Journal of Biomedical Science*, 2012. 19(1): p. 79)

miR genes are transcribed by pol II or III and generate primary miR (pri-miR) in the nucleus, which is then processed by the microprocessor complex (Drosha-DGCR8) and formed into pre-miR with a ~60-100-nucleotide hairpin structure. Exportin-5-Ran-GTP exported the pre-miR out of the nucleus into the cytoplasm, where the RNase III Dicer and TRBP cleave the hairpin loop off the pre-miR and transform it into ~22-nucleotide miR/miR duplex. The passenger strand (miR*) is released and then degraded quickly, while the guide strand (mature miR) is incorporated into a miRISC and binds to the sequence of its target mRNAs base-pairing and induces mRNA degradation or translational repression [204].*

1.3.4.2 Known roles of microRNA in atherosclerosis

MicroRNAs are crucial regulators in the cardiovascular system. Abnormal expression of microRNAs has been shown in development of atherosclerosis [223].

MicroRNAs in endothelial cells

Blood vessels constantly undergo various hemodynamic forces, which have prominent effects on the maturity of the vessels. The monolayer vascular EC are constantly subjected to fluid shear stress of the blood flow, and their dysfunction induces the initial step of atherosclerosis. Many studies have demonstrated participation of microRNA in the regulation of vascular EC function. Goretti *et al*. and Zampetaki *et al.* demonstrated a role of miR-126 in maintaining vascular EC function and miR-16 can promote the capacity of EPC to repair injured tissues [224, 225]. It has been identified that oscillatory shear stress (OSS) stimulates miR-21 expression in EC and causes an inflammatory response [226], and pulsatile shear stress (PSS) decreases miR-92a expression in EC and results in EC dysfunction [227]. Sun *et al.* indicated that miR-181b can inhibit EC inflammatory responses [228]. Caporali *et al.* reported that deregulation of miR-503 contributes to diabetes-induced impairment of endothelial function [229]. MiR-663 [230], miR-19a and miR-23b [231, 232] have all demonstrated that they play a role in regulating EC inflammation and proliferation [204].

miRNAs regulate VSMC

Vascular smooth muscle cell (VSMC) proliferation and migration play an important role in the development of atherosclerosis. Wang *et al.* reported that the inhibition of miR-21 significantly reduced VSMC proliferation and migration [233]. Sun *et al.* showed miR-146a promoted VSMC proliferation *in vitro* and neointima formation

[234]. Huang *et al.* demonstrated miR-10a was a regulator in SMC differentiation [235]. Recent studies have proven the role of miR-143/miR-145 in VSMC differentiation and vascular diseases [236-240]. Elia *et al.* revealed that, in the aorta of apolipoprotein E (ApoE) knockout mice, miR-143 and miR-145 levels were downregulated, and hence suggested that miR-143 and miR-145 were critical in VSMC differentiation and vascular disease [239]. Studies have indicated that miR-26a [241], miR-1/133 [242, 243], miR-24 [244] and miR-208 [245] play important roles in controlling VSMC during atherosclerosis.

MicroRNAs in atherosclerotic plaques

Raitoharju *et al*. [246] firstly, indicated that in human atherosclerotic plaques, miR-21, -34a, -146a, -146b-5p, and −210 were significantly expressed, which induced the down-regulation of numerous predicted targets. Santovito *et al.* demonstrated miR-145 expression was upregulated in human atherosclerotic plaques [247]. In atherosclerotic pathogenesis, miR-34 can cause apoptosis and cell cycle arrest and target at the genes associated with VSMC proliferation and cholesterol metabolism [204].

1.3.4.3 Known roles of microRNA in restenosis

Restenosis in coronary arteries after PCI is caused by the medial SMC de-differentiation and proliferation leading to neointimal hyperplasia. Many miRNAs are involved in this process of vascular remodeling, Ji *et al.* [248] firstly, demonstrated miR expression in the rat balloon injured carotid artery, and revealed a significant overexpression of miR-21 in the neointima. Lin *et al*. identified that miR-21 played a role in enhancing VSMC proliferation and anti-apoptosis [249].

Laconetti *et al.* displayed with the rat carotid artery balloon injury model that inhibition of miR-92a can improve endothelial regeneration and reduce neointima formation after vascular injury [250]. Liu *et al.* [251] identified increased miR-221 and miR-222 expression and decreased expression of target genes in balloon-injured rat carotid arteries. When expression of miR-221 and miR-222 were downregulated, VSMC proliferation and neointimal growth decreased in the rat carotid artery after angioplasty.

Recently, it has been shown by microarray studies that miR-191 is downregulated after vascular injury [248]. This miR has been linked to the regulation of Egr-1 expression in the context of breast cancer and prion disease [252, 253]. Egr-1 is an immediate early gene that is known to be poorly expressed in healthy vessels but is rapidly upregulated in response to mechanical or physiological stress of the vascular wall [254-257] and hence demonstrates a reciprocal relationship with the expression of miR-191 in this context. Egr-1 is known to regulate a cascade of downstream genes involved in proliferation and migration and has been shown to have a role in intimal hyperplasia [256, 258, 259]. As yet, a direct ability of miR-191 to alter Egr-1 expression *in vivo* and to influence restenosis has not been examined.

1.3.5 Thesis aims

As discussed, coronary artery disease (CAD), underpinned by atherosclerosis, remains a leading cause of morbidity and mortality, particularly in the Western World. Although the advent of percutaneoustransluminal coronary angioplasty (PTCA) provided a fundamental change in the treatment of CAD and drug-eluting

stents (DES) have brought about marked improvement, restenosis and late stent thrombosis remain problematic. Coronary Artery Bypass Grafting (CABG) has been acknowledged as the most effective way to treat CAD, however, saphenous vein graft failures are still a problem due to stenosis and impede the longer term success of CABG. The pathogenesis of these problems has been identified and demonstrated by many studies. Vascular smooth muscle cells (VSMC) proliferation and migration is the primary driver of restenosis after PCI and vein graft failure after CABG. Endothelial dysfunction also plays an important role in both restenosis and late thrombosis following PCI. Therefore, the key to prevent restenosis is to suppress SMC proliferation and migration, and in order to solve the problem of late thrombosis, it is of great significance to enhance re-endothelialisation. The broad aim of work in this thesis is to seek more effective strategies to inhibit SMC hyperplasia and intimal thickening while promoting re-endothelialisation. To this end, we have deployed the rat carotid artery balloon injury model and rabbit artery bypass grafting model to investigate the effects of various interventions on reducing SMC proliferation and migration as well as enhancing re-endothelialisation, hoping this could bring about an improvement to current therapeutics.

More specifically, the effects of three kinds of bio-molecules on prevention of restenosis are investigated in this thesis. Firstly, a novel “cocktail” consisting of a combination of VEGF-A, VEGF-D and cRGD, which has not been studied on SMC and EC in response to injury, is tested in the rat carotid balloon injury model. Secondly, the efficacy of the DNAzyme, Dz13, targeting c-Jun is tested in the rabbit autologous vein bypass graft model using the lipid-based transfection agent 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) / 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Lastly, the anti-restenotic potential of miR-191, a

natural microRNA inhibitor of the immediate early gene Egr-1, is examined in the balloon-injured rat carotid artery model.

In particular, this thesis is to test the following hypotheses:

A. Whether a novel cocktail of VEGF-A, VEGF-D and cRGD can inhibit intimal thickening and accelerate re-endothelialisation after vascular injury.

1. Whether the cocktail can stimulate EC and EPC growth, inhibit SMC proliferation *in vitro*.
2. Whether the cocktail can prevent intimal thickening, and accelerate re-endothelialisation *in vivo*.

B. Whether NO plays an important role in the mechanism mediating the effects of the cocktail.

1. Whether the cocktail can increase NOS3 expression after injury *in vitro.*
2. Whether the cocktail can increase NOS3 expression after injury *in vivo.*
3. Whether the NOS3 inhibitor nitro-L-arginine methyl ester (L-NAME) can prevent cocktail-mediated effects *in vitro* and *in vivo*.

C. Whether Dz13 targeting c-Jun in a DOTAP/DOPE formulation can inhibit vein graft stenosis after autologous end-to-side transplantation in rabbits.

1. Whether Dz13 in DOTAP/DOPE can reduce serum-inducible VSMC proliferation.
2. Whether Dz13 in DOTAP/DOPE can reduce c-Jun expression *in vitro*.
3. Whether DOTAP/DOPE can be used as a transfection agent effectively delivering Dz13 *in vivo*.
4. Whether Dz13 in DOTAP/DOPE can reduce intimal hyperplasia after

autologous vein bypass grafting.

5. Whether Dz13 in DOTAP/DOPE can decrease c-Jun expression in vein grafts after transplantation.

D. Whether Pre-miR191, a natural microRNA inhibitor of Egr-1, can inhibit neointima formation in rat carotid artery balloon injury model.

1. Whether topical application of pre-miR191 can attenuate neointima formation after balloon injury in rats.

2. Whether topical application of pre-miR191 can suppress the expression of injury induced Egr-1 in the rat carotid arteries after balloon injury.

2. Chapter II

Materials and Methods

2.1 Materials

2.1.1 Biomolecules investigated in this thesis

Cocktail

The cocktail referred to in this work is a combination of vascular endothelial growth factors-A, and -D (VEGF-A, VEGF-D), and cyclic Arg-Gly-Asp peptide (cRGD). The stock VEGF-A (Sigma, NSW, AU; Human, Recombinant, expressed in *E. coli*) solution and VEGF-D (BioScientific, NSW, AU; Mouse, Recombinant, expressed in *Sf21* cells) solution were prepared respectively by mixing 10 µg VEGF-A with 200 µL dH_2O and 25 µg VEGF-D mixed with 500 µL PBS. The cRGD (Auspep, NSW, AU) structure is H-Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala-OH (Disulfide bond between Pen^2 and Cys^9) and the stock solution was obtained by mixing 5 mg cRGD together with 500 µL dH_2O.

For *in vitro* experiments, the cocktail was suspended in medium to get a final concentration of 50 ng/mL (VEGF-A and VEGF-D), 0.2 µM (cRGD). For the *in vivo* experiments, the cocktail was suspended in Pluronic F127 gel (BASF, 25% w/v in sterile saline), maintained at 4°C, to obtain a final concentration of 100 ng/ml or 2500 ng/ml (VEGF-A and VEGF-D), 0.4 or 10 µM (cRGD), to obtain low or high dose cocktail, respectively.

DNAzymes

DNAzymes with 3′-3′–linked inverted T used in these experiments were produced and high-performance liquid chromatography–purified by TriLink Biotechnology. The sequences of DNAzyme:

Dz13: 5’-CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG-invT-3’;

Dz13.G>C: 5’-CGGGAGGAAGGCTACCTACAACGAGAGGCGTTG-invT-3’.

FITC-Dz13: 5'-(FAM)CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG-invT-3'

DOTAP and DOPE

The cationic liposomal formulation containing 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) / 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in powder form were provided by Avanti Polar Lipids.

MicroRNAs

Precursor of hsa-miR-191 (Pre-miR191) was ordered from Applied Biosystems. The sequence is

5'CGGCUGGACAGCGGGCAACGGAAUCCCAAAAGCAGCUGUUGUCUCCAGA

GCAUUCCAGCUGCGCUUGGAUUUCGUCCCCUGCUCUCCUGCCU-3'

A total amount of 30 µg or 60 µg of the Pre-miR191 was suspended in Pluronic F127 gel (BASF, 20% w/v in sterile saline) with the transfection agent, Lipofectamine (Invitrogen), added at a final concentration of 1% (w/v). The solution was maintained at 4°C for 2 h prior to the application of 200 µL to the balloon injured adventitial surface.

2.1.2 Media, buffers and solutions

MCDB 131 medium

MCDB 131 medium (Invitrogen, Gaitherdburg, MD) contained 10% (v/v) foetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF) (Sigma E9644), 2 mM L-glutamine, 1 µg/mL hydrocortisone (Sigma H0135), and 5 U/ml penicillin/streptomycin.

Waymouth's MB752/1 medium (WM)

Serum-free Waymouth's MB752/1 medium was prepared by mixing one vial of Waymouth's MB752/1 medium (Invitrogen, NY, USA) and 1 L of dH2O and adjusting to pH 7. Serum-containing medium contained 10% (v/v) foetal bovine serum (FBS), and 10 U/mL penicillin/streptomycin.

Medium 199

Medium 199 (Sigma, M4530) contained 20% (v/v) FBS and 5 U/mL penicillin/streptomycin.

RIPA cell lysis buffer

1x RIPA buffer was prepared by mixing 50 mM Tris-HCL pH 7.5 (Sigma, USA), 150 mM NaCl (Sigma, USA), 0.1 % (v/v) SDS, 1 % (w/v) Triton X-100 (Sigma, USA), 1 % deoxycholate (w/v) (ICN, Biomedical, Inc France), 5 mg/mL leupeptin (Sigma, USA), 1 % (v/v) aprotinin-Trasylol (Bayer, Germany), and 100 mM phenylmethylsulfanyl (PMSF) (Sigma, USA).

4x SDS protein loading buffer

4x SDS protein loading buffer was prepared by mixing 400 µL 0.05% (w/v) bromophenol blue (Sigma, USA), 500 µL 1 M Tris-HCl (pH 6.8) (Sigma, USA), 900 µL 20 % (w/v) SDS (INC, Biomedicals, Inc, France), and 800 µL 100% (v/v) glycerol (Merck, Germany), together with 500 µL dH_2O.

SDS running buffer

SDS running buffer was prepared by mixing 6 g Tris-base (INC, Biomedicals, Inc, France), 2 g SDS (INC, Biomedicals, Inc, France) and 28.8 g glycine (Sigma, USA) with dH_2O up to 1 L.

Transfer buffer

I L transfer buffer was made of 6 g Tris-base (INC, Biomedicals, Inc, France), 28.8 g glycine (Sigma, USA), 200 mL methanol and dH_2O.

PBS (10 x)

5 L PBS (10x) was prepared by mixing one vial of Dulbecco's Phosphate Buffered Saline (GIBCO TM, Invitrogen Corporation, USA) together with dH_2O.

T20-PBS

0.05 % (v/v) T20-PBS was prepared by mixing 1 mL Tween-20 (Sigma, USA) with 1 x PBS up to 2 L.

Antigen retrieval buffer

Citrate buffer contained 2.1 g citric acid, 1 L distilled water, pH adjusted to 6.0 by adding 2M NaOH.

Tris-EDTA buffer contained 1.21g Tris, 0.37g EDTA, 1 L distilled water (mix to dissolve, pH 9.0), and 0.5 mL Tween 20.

EDTA buffer contained 0.37g EDTA, 1 L distilled water (mix to dissolve, and adjust pH to 8.0 using 1M NaOH), and 0.5 mL Tween 20.

2.2 Methods

2.2.1 *In Vitro* experimental procedures

2.2.1.1 HMEC and SMC culture conditions and proliferation

The immortalised human microvascular endothelial cell line, HMEC-1 (American Type Culture Collection, Rockville, MD) was cultured in MCDB 131 medium. This was routinely cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Rat aortic smooth muscle cells (RASMC) were obtained from a commercial source (Cell Applications Inc., San Diego, CA) and cultured in Waymouth's MB752/1 medium containing serum. The growth environment was humidified with 5% CO_2 at 37°C. Cells were used between passages 4 and 8.

2.2.1.2 Proliferation assays

In HMEC proliferation assays, HMEC were seeded in 96-well plates and arrested in serum-free medium for 24 h, then treated with cocktail (VEGF-A, VEGF-D and cRGD) for 48 h in 10% (v/v) FBS containing medium without the addition of EGF. For cell counting conducted using an automatic counter (Beckman Coulter), cells were harvested using trypsin before counting. Where specified cell growth was also quantified in real time using the Xcelligence system (ACEA Biosciences, Inc).

In RASMC proliferation assays examining the effect of "cocktail" on cell growth, 5000 cells were seeded in 96-well plates and arrested in serum-free medium for 24 h. Cells were then treated with cocktail (VEGF-A, VEGF-D and cRGD) for 48 h. Trypsin/EDTA (0.02 %, w/v, BioWhittaker) was used to harvest the cells and an automatic counter (Beckman Coulter) was used to count the suspension. Alternatively, the Xcelligence system (ACEA Biosciences, Inc) was also used to quantify cell growth in real time for designated experiments.

In RASMC proliferation assays examining the effect of DNAzyme on growth, the Xcelligence system (ACEA Biosciences, Inc) was used to quantify cell growth in real time. SMC were seeded in 96-well plates and arrested in serum-free medium for 24 h, then treated with Dz13 or Dz13.G>C mutant in a DOTAP/DOPE

transfection mix (DNAzyme/carrier ratio: 1.33/1, 0.6 μM DNAzyme) for 24 h then changed to medium containing 5% (v/v) FBS.

2.2.1.3 RASMC injury assays

RASMC were grown in 6-well plates to 90% confluence then arrested in serum-free medium for 24 h. A sterilised comb of headless stainless steel pins was used to scrape the cell layer 20 times over a period of 30 sec [260]. Floating cells were removed and remaining cells were treated with cocktail. Cells were then harvested at appropriate time points for analysis of RNA and protein.

2.2.1.4 HMEC and RASMC wound scraping model

HMEC or RASMC were grown in 6-well plates to 90-100% confluence then arrested in serum-free medium for 6 h. A sterilised toothpick was used to scrape the cell monolayer and photos of the fresh scrapes were taken using a Nikon inverted microscope. Cells were then treated with cocktail or vehicle and photos taken 12 h, 24 h and 48 h after the treatment. Cells regrowing within the denuded zone were counted using Image-Pro Plus software.

2.2.1.5 Endothelial progenitor cell (EPC) Isolation and differentiation assay

A 25mL sample of human blood (Buffy Coat, from Australian Red Cross, Agreement No. 09-03NSW-17) was diluted with saline (1:1) in a 50 mL centrifuge tube. Then 15 mL Ficoll-Paque Plus (GE Healthcare Bio-sciences, 17-1440-03) was carefully added to the bottom of the centrifuge tube. Centrifugation was conducted at 500 g for 20 min at 18-20°C.

After centrifugation, the lymphocyte layer was removed using a clean Pasteur pipette from the interface of the plasma and Ficoll-Paque Plus layers and transferred to a clean centrifuge tube for washing and removal of platelets. Cold saline was added to the centrifuge tube up to 50 mL and the cells gently resuspended. Centrifugation was conducted at 300 g for 10 min at 4°C and the supernatant was removed. These centrifugation procedures were repeated 3-4 times until the supernatant became clear. Then the lymphocytes were suspended in Medium 199 (Sigma, M4530) containing 20% (v/v) FBS and 5 U/mL penicillin/streptomycin.

The lymphocytes were plated on culture dishes coated with 10 μg/ml fibronectin (Sigma F2006). After 4 days, non-adherent cells were removed by washing with PBS. The left were re-plated on new fibronectin-coated dishes and treated with vehicle or cocktail. Early EPC colonies appeared within 7 days of culture. Colonies were defined as a cell mass with small round cells at the centre and elongated spindle-shaped cells sprouting at the periphery.

These cells were then assayed by co-staining with DiI-AcLDL (Biomedical Technologies, BT902) and FITC-conjugated UEA-1 Lectin (Sigma, L9006) the following day. The cells were first incubated with DiI-AcLDL (10ug/ml) at 37°C for 4 h, then fixed with 2% (w/v) paraformaldehyde for 10 min. After washing with PBS, the cells were reacted with FITC-conjugated UEA-1 Lectin (10 μg/ml) for 1 h. The photos were taken under fluorescence microscopy (DiI excitation: 549 nm emission: 656 nm; FITC excitation: 494 nm emission: 518 nm).

2.2.1.6 Total RNA isolation

Total RNA was prepared from HMEC that were injured and treated with or without cocktail using the RNeasy Mini Kit (Qiagen). Briefly, HMEC were grown in 6-well plates and washed twice with cold phosphate-buffered saline (PBS). Cell lysates were collected using RLT buffer. One volume of 70% (v/v) ethanol was added, the cells ruptured using repeated withdrawal into an insulin syringe and the mixture subjected to a column as described (RNeasy Mini Kit, Qiagen) and centrifuged at 9,000 g for 15 sec. The column was washed 3 times separately with RW1 buffer (RNeasy Mini Kit, Qiagen), RPE buffer (RNeasy Mini Kit, Qiagen) and RPE buffer, and then placed in a new tube and centrifuged at 9,000 g for 1 min. RNase free water was added and centrifuged at 9,000 g for 1 min. Quantification of RNA was done by reading the absorbance at 260 nm (Nanodrop). Purity was confirmed by obtaining the A260/A280 and A260/A230 ratios. Only samples with ratios between 1.8-2.0 were used. Total RNA was stored at -80°C.

2.2.1.7 Reverse transcription

Oligo (dT) 15 primers (Sigma) and Superscript II reverse transcriptase (Invitrogen) were adopted to reverse-transcribe RNA to cDNA. Briefly, 0.5 µg total RNA was incubated with Oligo (dT)15 (Sigma) and deoxyribonucleoside triphosphate (dNTP) mix (10 mM each of dATP, dGTP, dCTP, and dTTP; Roche), and incubated at 65°C for 5 min, followed by quick cooling on ice. 5X First-strand buffer, 0.1M dithiothreitol (DTT) (Sigma), and RNAsin® RNase inhibitor (Promega) were added and the reaction was incubated at 42°C for 2 min. Superscript II (Invitrogen) was added, followed by another 50 min of incubation at 42°C. The reaction was terminated by incubating at 70°C for 15 min. cDNA was stored at -20°C.

2.2.1.8 Quantitative real time RT-PCR (QRT-PCR)

Total RNA was isolated and reverse-transcribed as described above. PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems), forward and reverse primers (Nitric oxide synthase 3, Qiagen Catalog no.: 330001 PPH01298E), H_2O and appropriate cDNA. Corbett Rotor Gene RG-6000 system and appropriate software were used to perform PCR amplification. The conditions were set up as following: initial hold at 50°C for 2 min, activation at 95°C for 10 min, 40 cycles at 95°C for 10 sec, then at 60°C for 15 sec and 72°C for 10 sec. The Ct (threshold cycle) value of NOS3 amplification was normalised to that of β-actin. Melt curve analysis and the use of ethidium bromide-stained agarose gels were used to ensure amplification of single PCR products of correct size without primer-dimer formation. Primer efficiency curve was generated (Reaction efficiencies = 0.98, and R values = 0.98916, for NOS3).

2.2.1.9 Total protein isolation

Cells growing in 100-mm Petri dishes were washed twice with ice-cold 1XPBS then collected by scraping in 500 µL of RIPA cell lysis buffer. The samples were then frozen on dry ice and thawed to facilitate cell lysis, followed by centrifugation at 18,000 g, 4^{o}C for 10 min. The supernatant was transferred to a fresh tube, and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Thermo Scientific).

2.2.1.10 Western blot analysis

Protein samples were combined with 6 µL 4X Sodium Dodecyl Sulfate (SDS) loading buffer and 2 µL of 0.5 M DTT then boiled for 5 min. Samples were snap-cooled on ice and 2 µL of 0.5 M iodoacetamide (IAM) (Sigma) was added. The

proteins were resolved on an SDS-polyacrylamide gel, consisting of a 4%-20% gradient gel (BIO-RAD Mini-PROTEAN TGX^{TM} Cat# 456-1094). Proteins were separated using a Bio-Rad Trans-Blot® Mini cell system with 1XSDS running buffer at 100V. Proteins were electrotransferred to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore) pre-soaked in 100% ethanol (Merck). Transfer was performed in pre-cooled transfer buffer at 90 V for 2 h. Transfer was confirmed by staining the gel with Gel Code Blue stain reagent (Pierce). The membranes were blocked 1 h with 5% (w/v) skim milk in 0.05% (v/v) Tween 20 (Sigma) in PBS with gentle shaking on a platform shaker at room temperature. Then membranes were incubated with the primary antibody (1:2000, v/v, Anti-eNOS BD 610296; or 1:3000, v/v, Anti-c-Jun, Santa Cruz H-79) diluted in either 5% skim milk in Tween 20 PBS at 4^{o}C overnight. The washes were repeated, and the membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (DAKOCytomation, Denmark) diluted in 5% skim milk-Tween 20 PBS (1:1000, v/v) for 1 h. The membrane was washed three times and chemiluminescence detected using the Western Lightning Chemiluminescence system (PerkinElmer, MA, USA) and Hyperfilm-MP (Amersham Pharmacia Biotech, Eng, UK).

2.2.1.11 DNAzyme transfection of RASMC

To determine the optimal conditions for Dz13 transfection into RASMC, FITC-labelled Dz13 was used. SMC were seeded in 4-chamber culture slides (BD Falcon^{TM}) and arrested in serum-free medium for 24 h, and then incubated in transfection solution that contained 0.6 μM FITC-Dz13 (TriLink BioTechnologies, Inc) and DOTAP/DOPE (Avanti Polar Lipids) (DNAzyme/carrier ratio: 1.33/1) in serum-free medium at 37^{o}C for 24 h. Slides were washed by PBS and fixed in 10% (w/v) neutral buffered formalin (Sigma-Aldrich) for 15 min and imaged under

fluorescence microscopy (excitation: 494 nm, emission: 518 nm).

2.2.2 *In vivo* experimental procedures

2.2.2.1 Rat carotid artery balloon injury model

In the cocktail study, twenty-six adult Sprague-Dawley (SD) rats (500-550 g) were divided into five groups, i.e. gel with balloon injury control (n=6), cocktail at low concentration (n=4), cocktail at high concentration (n=6), cocktail (high concentration) with nitro-L-arginine methyl ester (L-NAME) (n=4) and L-NAME with balloon injury (n=6). In the miR-191 study, sixteen adult SD rats were divided into three treatment groups, pre-CTL (n=4), pre-191 (n=7) and vehicle (n=5). The rats were anaesthetised with a mixture of ketamine (60 mg/kg body weight)/xylazine (8 mg/kg). Balloon injuries were produced as follows. The left common carotid artery was isolated through a midline cervical incision to expose a 3 cm segment of the artery from the bifurcation and the femoral artery was exposed. A 2F balloon embolectomy catheter was introduced through the femoral artery and advanced through into the left common carotid artery. The balloon was then inflated with 0.05 ml of saline and withdrawn 5 times with rotation to remove the endothelial cells lining the vessel. The catheter was removed and the side branch of the femoral artery tied off. In the miR-191 study, 30-60 μg of the agents (pre-191 or pre-CTL) suspended in Pluronic F127 gel (BASF Corporation, 20-25% w/v in sterile saline) with 1% (w/v) of the transfection agent, Lipofectamine RNAiMax (Invitrogen), was applied to the perivascular surface of the carotid artery. In the cocktail study, a volume of 200 μl of cocktail (high or low concentration) in Pluronic gel, or Pluronic gel alone (as vehicle), was applied to the perivascular surface of the carotid artery. Following the rapid solidification of the Pluronic gel around the artery at body temperature, the wound was closed and the animals were placed in a warm

environment for recovery. The rats recovered and were returned to the animal house and kept in individual cages to avoid injury. (**Figure 2.1A&B)** At the end of the study, the rats were euthanised with CO_2. A cut was made in the upper abdomen and extended up into the chest to expose the heart. A small needle (21 G) was inserted into the right ventricle (right sided chamber) of the heart to remove as much blood as possible. The right atrium of the heart was cut off and 50 mL heparin saline (1 IU/mL) was injected through the left ventricle. 50 mL of 10% (w/v) neutral buffered formalin solution, which acts as a preservative of the vessel, was injected to pressure fix the arteries until no blood remained. Both carotid arteries were removed for analysis. Common carotid artery (from aorta to the bifurcation of internal and external carotid artery) was removed and fixed with 10% (w/v) neutral buffered formalin solution. The artery was equally divided into 4 segments and embedded with paraffin in one block. Artery cross sections were stained (Verhoeff van Geison elastin stain) and examined in terms of medial (M) and intimal (I) areas, as well as the ratio (I/M area) for each sample. A mean I/M area +/- Standard Error (SE) was determined for each experimental group as primary endpoint.

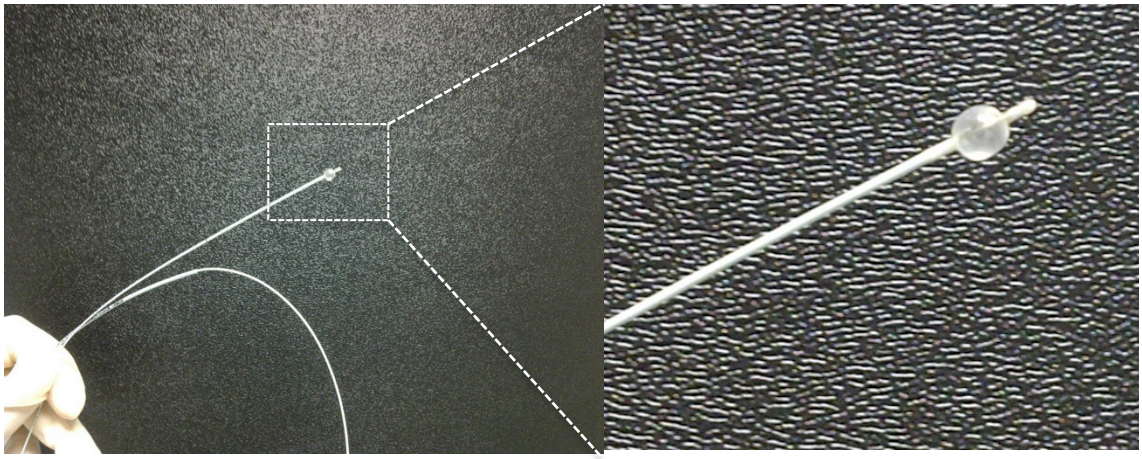


Figure 2.1A *2F balloon embolectomy catheter*

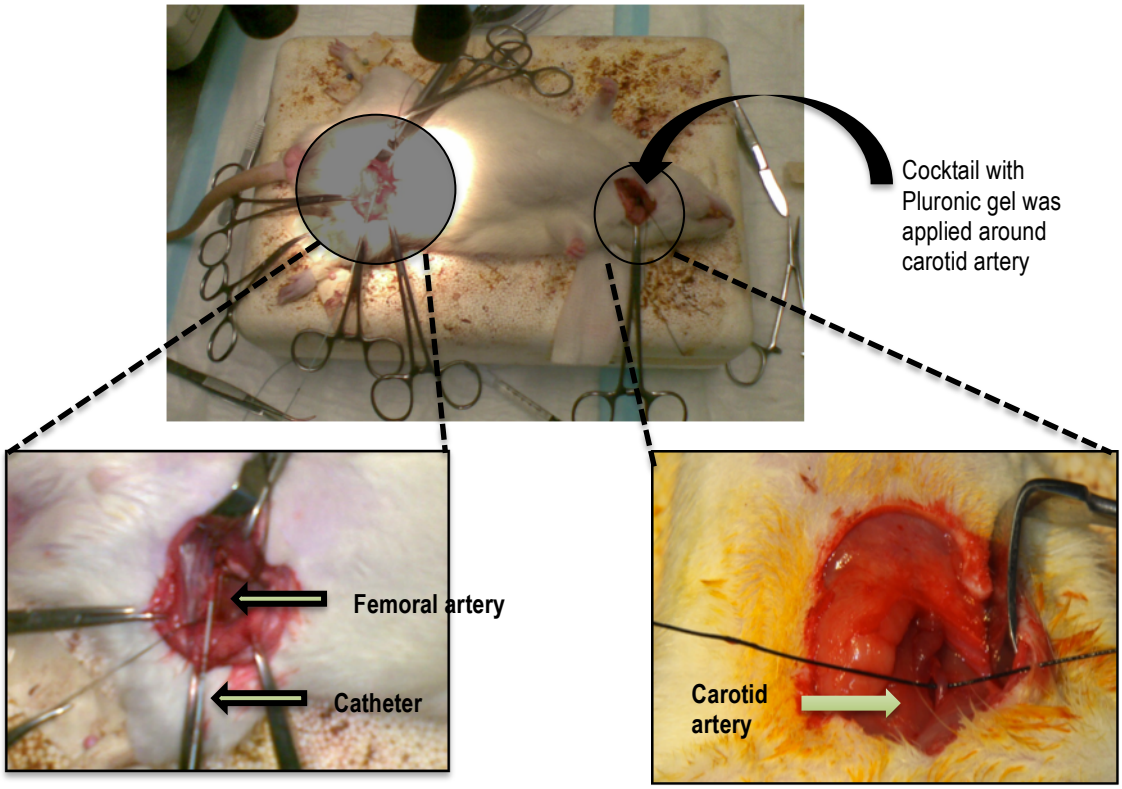


Figure 2.1B *Rat carotid artery balloon injury model*

2.2.2.2 Rabbits vein autologous end-to-side transplantation bypass graft model

Adult male New Zealand White rabbits (approximately 2.5 kg, on normal diet) were used for the experiments with 5 per group. The rabbits were anaesthetised with an intramuscular injection of ketamine (25 mg/kg) and xylazine (Ilium Xylazil-20, 5 mg/kg) (Parnell Laboratories, Australia) and anaesthesia was maintained throughout the operation via mask with a mixture of 2% isofluorane and 2% O_2.

After midline incision, a 2cm segment of the right external jugular vein was carefully exposed, flushed and kept moist in heparinised saline (100U heparin/mL, David Bull Labs, Australia). The right common carotid artery was dissected and clamped at the proximal and distal ends. Along the side of the artery an incision was made and flushed with heparinised saline containing 1% (w/v) lignocaine. The vein was separated from the surrounding tissues, and then the 2cm vein segment was extracted, flushed with 5 mL heparinised saline, and put into a 500 µL tube containing 300 µL vehicle solution which comprised 37.5 µL (375 µg) DOTAP/DOPE, 5 µL 1 mM $MgCl_2$ in DMEM (Life Technologies) medium, pH 7.4 with or without 500 µg Dz13, Dz13.G>C or just the vehicle. The veins were incubated with DNAzyme at 37°C for 30 min.

An 8/0 Proline uninterrupted suture under 3.5x magnification (Zeiss, Germany) was used to make a reversed vein attachment end-to-side to the artery (proximal end of the vein to the distal part of the artery and vice versa). The artery segment bypassed by the vein graft was ligated and cut so that unidirectional blood flow was ensured. After graft anastomosis, the incision was closed with 4/0 silk suture (Silkam, Johnson & Johnson).

When completing the operation, the patency of anastomosis was confirmed by palpable pulsatile blood flow through the vein. Before removing the cannula, 50 mL normal saline was given intravenously. The rabbits were placed on their sides under warm light and given oxygen until they completely recovered from anaesthesia (**Figure 2.2A**).

The vein grafts were collected 28 days after the transplantation. The rabbits were anaesthetised and a midline incision was conducted to expose the vein graft, which was then dissected from surrounding tissues. Cannulas were put through the proximal and distal carotid segments and the vein was perfusion-fixed under distending pressure with 10% (w/v) formalin. The grafts were removed and placed in 10% (w/v) formalin (Ambion, USA) overnight and stored in 70% (v/v) ethanol. At the end of study, the rabbits were euthanized with an intravenous pentobarbital overdose (120 mg/kg).

Vein graft cross sections were stained (Verhoeff van Geison elastin stain) and examined for intimal and total vessel areas. The ratio of intimal to total vessel area was obtained for each sample. A mean intimal/total vessel area +/- SEM was determined for each experimental group. Differences between groups were tested for statistical significance using Student's (unpaired) t-test and considered significant at $p<0.05$.

Ex vivo optical coherence tomography (OCT) was also performed on explanted vein grafts using the ILUMIEN™ PCI Optimisation System (St Jude Medical). Image acquisition was performed (pull back speed of 10 mm/s) using the Dragonfly catheter which was placed intraluminally in the vessels and continuously flushed

with normal saline (**Figure 2.2B**). The pull-back segment was analysed offline and frames demonstrating the minimum luminal area were used for analysis. Neointimal area and total vessel area were evaluated offline by 2 blinded observers.

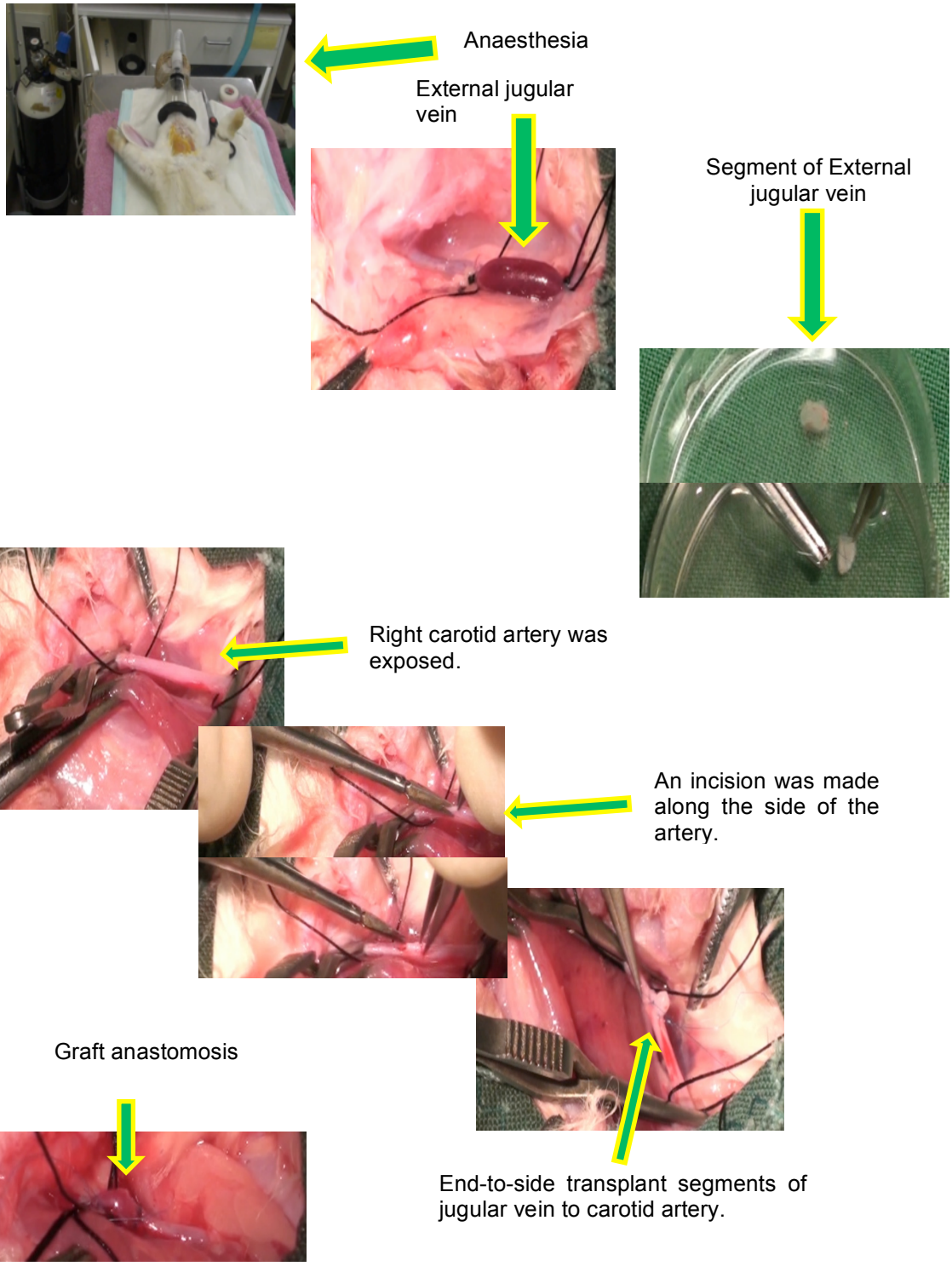


Figure 2.2A *Rabbits vein autologous end-to-side transplantation bypass graft model*

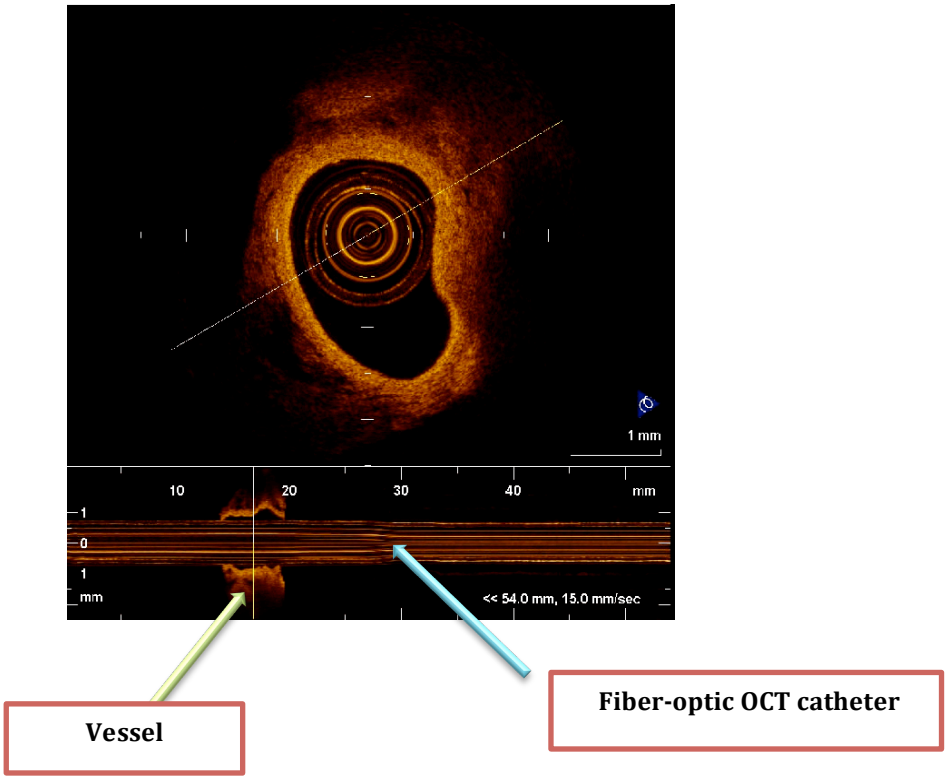


Figure 2.2B *Ex vivo optical coherence tomography (OCT)*

2.2.2.3 DNAzyme transfection of veins

Prior to transplantation, veins were transfected with Dz13. FITC-labelled Dz13 was firstly used to ensure optimal transfection conditions for Dz13 uptake. Veins were incubated in 300 µl transfection solution that contained 500 µg FITC-Dz13 (TriLink BioTechnologies, Inc) and 37.5µl DOTAP/DOPE (Avanti Polar Lipids) at 37^{o}C for 30 min, then rinsed in PBS pH 7.4 and snap frozen with OCT (Sakura Tissue-Tek® 4583) on dry ice. The blocks were sectioned and 5 µm sections placed onto slides and fixed in 95% (v/v) ethanol. The slides were stained with DAPI (Invitrogen, USA) to identify nuclei under fluorescence microscopy (FITC excitation: 494 nm, emission: 518 nm; DAPI excitation: 358 nm, emission: 461 nm).

2.2.2.4 Immunohistochemistry

Immunohistochemical analysis was performed with CD31 antibody (Abcam, ab28364, 1:200), Ki67 antibody (Abcam, ab16667, 1:50), c-Jun antibody (Santa Cruz H-79, 1:250), PCNA antibody (Santa Cruz PC10, 1:200) and EGR1 antibody (Cell Signaling, 1:40) on consecutive 5 µm paraffin-embedded sections of formalin-fixed balloon-injured rat carotid arteries or rabbit vein bypass graft. Primary antibodies were omitted as negative controls.

In staining, sections were first deparaffinised by placing them in xylene for 3 x 5 min, 100% ethanol for 2 x 3 min, and 70% (v/v) ethanol for 1 x 3 min, and then rehydrated in distilled water for 2 x 5 min.

For CD3l, PCNA and EGR1 staining, antigen retrieval was performed by placing the slides into a glass jar and adding sufficient citrate buffer and heating it up to 95°C for 30 min in a microwave. For Ki67 staining, Tris-EDTA buffer was used. For

c-Jun staining, EDTA buffer, pH 8.0 containing Tween 20 was used. After retrieval, slides were cooled to room temperature for 20 min and then rinsed in TBS for 5 min.

Endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min, and then rinsed in TBS for 2 x 5 min. Non-specific staining was inhibited with KPL normal goat serum (KPL 71-00-27) for 30 min. For the primary antibodies, anti CD31 antibody and PCNA antibody were diluted in 0.05% (w/v) saponin, 1% (w/v) BSA/PBS at 1:200 (v/v) and incubated at 4°C overnight respectively; anti Ki67, c-Jun and EGR1 antibody were diluted in 0.05% (w/v) saponin, 1 % (w/v) BSA/PBS at 1:50 (v/v), 1:250 (v/v) and 1:40 (v/v) respectively and incubated at 4°C overnight.

After rinsing in TBS for 2 x 5 min, the CD31, Ki67 and EGR1 staining slides were incubated in KPL goat anti-rabbit secondary antibody (KPL 71-00-30) at room temperature for 1 h, the c-Jun and PCNA staining slides were incubated with Dako goat anti-mouse secondary antibody (Dako E0433) at 1:250 dilution at room temperature for 1 h, and then rinsed again in TBS for 2 x 5 min. PL strepavidin peroxidase reagent (KPL 71-00-38) was used to incubate the slides for 30 min, and then rinsed in TBS for 2 x 5 min. DAKO liquid diaminobenzidine (DAB) chromagen (DAKO k3468) (1 drop DAB/mL of buffer substrate) was applied for 5 min, then the slides were rinsed in water for 5 min.

Counter staining was performed with Modified Harris Haematoxylin for 30 sec. The slides were rinsed in water, followed by 5 dips in Scotts Blue reagent then a water rinse. Sections were dehydrated in 70% (v/v) ethanol for 1 x 3 min, 100% ethanol

for 2 x 3 min and xylene for 3 x 5 min prior to coverslipping. Due to poor staining with Egr-1 these sections were not counterstained.

2.2.2.5 TUNEL stain

Artery cross sections were also stained for apoptosis markers using the TUNEL Apoptosis Detection Kit (Millipore, 17-141). Both negative and positive controls tissues were from uninjured arteries, however, the positive control was additionally treated with DNase I (Millipore, 17-141h) to generate cleaved DNA which acted as a positive substrate for the TUNEL reaction.

The staining was performed according to the manufacturer's protocols. Briefly, sections were first deparaffinised by placing them in xylene for 3 x 5 min, 100% ethanol for 2 x 3 min, 70% (v/v) ethanol for 1 x 3 min and then rehydrated in distilled water for 4 x 2 min. The sections were rinsed with 500 µL/section of PBS at 37°C for 30 min and then covered with 250 µL/section of Proteinase K working solution (Millipore, 17-141a) and incubated at 37°C for l5-30 min to help expose the DNA for end-labelling with TdT (Terminal deoxynucleotidyl Transferase). The Proteinase K digestion was stopped by washing sections with PBS for 4 x 2 min. The sections were then equilibrated with 50 µL/section of TdT Buffer (Millipore, 17-141c) for 5-l0 min and then incubated with 50 µL of TdT ending-labeling cocktail (mixing TdT buffer, Biotin-dUTP, and TdT at a ratio of 90:5:5, respectively) at 37°C for 60 min. TdT end-labeling cocktail was removed and the sections were immersed in TB Buffer (lx) at room temperature for 5 min to stop the reaction. After being rinsed with PBS for 4 x 2 min, 50 µL, Blocking Buffer was applied to each section and incubated at room temperature for 20 min. 1 part Avidin-FITC was diluted with 9 parts of

Blocking Buffer to prepare sufficient Avidin-FITC. A volume of 50 µL of Avidin-FITC solution was then applied to each sample section, which was then incubated at 37°C in the dark for 30 min. The sections were rinsed with PBS for 2 x 15 min in the dark at room temperature prior to coverslipping. The sections were viewed with fluorescent microscope (excitation: 494 nm, emission: 518 nm). Slides were visualised by fluorescent microscopy with an Olympus Bx53 Microscope and positive cells were counted.

2.3 Statistics

Data were expressed as mean ± SEM. Differences between groups were tested for statistical significance using the Mann-Whitney U test for two group comparisons and Kruskal Wallis test for multiple comparisons. Significance was considered significant at $p<0.05$. Statistics were calculated with GraphPad Prism v5 (GraphPad Software, San Diego, CA, USA). Power calculations were completed using software G*Power 3.1 to determine animal numbers per group.

3. Chapter III

Inhibition of Intimal Thickening and Accelerated Re-endothelialisation After Vascular Injury using a Novel cocktail of VEGF-A, VEGF-D and cRGD

3.1 Introduction

Coronary artery disease (CAD) is the leading cause of death in the world [3, 8, 261] and this is likely to remain unchanged for the next 20 years [1]. Percutaneous coronary intervention (PCI) and drug–eluting stents (DES) have provided a fundamental change in the treatment of the disease. Compared with bare metal stents (BMS), DES exhibit reduced rates of restenosis and a reduced requirement for repeated revascularisation [30, 31]. Although the efficacy and safety of DES has been demonstrated [262], there still remain significant challenges. First, restenosis may still occur, especially in long lesions, bifurcation lesions, small coronary arteries, multi-vessel disease, and in the setting of diabetes [262]. In clinical and histological studies of DES, continuous neointimal growth in the extended phase follow up has been observed as a cause of late in-stent restenosis [263, 264]. Second, late stent thrombosis associated with DES has become significant [48, 56-58], with a 0.25-1% annual incidence rate during 1.25 to 4 years follow-up [56, 58, 265, 266]. Continuation of dual-anti-platelet therapy is required for one or more years after DES placement [51, 267].

The pathogenesis of restenosis after PCI is due mainly to vascular smooth muscle cell (SMC) proliferation and migration [268]. Current clinical approaches, such as DES with Sirolimus and Taxol, inhibit SMC growth, but also inhibit endothelial cell (EC) function [269]. Dysfunctional or absent endothelium is an undesired consequence of interventional revascularisation [155]. Analysis of human coronary artery stents indicate that impaired re-endothelialisation is an important feature of stent thrombosis following DES implantation, and it may increase the risk beyond 30d [53-55]. In animal models, DES inhibits proliferation and migration of coronary EC, induces apoptosis of endothelial progenitor cells (EPC), and reduces vascular

endothelial growth factor (VEGF) expression [270]. The key to solving these problems is to inhibit SMC proliferation and migration while enhancing re-endothelialisation. Current approaches use single agent therapy. Agents that have the potential to reduce restenosis and circumvent late thrombosis include inducers of heme oxygenase-1 (HO-1) like probucol [271, 272], as well as other stimulants such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and cyclic Arg-Gly-Asp peptide (cRGD) [193, 273].

The VEGFs are a family of angiogenic growth factors, including VEGF-A, -B, -C, -D, -E and PlGF (placenta growth factor) [105]. These stimulate EC proliferation and accelerate repair. VEGF-A binds two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 while VEGF-D binds VEGFR-2 and VEGFR-3. These receptors are expressed almost exclusively on EC [155]. The RGD sequence in extracellular matrix proteins is a basic unit of the cellular recognition system, which is recognised by integrins, such as avß3, a5ß1, avß1, avß5 and aIIßIII [176]. cRGD can accelerate endothelialisation through attracting EPC and stimulating their differentiation [193]. cRGD is selective for avß3 expressed on growth factor-activated EC [194] and promotes their migration to VEGF [195].

Here the effects of a unique combination of VEGF-A, VEGF-D and cRGD on EC and SMC proliferation and wound repair, and EPC differentiation were evaluated. The effects of the cocktail on intimal hyperplasia and re-endothelialisation in rat carotid arteries after balloon injury were also investigated. Further, it was examined that the involvement of endothelial nitric oxide synthase (eNOS, NOS3) in this process as NOS3, which catalyzes the production of nitric oxide (NO) from L-arginine, is a critical regulator of vascular homeostasis and dysfunction.

3.2 Results

3.2.1 Effects of VEGF-A, VEGF-D, cRGD on EC and SMC proliferation

Cultured EC and SMC were treated with VEGF-A, VEGF-D or cRGD in the presence of FBS. Cell numbers were quantified after 48 h using an automated Coulter counter. VEGF-A increased EC proliferation at 50 ng/mL compared with FBS alone however had no effect on SMC proliferation at 5-100 ng/mL **(Figure 3.1**). FGF-2 was used as a positive control with both cell types (**Figure 3.1**). VEGF-D also increased EC proliferation at 500 ng/mL compared with FBS alone. However VEGF-D significantly reduced SMC proliferation at the same concentrations **(Figure 3.2**). cRGD also significantly increased EC proliferation (at concentrations greater than 200 nM) while reducing SMC proliferation (at concentrations greater than 20 000 nM) relative to FBS alone (**Figure 3.3)**.

The preceding results demonstrated the independent effects of VEGF-A, VEGF-D and cRGD on EC and SMC growth. Next, the 3 factors as a cocktail (combination of VEGF-A 50 ng/mL, VEGF-D 50 ng/mL and cRGD 200 nM) were used in a proliferation assay using the xCELLigence system that quantifies cell growth in real time. The cocktail significantly increased EC growth after 3 days compared with the FBS control (**Figure 3.4**). Conversely, the cocktail significantly decreased SMC growth over the same time frame (**Figure 3.4**).

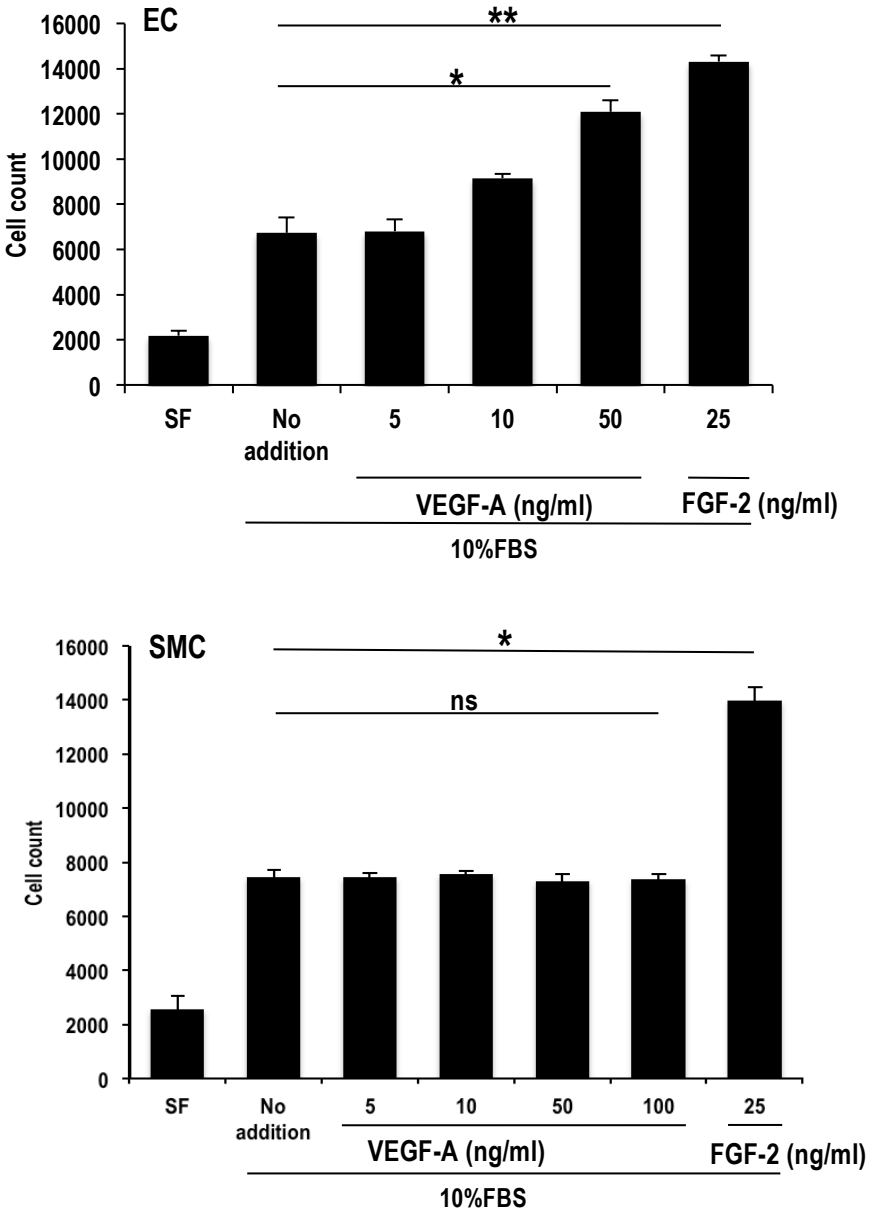


Figure 3.1 *Effects of VEGF-A on EC and SMC proliferation. Serum-arrested EC and SMC were treated with VEGF-A for 48 h in presence of serum and cells counted using automated Coulter counter. FGF-2 was used as a positive control. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ns=not significant.*

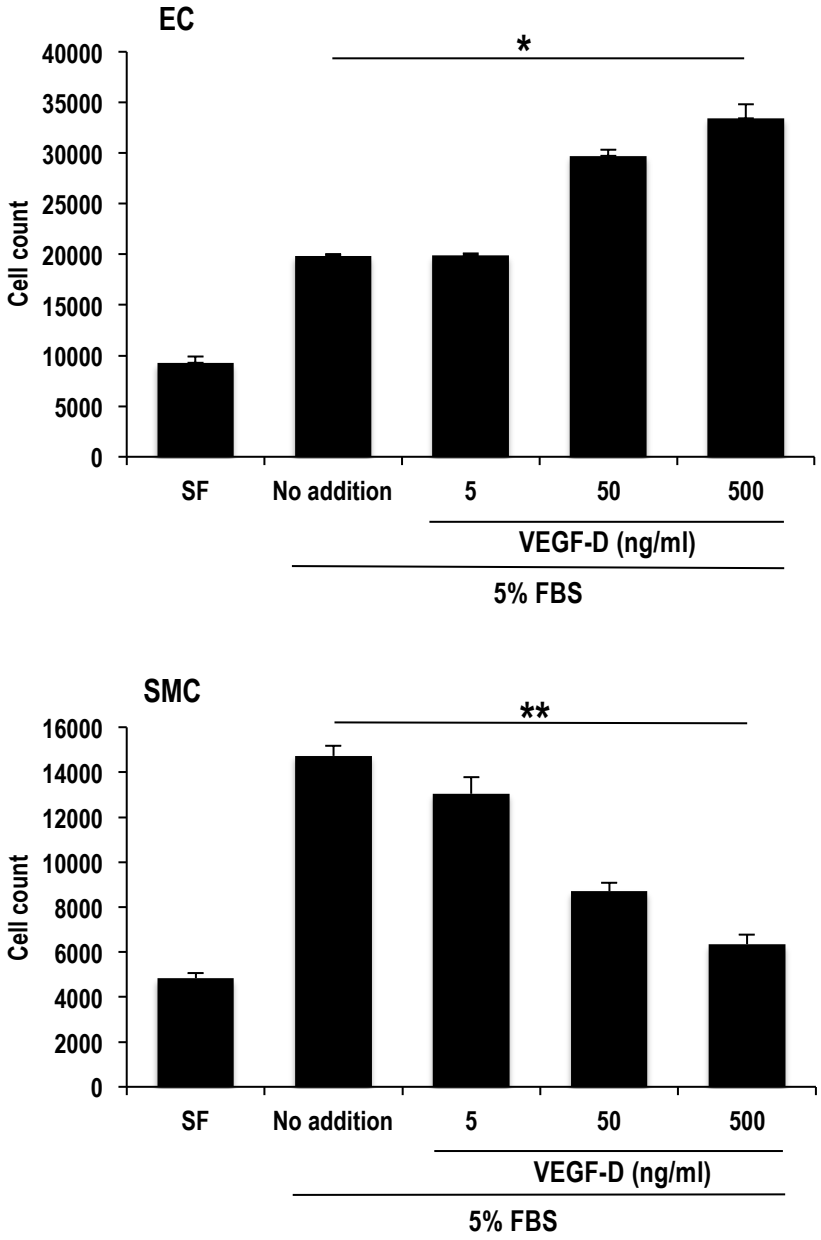


Figure 3.2 *Effects of VEGF-D on EC and SMC proliferation. Serum-arrested EC and SMC were treated with VEGF-D for 48 h in presence of serum and cells counted using automated Coulter counter. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01.*

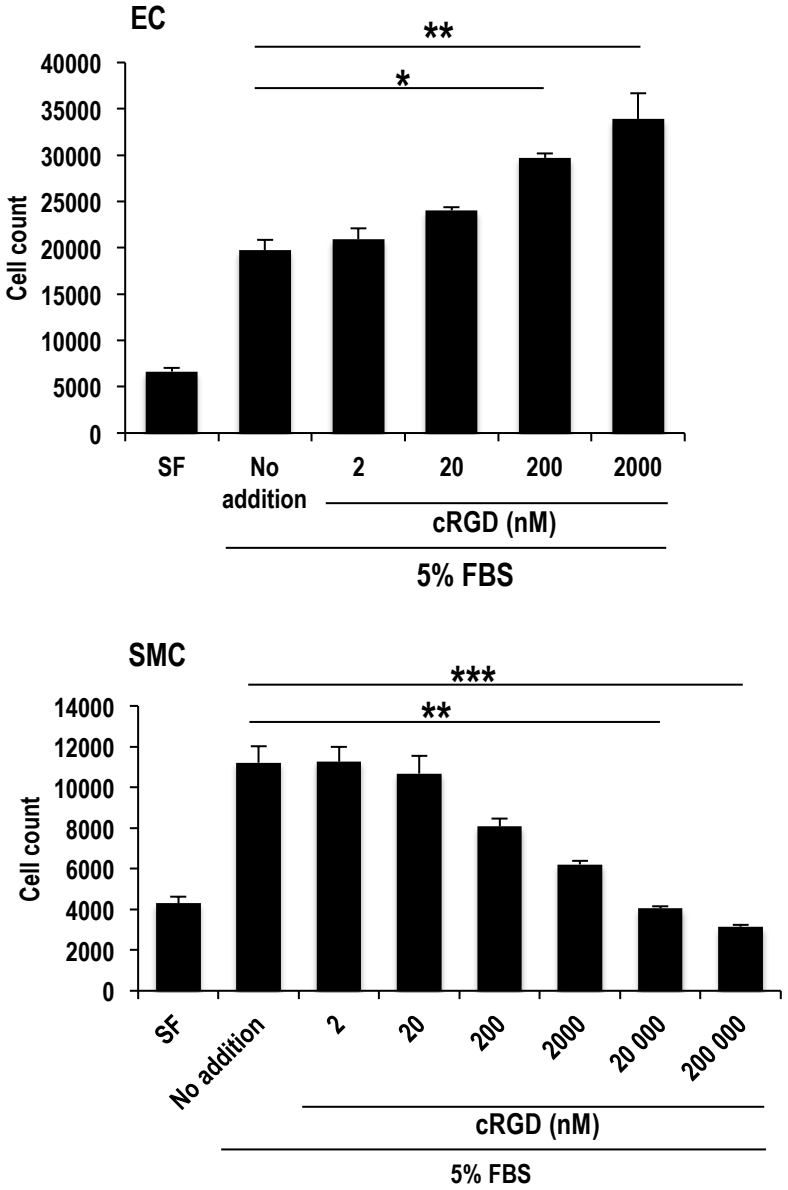


Figure 3.3 *Effects of cRGD on EC and SMC proliferation. Serum-arrested EC and SMC were treated with cRGD for 48 h in presence of serum and cells counted using automated Coulter counter. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001.*

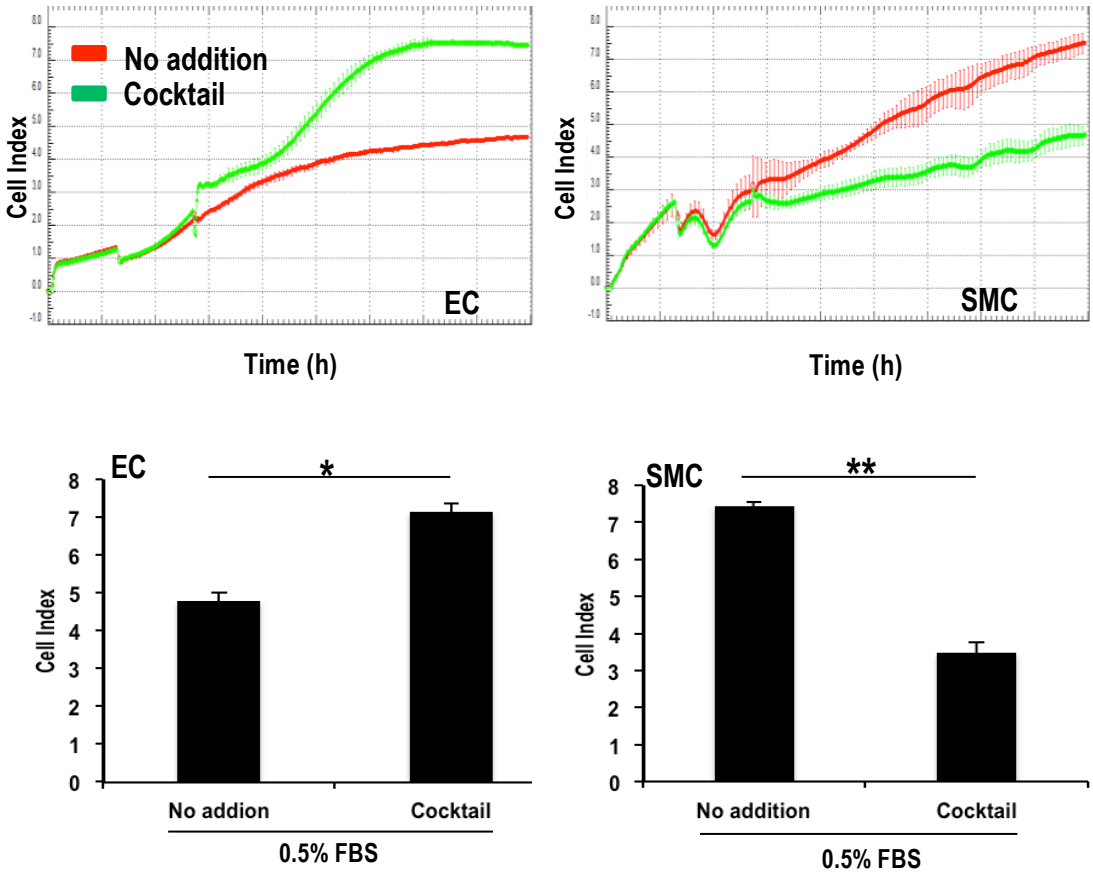


Figure 3.4 *Effects of Cocktail on EC and SMC proliferation. Serum-arrested EC and SMC were treated with cocktail (VEGF-A/D 50 ng/mL plus cRGD 200 nM) in the presence of serum and cell proliferation monitored by xCELLigence (Roche) over a 72 h period. Cell index represents a quantitative measure of cell number present in a well of a plate. Data represent mean ± SEM of 4-5 independent experiments. Statistical differences were determined using the non-parametric Mann-Whitney test. *p<0.05, **p<0.01.*

3.2.2 Effects of VEGF-A, VEGF-D, cRGD and cocktail on EC and SMC regrowth after injury

Next the effects of VEGF-A, VEGF-D, cRGD or their combination were investigated in an *in vitro* model of EC and SMC wound repair in which the cultures are scraped with a toothpick and repopulation of the denuded area monitored. The cocktail treatment significantly increased EC repair compared with the FBS (0.5%, v/v) control while VEGF-A (50 ng/mL), VEGF-D (50 ng/mL) or cRGD (200 nM) alone had no effect on EC repair. (**Figures 3.5A&B).** The cocktail stimulated EC wound regrowth to a similar extent as 5% FBS. In SMC, the cocktail significantly inhibited wound repair while VEGF-A (50 ng/mL), VEGF-D (50 ng/mL) or cRGD (200 nM) alone had no effect on wound regrowth compared to the FBS (0.5%, v/v) control. (**Figures 3.6A&B).**

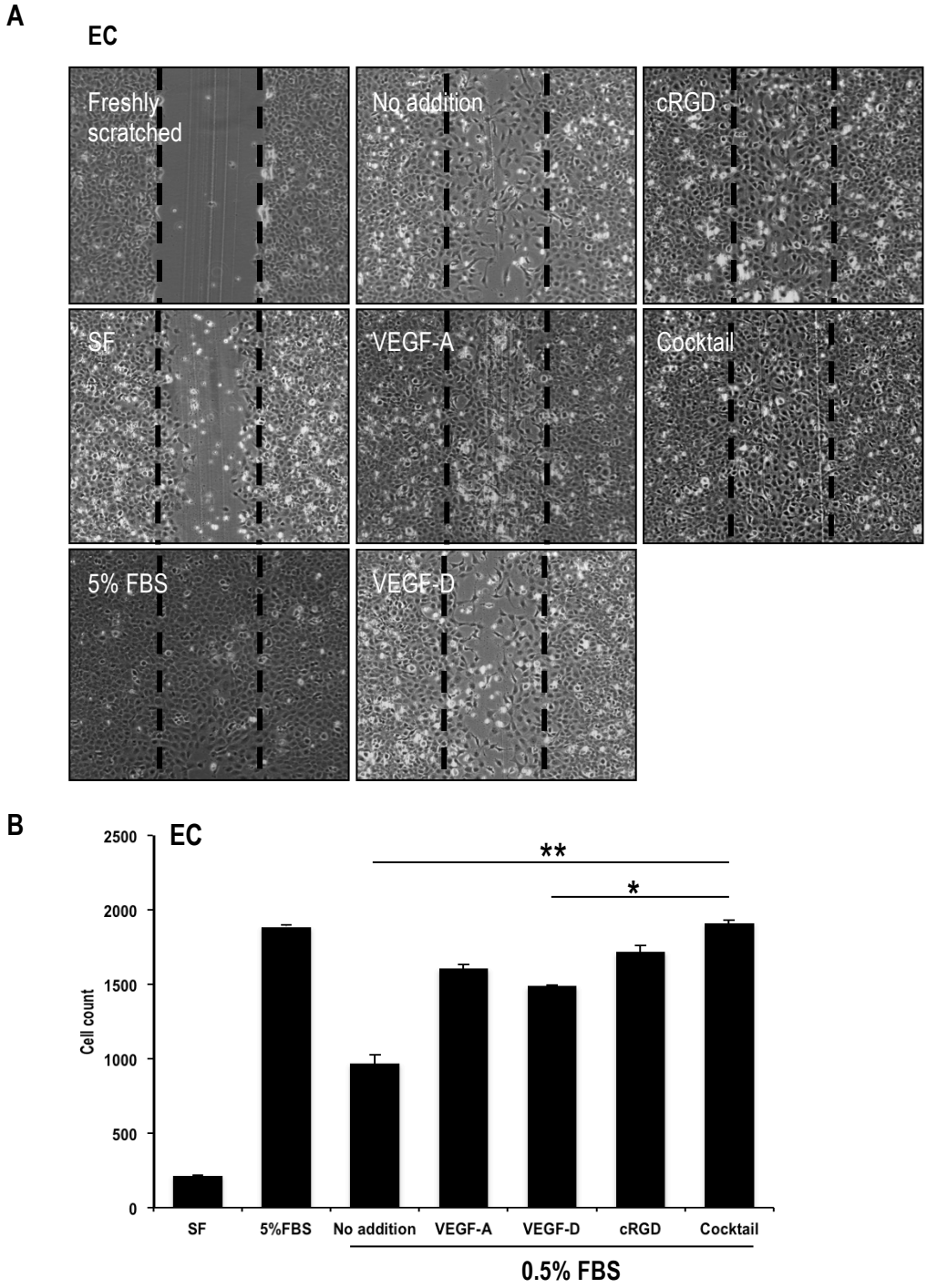


Figure 3.5 *Cocktail effects on EC wound repair. (**A,B**) EC monolayers were serum-arrested then scraped with a sterile toothpick and treated individually with VEGF-A or VEGF-D (50 ng/mL), cRGD (200 nM) or the cocktail combination (VEGF-A/D 50 ng/mL plus cRGD 200 nM) in the presence of 0.5% (v/v) serum and regrowth into the denuded area monitored over a 48 h period. Representative photomicrographs (**A**) were taken at 100X magnification. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01.*

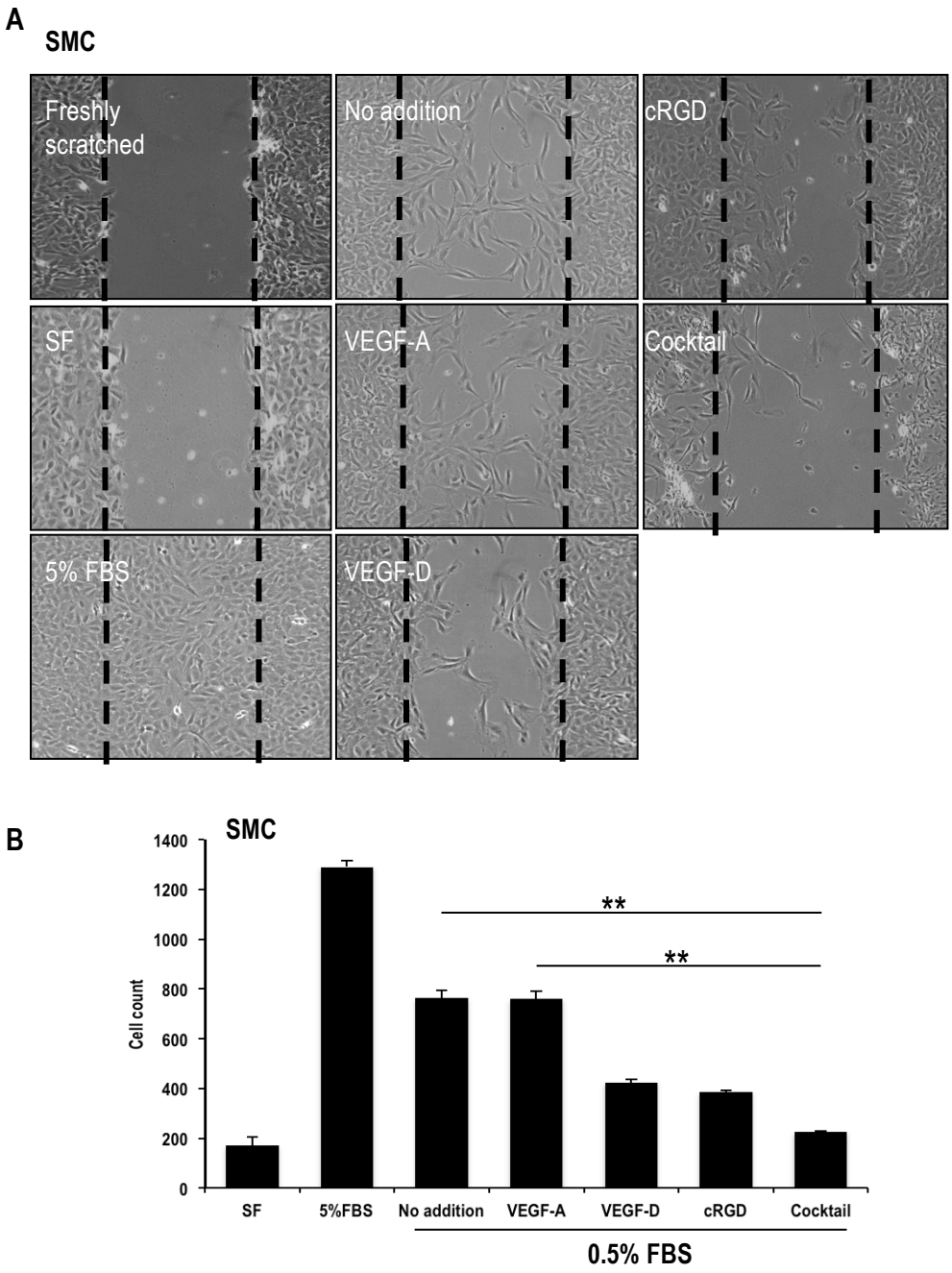


Figure 3.6 *Cocktail effects on SMC wound repair. (**A,B**) SMC monolayers were serum-arrested then scraped with a sterile toothpick and treated individually with VEGF-A or VEGF-D (50 ng/mL), cRGD (200 nM) or the cocktail combination (VEGF-A/D 50 ng/mL plus cRGD 200 nM) in the presence of 0.5% (v/v) serum and regrowth into the denuded area monitored over a 48 h period. Representative photomicrographs (**A**) were taken at 100X magnification. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. **p<0.01.*

3.2.3 Effects of VEGF-A, VEGF-D, cRGD and cocktail on EPC differentiation

To determine the effect of the cocktail and individual components on EPC differentiation, isolated peripheral blood mononuclear cells were treated with 50 ng/mL VEGF-A or VEGF-D, 200 nM cRGD or the cocktail (combination of VEGF-A 50 ng/mL, VEGF-D 50 ng/mL and cRGD 200 nM) in the presence of 20% *(v/v)* FBS for 10 days. Differentiated EPC were classified according to morphology and double staining with both DiI-AC-LDL and FITC-conjugated UEA-1 lectin. EPC differentiation increased significantly relative to the FBS control (38.6%) in the presence of cRGD (73.5%, $p<0.05$) (**Figure 3.7A&B**). EPC differentiation increased in response to the cocktail (85.5% versus 38.6% FBS control, $p<0.01$) (**Figures 3.7A&B**). VEGF-A and VEGF-D alone had no significant effect on EPC differentiation (**Figures 3.7A&B**).

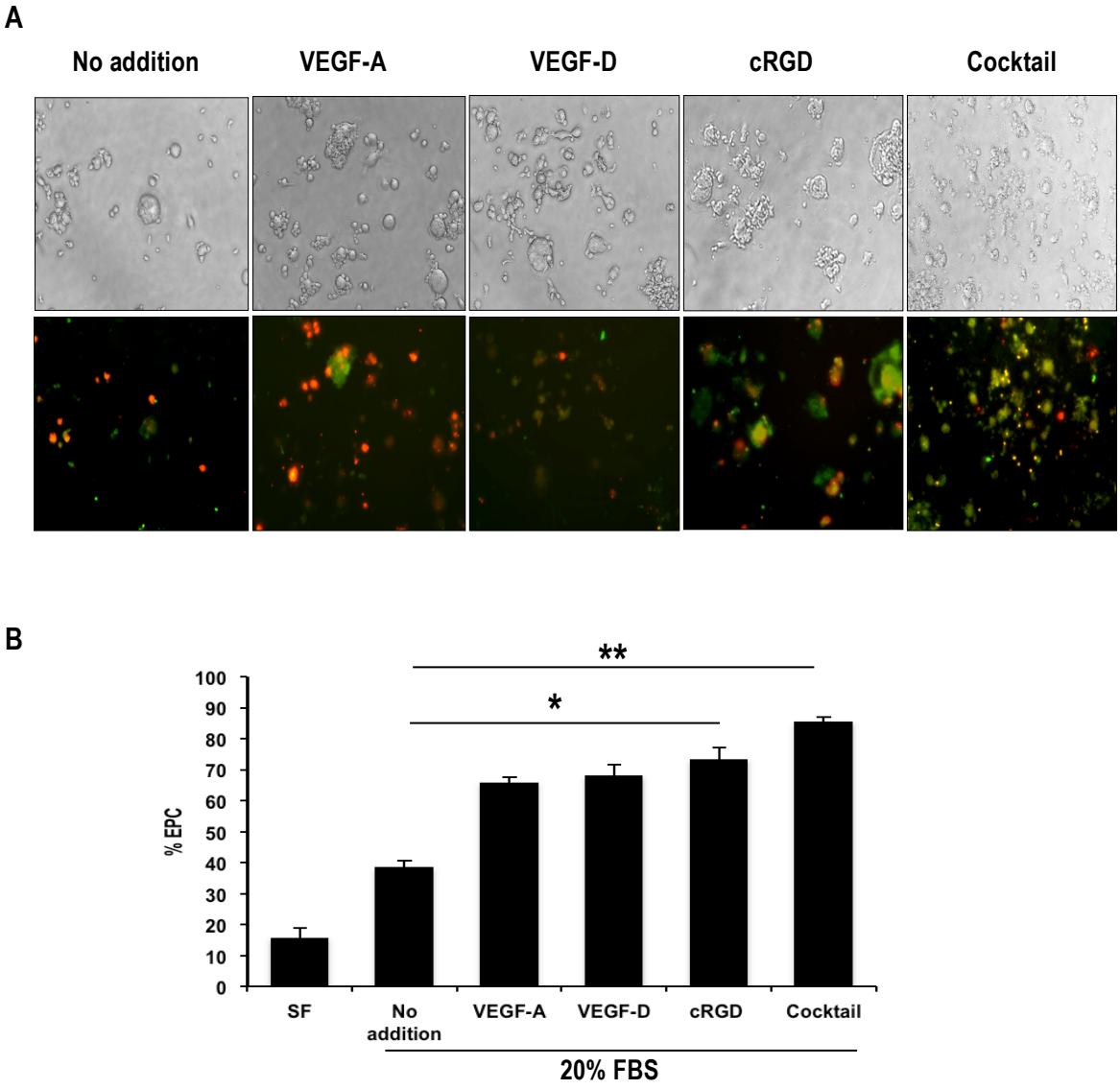


Figure 3.7 *Cocktail stimulates EPC differentiation. (**A,B**) Isolated peripheral blood mononuclear cells were treated individually with VEGF-A or VEGF-D (50 ng/mL), cRGD (200 nM) or the cocktail combination (VEGF-A/D 50 ng/mL plus cRGD 200 nM) in the presence of 20% (v/v) serum over a 10 days period. EPC were identified by morphological changes and positive co-staining with both DiI-AcLDL and FITC-conjugated UEA-1 Lectin. Representative photomicrographs (**A**) were taken at 200X magnification. Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01.*

3.2.4 Role of NO signaling in cocktail-mediated EC wound repair

An *in vitro* mechanical injury model was used to investigate the effect of cocktail on the EC response to injury and the role that endothelial NOS (NOS3) activity may play in the stimulatory actions of the cocktail *in vitro*. EC were grown in monolayer, and arrested in serum-free medium for 24 h. A comb of stainless steel pins was used to repeatedly scrape the cell layer. The injured EC were immediately treated with cocktail or left untreated for 4 h. Total RNA and protein was harvested 4 h after injury. Quantitative real-time RT-PCR analysis measured NOS3 mRNA levels relative to beta-actin. The results showed that the NOS3 mRNA levels at 4 h after injury increased in the cocktail-treated group compared with the control group (**Figure 3.8A**). Exposure of injured EC to cocktail for 4 h also led to increased levels of NOS3 protein expression (**Figure 3.8B**).

The role of NO production in the mechanism of cocktail inhibitory actions was further investigated in the *in vitro* scraping model of EC wound repair using the NOS inhibitor nitro-L-arginine methyl ester (L-NAME). It was demonstrated that L-NAME at 100 μM completely reversed the cocktail-stimulated EC wound repair in this *in vitro* model (**Figures 3.9A&B**).

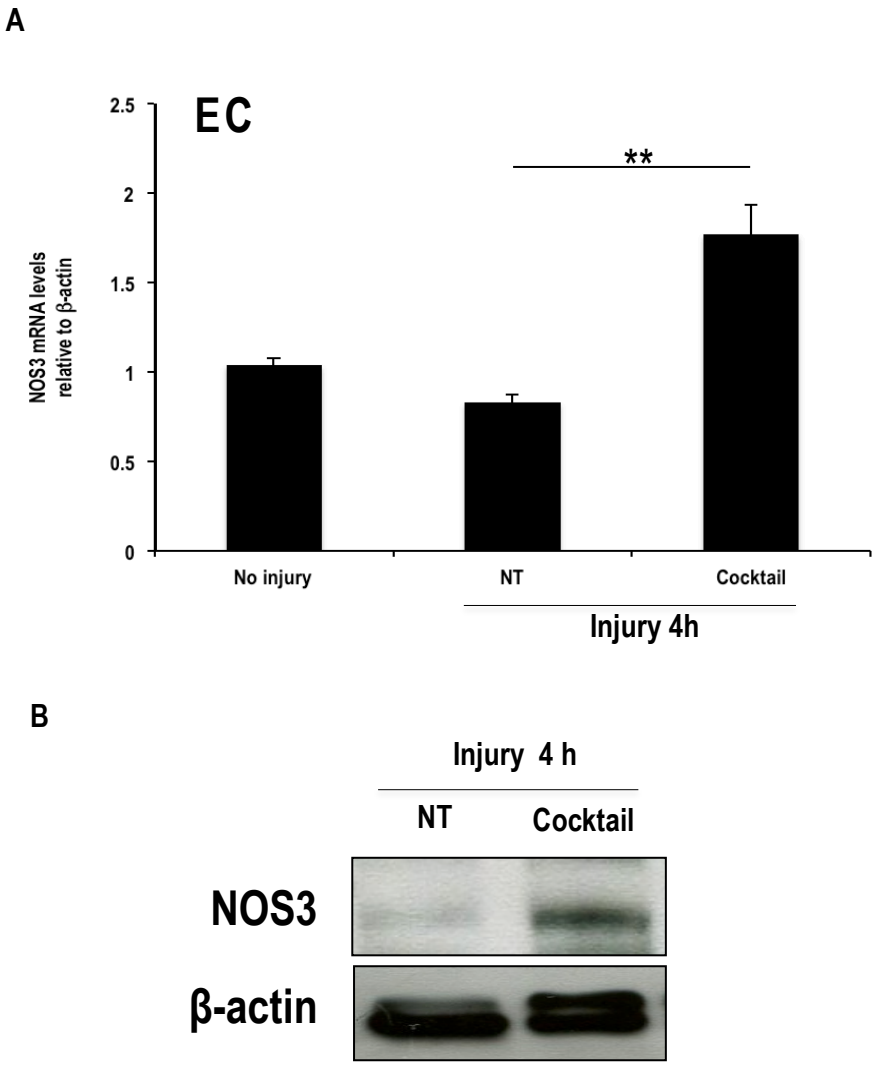


Figure 3.8 *Cocktail stimulates NOS3 expression in EC. Expression of NOS3 mRNA (**A**) and protein (**B**) levels were determined in EC subject to scraping injury with or without cocktail treatment (VEGF-A/D 50 ng/mL plus cRGD 200 nM) for 4 h by real-time qPCR or Western analysis, respectively. NOS3 mRNA levels were normalised to beta-actin expression (**A**) and represent mean ± SEM of 3 separate experiments performed in triplicate. (**B**) Representative Western blot of NOS3 protein expression in response to injury and cocktail with beta-actin used as a loading control. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. **p<0.01.*

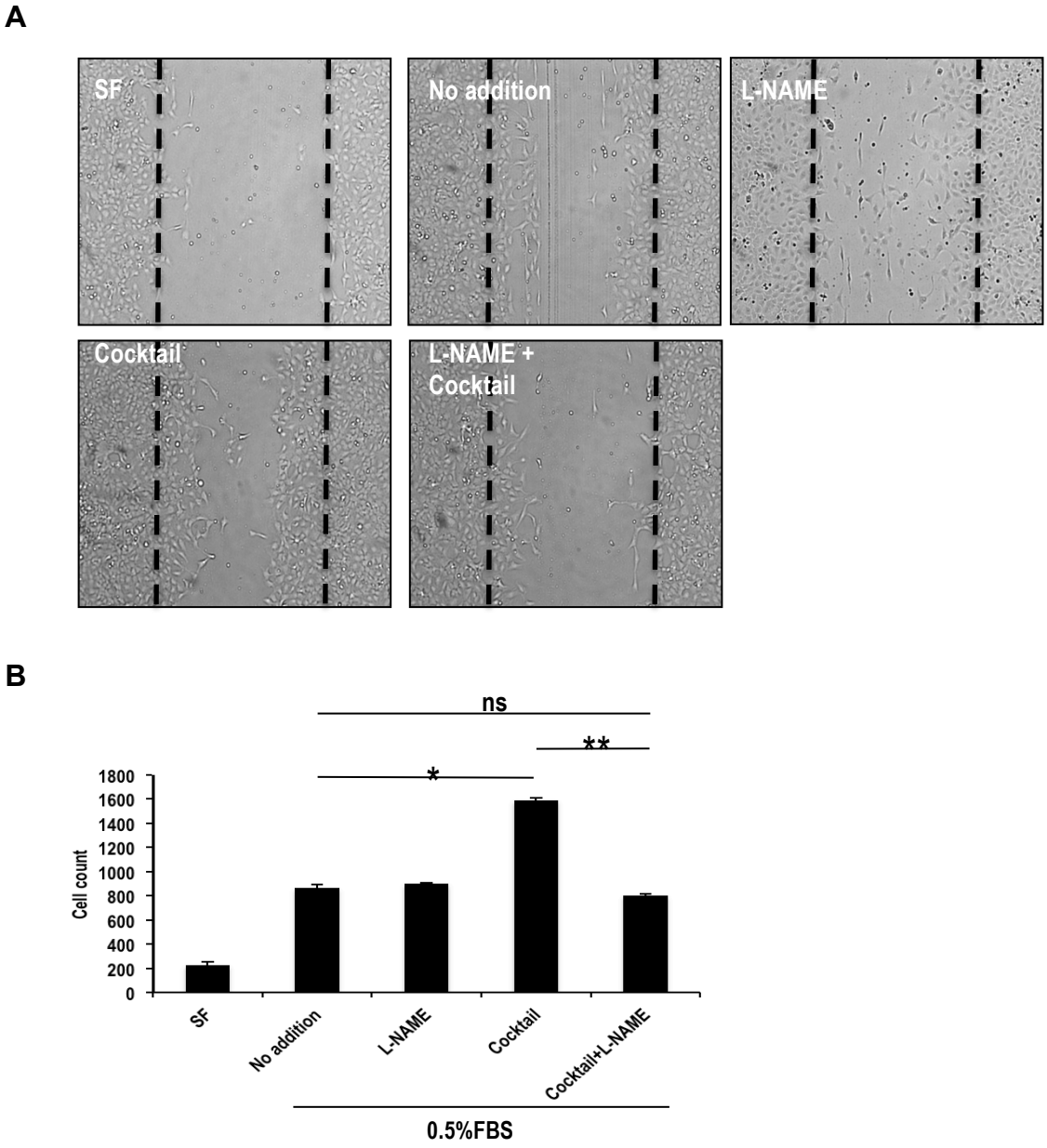


Figure 3.9 *L-NAME reverses the cocktail-stimulated EC wound repair. EC monolayers were serum-arrested then scraped with a sterile toothpick and treated with cocktail (VEGF-A/D 50 ng/mL plus cRGD 200 nM) in the presence of 0.5% (v/v) serum with or without the NOS inhibitor L-NAME (100 μM) and regrowth into the denuded area monitored over a 48 h period. (**A**) Representative figures of scratched injured EC. (**B**) Data represent mean ± SEM of 4 independent experiments. Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ns=not significant.*

3.2.5 Effects of cocktail on intimal thickening and re-endothelialisation in balloon- injured rat carotid arteries and role of NOS activity

Balloon injury to rat carotid arteries was next performed to determine the effect of the cocktail on intimal thickening. The carotid arteries were removed for morphometric analysis at 14 days post-injury. Artery cross sections were stained for elastin and were examined for intima (I) and media (M) cross-sectional areas and I/M ratios were calculated for each sample (**Figure 3.10A**). At 14 days, the intimal area and I/M ratios in the cocktail-treated group (high concentration) were significantly lower than that of the vehicle-treated group (**Figure 3.10B**). This inhibitory effect was reversed when animals were co-treated with cocktail (high concentration) and the NOS inhibitor L-NAME that completely prevented cocktail-mediated neointimal inhibition (**Figure 3.10B**).

To evaluate the effect of the cocktail on re-endothelialisation *in vivo*, endothelial coverage of the neointima was assessed by immunostaining for CD31. At 14 days, re-endothelialisation in the cocktail group was at least 4-fold greater than that of the control group, 19-fold greater than that of the cocktail with L-NAME group and 20-fold greater than that of L-NAME only group (**Figure 3.11**).

Consistent with the attenuating effects of L-NAME on cocktail-mediated EC effects both *in vitro* and *in vivo* and hence a role for NO signaling, NOS3 expression in cocktail-treated arteries was approximately 9-fold greater than that in the vehicle control group 14 days after balloon injury (**Figure 3.12)**. Similarly, co-treatment with L-NAME dramatically reduced cocktail-stimulated NOS3 expression (**Figure 3.12**).

The effect of the cocktail on SMC proliferation *in vivo* was further examined by the expression of the proliferation marker ki67. At 14 days, ki67 staining in the high cocktail group was significantly lower than that in the control group (**Figure 3.13**). Effects in the L-NAME group were not investigated.

Thus, taken together our findings reveal that a cocktail containing VEGF-A, VEGF-D and cRGD stimulates EC growth and EPC differentiation, inhibits SMC proliferation, prevents intimal thickening, stimulates NOS3 expression and remarkably, accelerates re-endothelialisation.

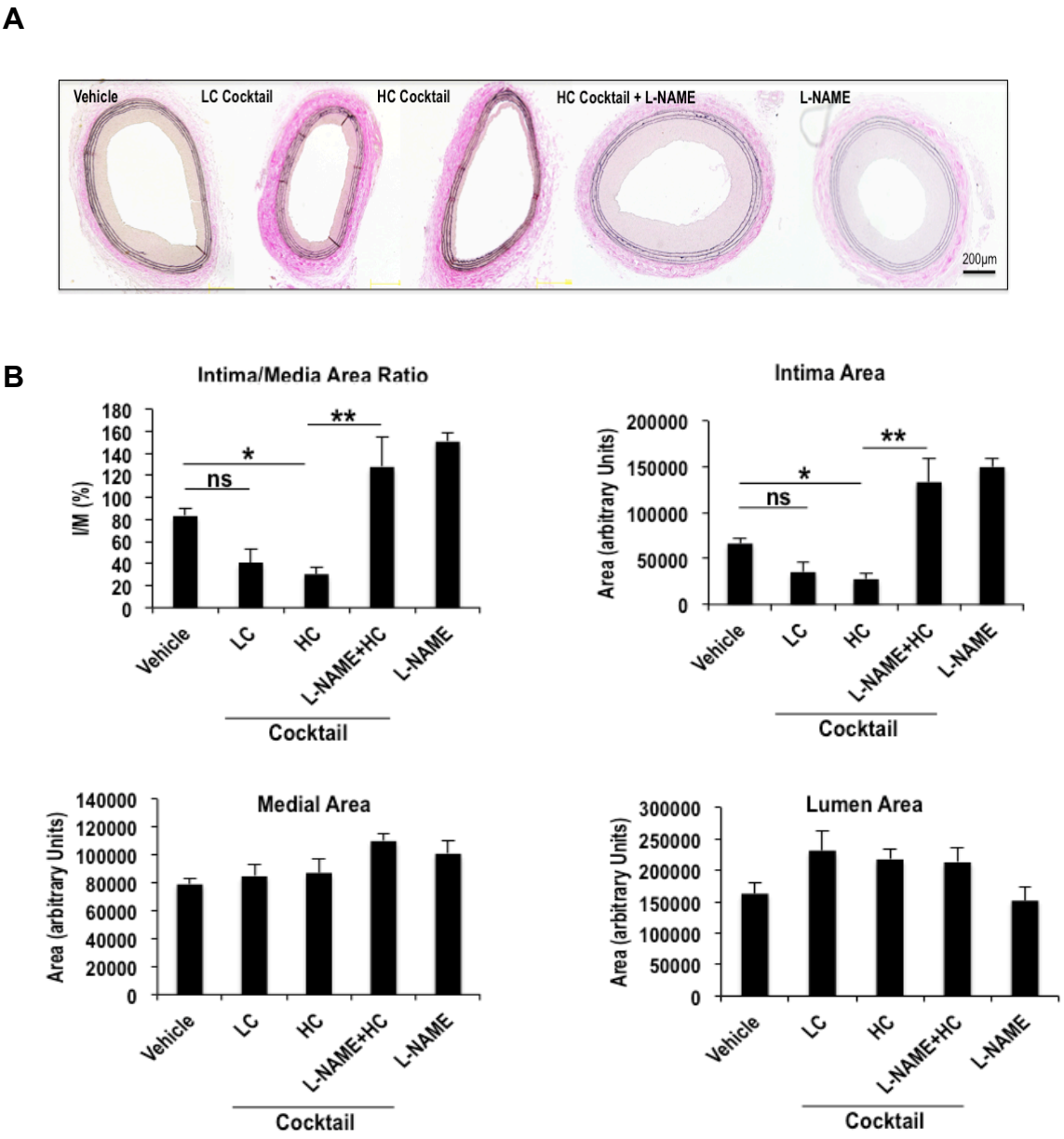


Figure 3.10 *Cocktail inhibits neointima formation in the rat carotid injury model. The left carotid arteries of Sprague-Dawley rats were subject to balloon injury and treated locally by Pluronic gel with vehicle (Pluronic gel only, n=6) or cocktail at low concentration (LC: VEGFA/D at 100 ng/mL, cRGD at 400 nM; n=4) or high concentration (HC: VEGFA/D at 2500 ng/mL, cRGD at 10 μM; n=6), with or without subcutaneous injections of L-NAME (3 mg/kg/day; n=4). An L-NAME alone treatment group with injury but no cocktail was also included (n=6). Neointima formation was examined after 14 days. (**a**) Representative photomicrographs (100X magnification) of Verhoeff van Gieson (elastin) stained carotid arteries 14 days after injury and treatment. (**b**) Intimal, medial and luminal areas were quantified using ImageProPlus software and mean ± SEM determined (n=4-6). Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ns=not significant.*

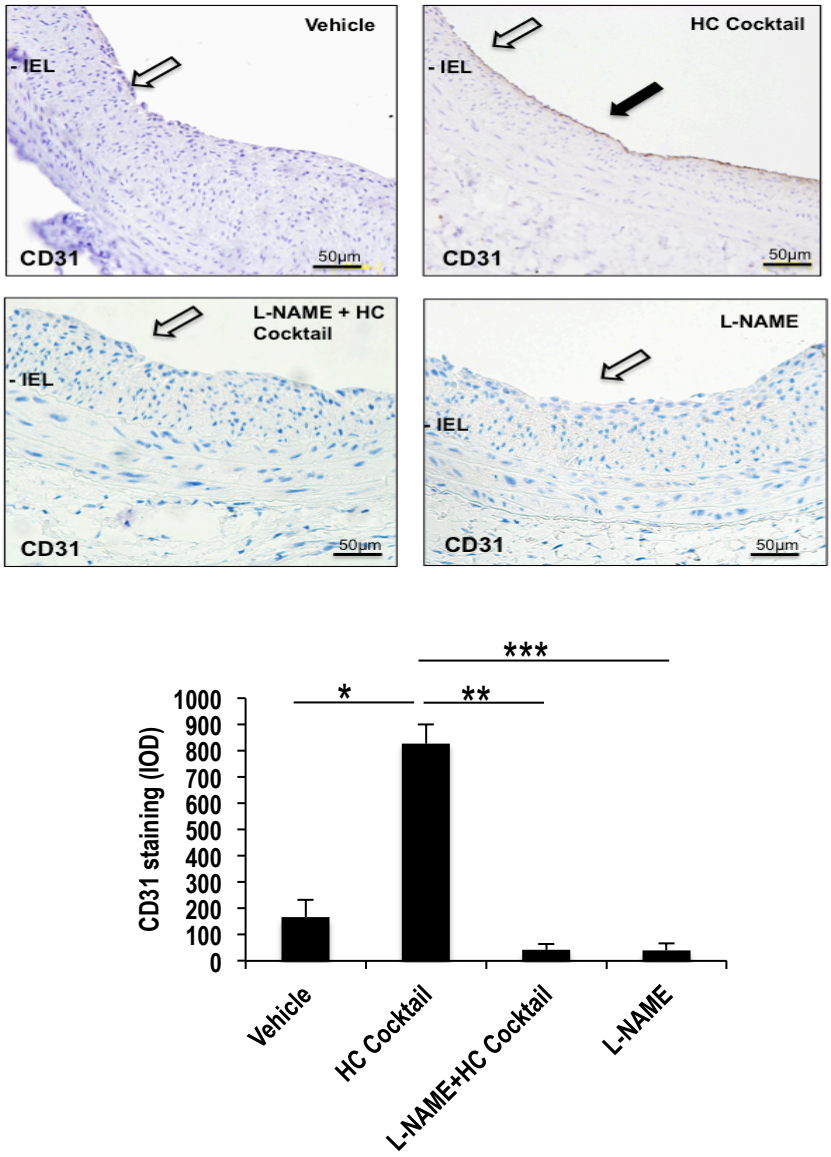


Figure 3.11 *Cocktail increases re-endothelialisation in injured rat carotid arteries. Re-endothelialisation was examined by immunohistochemical staining of the CD31 marker in fixed and embedded rat carotid artery sections taken 14 days after balloon injury and treatment with high concentration cocktail (HC: VEGFA/D at 2500 ng/mL, cRGD at 10 µM; n=6), with or without subcutaneous injections of L-NAME (3 mg/kg/day; n=4 or 6). Protein labeling was visualised with DAB. Sections were counterstained with haematoxylin. Photomicrographs show representative sections of neointimal area at 400X magnification. Open arrows indicate lack of endothelial staining. Closed arrows indicate positive staining. Integrated Optical Density (IOD) of positive staining was determined using software image-pro plus (Cybernetics) and mean ± SEM, n=4-6. (IOD = Area x Density) Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test.*p<0.05, **p<0.01, ***p<0.001.*

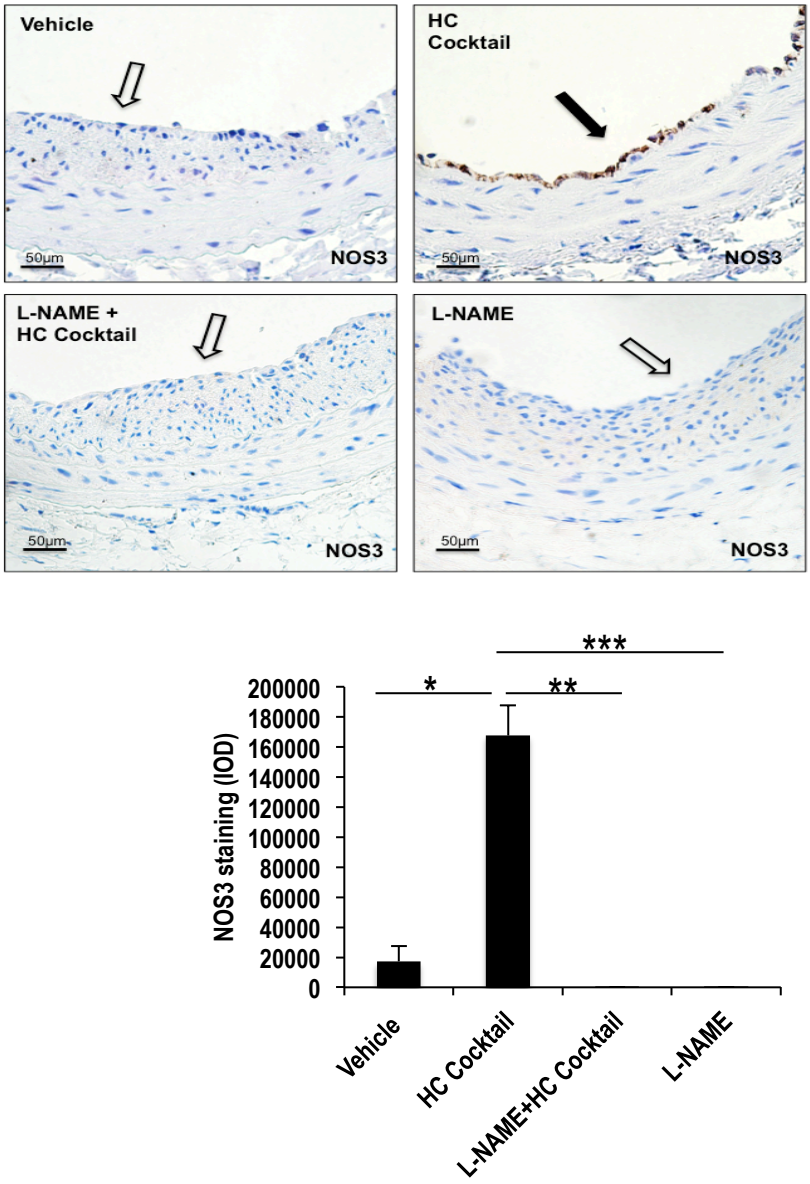


Figure 3.12 *Cocktail increases NOS3 expression in injured rat carotid arteries. NOS3 expression was examined by immunohistochemical staining of the NOS3 marker in fixed and embedded rat carotid artery sections taken 14 days after balloon injury and treatment with high concentration cocktail (HC: VEGFA/D at 2500 ng/mL, cRGD at 10 µM; n=6), with or without subcutaneous injections of L-NAME (3 mg/kg/day; n=4 or 6). Protein labeling was visualised with DAB. Sections were counterstained with haematoxylin. Photomicrographs show representative sections of neointimal area at 400X magnification. Open arrows indicate lack of NOS3 staining. Closed arrows indicate positive staining. Integrated Optical Density (IOD) of positive staining was determined using software image-pro plus (Cybernetics) and mean ± SEM, n=4-6. (IOD = Area x Density). Statistical differences were determined using the non-parametric Kruskal Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001.*

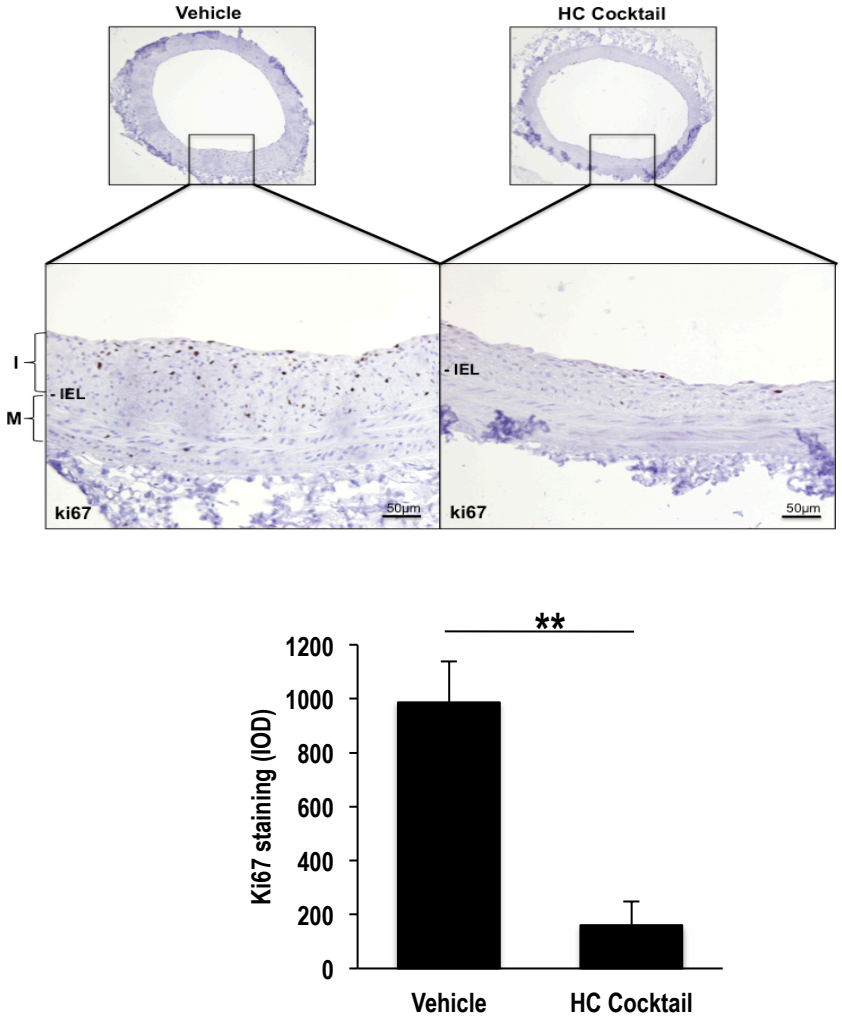


Figure 3.13 *Cocktail decreases expression of the proliferation marker ki67 in injured rat carotid arteries. Expression of the proliferation marker ki67 was examined by immunohistochemical staining of the Ki67 marker in fixed and embedded rat carotid artery sections taken 14 days after balloon injury and treatment with Vehicle (Pluronic gel only, n=6) and high concentration cocktail (HC: VEGFA/D at 2500 ng/mL, cRGD at 10 µM; n=6). Protein labeling was visualised with DAB. Sections were counterstained with haematoxylin. Photomicrographs show representative sections of neointimal area at 100X (small boxes) and 400X magnification. Integrated Optical Density (IOD) of positive staining was determined using software image-pro plus (Cybernetics) and mean ± SEM, n=6. (IOD = Area x Density). Statistical differences were determined using the non-parametric Mann-Whitney test. **p<0.01.*

3.3 Discussion

The ideal treatment for the longer-term success of anti-restenosis strategies is an agent, or a combination of agents, that inhibits SMC proliferation, stimulates re-endothelialisation and promotes EPC recruitment. These cellular events would prevent restenosis and late stent thrombosis and help fill current unmet clinical needs. The present study employed the rat carotid artery balloon injury model to demonstrate that a cocktail comprising VEGF-A, VEGF-D and cRGD significantly reduces neointima formation and accelerates re-endothelialisation. The cocktail inhibits SMC proliferation, promotes EC growth and stimulates eNOS expression *in vitro* and *in vivo*. *In vitro* analysis revealed that a cocktail comprising VEGF-A, VEGF-D and cRGD could promote EC proliferation and wound repair and stimulate EPC differentiation. Moreover, the cocktail inhibited SMC growth and wound repair after mechanical injury. In the rat balloon injury model, SMC are activated and the endothelium is completely removed as a consequence of balloon catheter inflation, triggering a reparative response and neointima formation within 14 days [274, 275]. In the present study model, the adventitial application of cocktail at the time of injury both restrained intimal thickening whilst promoting repair of the endothelium.

The mechanisms mediating the effects of the cocktail *in vitro* and *in vivo* are likely to reflect a combination of the modes of action of its individual excipients. Typically in EC, VEGF-A and VEGF-D signal through the tyrosine kinase receptor VEGFR-2, expressed on the endothelial surface, leading to the stimulation of endothelial prostacyclin (PGI_2) and nitric oxide (NO) production [105, 173]. NO is vital for the maintenance of endothelial function and regeneration [276, 277]. Disturbances in the NO pathway can lead to endothelial dysfunction [278]. Besides, NO promotes EPC recruitment, mobilisation, and differentiation through the phosphatidylinositol-3

kinase (PI 3-K)/protein kinase B (PKB)/Akt/endothelial nitric oxide synthase (NOS3)/nitric oxide (NO) signaling pathway [276]. PGI_2 also has a beneficial effect on EPC function through an NO-dependent mechanism [279].

NO is known to suppress SMC mitogenesis and mitochondrial respiration leading to reduced SMC proliferation [173, 280-282]. Thus in our study, cocktail stimulation of re-endothelialisation *in vivo* may increase endothelial NO production and have a paracrine effect on SMC proliferation. On the other hand, our *in vitro* data suggests that VEGF-D functions on SMC directly. It has been shown that VEGF can stimulate VEGFR-2 expression in SMC [283] and that SMC can express NOS3 and produce NO [284]. These make it possible for VEGF-D to induce an autocrine loop involving VEGFR2 and NO and hence inhibit SMC mitogenesis independently of EC activities [285].

Further beneficial effects of the cocktail may be attributed to the cRGD peptide which is selective for the avß3 integrin expressed on growth factor-activated EC [194] and promotes EC migration to VEGF [195]. VEGFR-2 associates selectively with the avß3 receptor [286]. The cRGD peptide with high affinity for the avß3 receptor facilitates the recruitment of circulating EPC, and then promotes re-endothelialisation and maintenance of endothelial integrity and function [105]. cRGD has also been shown to limit injury-induced SMC migration through the blockade of dominant SMC integrin (ß3)-matrix interactions [287]. It is a limitation of the *in vivo* portion of this study that the true origins of the cells that promote re-endothelialisation cannot be definitively determined due to the high technical difficulty in identifying cellular lineage without grafting labeled-EPC from bone marrow. This was beyond the scope of this work. It can only be postulated that they

may constitute either EPC derived from the circulation or EC that have regrown from the ends of the injured area. It can be hypothesised that both of these could re-populate the endothelium in response to cocktail given the *in vitro* activities described in this chapter. Previous studies have demonstrated the ability of EC to regrow from uninjured regions of the vascular wall [288]. Similarly, EPC colonisation of the endothelium has also been demonstrated previously [193].

In our present study, the NOS inhibitor L-NAME prevented the stimulatory effects of the cocktail on EC activities both *in vitro* and *in vivo*. L-NAME reduced cocktail-inducible EC wound repair, cocktail-inducible re-endothelialisation after balloon injury and reversed cocktail-mediated inhibition of neointima formation. The *in vivo* role of L-NAME/NO in counteracting the inhibitory effects of the cocktail was somewhat confounded by the increase in neointima formation occurring in response to L-NAME alone. This was not unexpected given the systemic nature of the L-NAME delivery and since NO has a multitude of homeostatic effects on the vasculature as a whole and a significant role in maintenance of blood pressure. Although we did not measure blood pressure in this study, our dose of 3 mg/kg/day was consistent with a dose range of 2-10 mg/kg/day used in other balloon injury studies that reported no change in blood pressure in contrast to rat hypertension models which use L-NAME at a dose of 100mg/kg/day [289, 290]. Despite this, the residual ability of L-NAME to counteract the cocktail-mediated inhibition of neointima formation *in vivo* and the cocktail-mediated stimulation of EC growth *in vitro* suggests that the clinically favorable biological features of the cocktail are mediated at least in part by NO.

Prior studies have used these agents separately but not in combination. For example, the randomised double-blinded, placebo-controlled Kuopio Angiogenesis Trial (KAT) II found that post-angioplasty catheter-mediated intracoronary VEGF-A gene transfer was safe and that treatment increased myocardial perfusion. However, six months follow-up showed no differences in the restenosis rate or minimal lumen diameter between the VEGF-A gene transfer group and placebo control [291]. Decreased neointima formation was demonstrated in response to local adenoviral delivery of VEGF-D in a rabbit balloon denudation model [173]. Similarly, Ark Therapeutics developed a locally applied VEGF-D gene transfer approach for the prevention of graft-vein stenosis in dialysis patients and demonstrated a reduction in intimal hyperplasia [174]. Adventitial application of cRGD was shown to limit SMC migration and neointima formation in the balloon-injured rat carotid artery model [287]. In pig coronary arteries, stents coated with cRGD were also shown to inhibit neointima formation and promote re-endothelialisation [193].

The effects of individual components of the cocktail were synergistic. VEGF-A is a crucial molecule eliciting EC proliferation, migration and tube formation [157]. It is necessary for EC survival and can enhance EC growth [160]. It has been reported that it increases re-endothelialisation in animal balloon injury models [292] but previous studies on the use of VEGF-A to inhibit neointimalformation have so far produced conflicting results [291-293]. VEGF-D has provided more solid evidence for inhibition of neointimal formation [173, 174], but not a satisfactory re-endothelialisation effect [173]. VEGF-A binds two tyrosine kinase receptors (VEGFR-1 and VEGFR-2) while VEGF-D binds to VEGFR-2 and VEGFR-3 which suggests that VEGF-A and VEGF-D actions may be complementary and that the

combination of them in a cocktail may better act to prevent restenosis than their individual use. The mechanism of cRGD inhibiting neointimal formation and enhancing re-endothelialisation is different from VEGF-A/D which again suggests an additive complementary effect would be expected when all three compounds are combined. In this study, the concentration of both VEGF-D and cRGD as components used in the cocktail is much lower than the concentration of each individual usage that has elicited a significant result in vitro experiments, and this indicates the synergistic effects of the components of the cocktail.

It is a limitation of this Cocktail study that in vivo the Cocktail was not compared with its individual components, however the degree of re-endothelialisation and inhibition of neointimal formation in the presence of the cocktail suggests a synergistic effect that would be greater than any individual component alone. It has previously been reported that VEGF-A, VEGF-D and cRGD individually inhibited neointimal formation in animal balloon injury models [173, 193, 292]. In these studies, VEGF-A, VEGF-D and cRGD independently decreased neointimal formation up to 34%, 52% and 42%, respectively. In this study, neointimal formation was reduced up to 64% with the cocktail. Similarly, previous studies have demonstrated enhanced re-endothelialisationup to 0.83-fold and 1.4-fold with independent VEGF-A and cRGD treatments, respectively [193, 292], while VEGF-D did not influence re-endothelialisation at all in a rabbit balloon denudation model [173]. Compare to this the Cocktail study, where re-endothelialisation in the cocktail group was at least 4-fold greater than that of the control group.

It was not tested in this study whether other combinations of VEGFA/D and cRGD would have significant effects. For example, whether VEGF-D and cRGD alone

would have had enough synergistic effects without VEGF-A. The individual properties of each molecule as extrapolated from the literature were considered to add value to the cocktail and hence the cocktail was formed with all three molecules. It cannot be concluded however that perhaps some other combinations may also have been effective at promoting endothelialisation while also inhibiting SMC proliferation and migration. This may need to be further investigated in another study. It is worth mentioning that several other “cocktails” were tested in preliminary studies prior to the choice of combination of VEGF-A/D and cRGD. These cocktails were Cocktail 1 (VEGF-A and Dz13), Cocktail 2 (VEGF-A, FGF-2 and cRGD) and Cocktail 3 (VEGF-A/D, cRGD and adenovirus-YY1). As in vitro proliferation experiments showed Cocktail 1 inhibited EC proliferation and Cocktail 2 had no satisfactory inhibition effect on SMC proliferation, both Cocktail 1 and 2 were excluded. Cocktail 3 did not show better effects than the combination of VEGF-A/D and cRGD, thus Cocktail 3 was also excluded.

In summary, this study has demonstrated that a cocktail of VEGF-A, VEGF-D and cRGD significantly reduces SMC proliferation and promotes EC proliferation *in vitro*, and following balloon injury to rat arteries, inhibits SMC proliferation, neointima formation and accelerates re-endothelialisation. This combination may be useful for reducing in-stent or in-segment restenosis and late thrombosis.

4 Chapter IV

Inhibition of Vein Graft Stenosis After Autologous End-to-Side Transplantation in Rabbits Using a c-Jun Targeting DNAzyme in DOTAP/DOPE Formulation

4.1 Introduction

Coronary artery bypass grafting (CABG), which was first introduced in the 1960s [86-88], is the most common cardiac surgical procedure with around 1 million CABG procedures performed annually worldwide [89]. It is currently the accepted revascularisation strategy to treat appropriate patients with three-vessel or left main coronary artery disease [90, 91]. Especially for patients with diabetes and advanced coronary artery disease, CABG was superior to PCI with drug-eluting stents (DES) [294]. According to FREEDOM clinical trials (1900 patients), CABG significantly reduced rates of death and myocardial infarction compared with DES implantation [294]. Saphenous vein grafts remain the most common type of conduits that are used for CABG [92]. However, vein grafts have limited life expectancy [92][93, 94]. More than 40% vein grafts were occlusive at 10 years after CABG [95]. Vein graft failure results in worse clinical outcomes. A retrospective analysis showed death, nonfatal myocardial infarction (MI) or revascularisation in vein graft failure patients were significantly higher than that among the patients without vein graft failure [295].

Key to the pathogenesis of vein graft failure after surgery is vascular smooth muscle cell (SMC) proliferation. The resident cells in the grafted vein are exposed to a new microenvironment, with a static-to-shear insult, increased shear stand pulsatile stress, circumferential stress, and radial deformation [96]. The graft has to adapt to higher arterial pressures. SMC eventually accumulate in the intima [97] and proliferation causes intimal hyperplasia. A potential approach to address this problem is to effectively inhibit SMC proliferation and migration. The statin class of drugs has reduced atherogenesis, however there is no proven effective

pharmacologic agent to inhibit smooth muscle cell growth to prevent vein graft failure [148].

Dz13 is a DNAzyme bearing 9+9 arms and a 10-23 catalytic domain that binds and cleaves c-Jun mRNA. Our laboratory has previously demonstrated that c-Jun plays an important role in injury- and shear-inducible transcriptional networks implicated in SMC proliferation and intimal hyperplasia using Dz13 in the proprietary lipid blend FuGENE6 (Roche) [71, 148]. FuGENE6 is not authorised for clinical use thus preventing the clinical translation of Dz13 in this cardiovascular setting. As an alternative, the transfection agent 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) has been used in human gene therapy trials and is safe [296]. Dz13 in DOTAP/DOPE was recently used to cause regression of pre-existing neovascularisation in mice [297] and inhibited the growth of squamous cell carcinoma and basal cell carcinoma in murine models [298]. This current study set out to determine the effect of Dz13 delivered in a DOTAP/DOPE formulation on SMC growth *in vitro* and intimal thickening in a rabbit model of autologous end-to-side transplantation.

4.2 Results

4.2.1 Effect of Dz13 and Dz13.G>C in DOTAP/DOPE on serum-inducible vascular SMC proliferation

Growth-quiescent cultured SMC were treated with Dz13 or Dz13.G>C (0.6 μM) (the negative control containing a single mutation in the active site) in DOTAP/DOPE and cell numbers in medium-containing serum were determined using the Xcelligence system, which quantifies cell growth in real time. Dz13 in DOTAP/DOPE reduced SMC proliferation compared with Dz13.G>C over the course of 48 h **(Figure 4.1**).

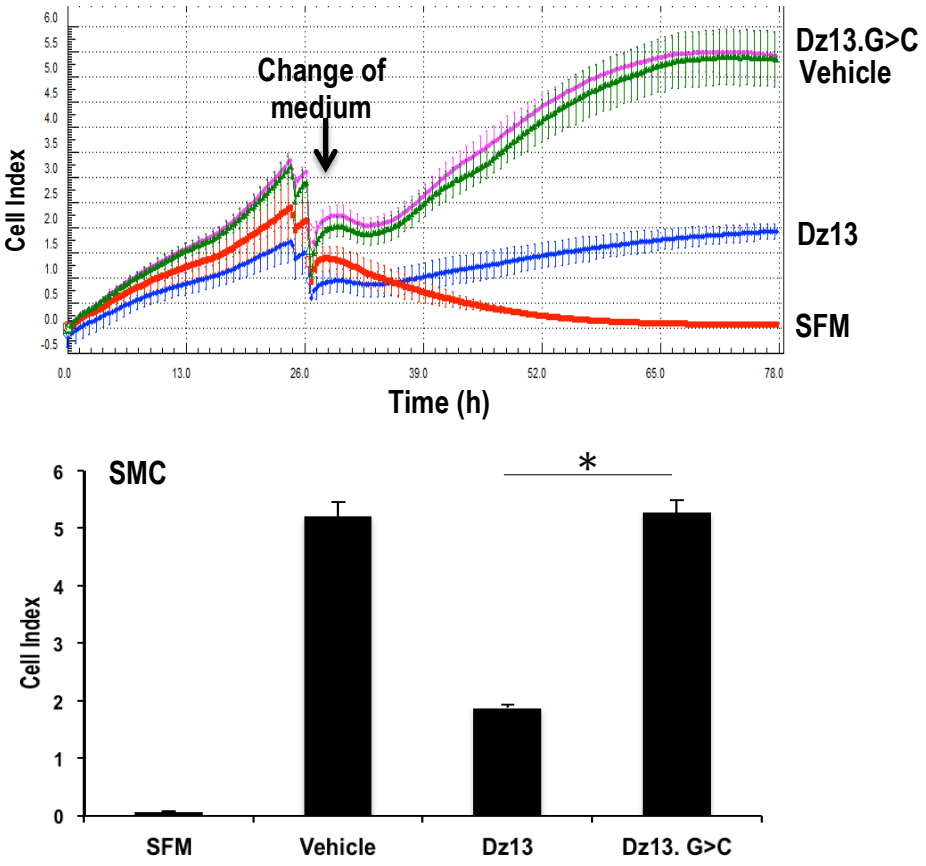


Figure 4.1 Effect of Dz13 in DOTAP/DOPE on SMC proliferation.

*SMC were grown in 96-well plates and rendered growth quiescent in serum-free medium for 24 h, then treated with Dz13 or Dz13.G>C in DOTAP/DOPE at 37°C for 24 h. The medium was then changed to 5% (v/v) FBS containing medium or serum-free medium. Cell populations were determined using the Xcelligence system. Dz13 in DOTAP/DOPE (0.6 μM) reduced SMC proliferation compared with Dz13.G>C. Differences between groups were tested for statistical significance using Kruskal Wallis test (Dunn's Multiple Comparison test). SFM denotes serum-free medium. * indicates statistical significance P<0.05.*

4.2.2 Dz13 in DOTAP/DOPE reduces c-Jun expression in vitro

Growth-quiescent SMC were treated with Dz13 or Dz13.G>C in DOTAP/DOPE for 24 h and incubated in medium-containing serum for another 2 h. Western blotting demonstrated that Dz13 in DOTAP/DOPE reduced levels of c-Jun protein compared with Dz13.G>C treatment **(Figure 4.2).** Imaging under fluorescence microscopy *demonstrates typical SMC uptake of FITC-labeled Dz13 under 400X magnification 24 h after transfection with DOTAP/DOPE.* (**Figure 4.2**).

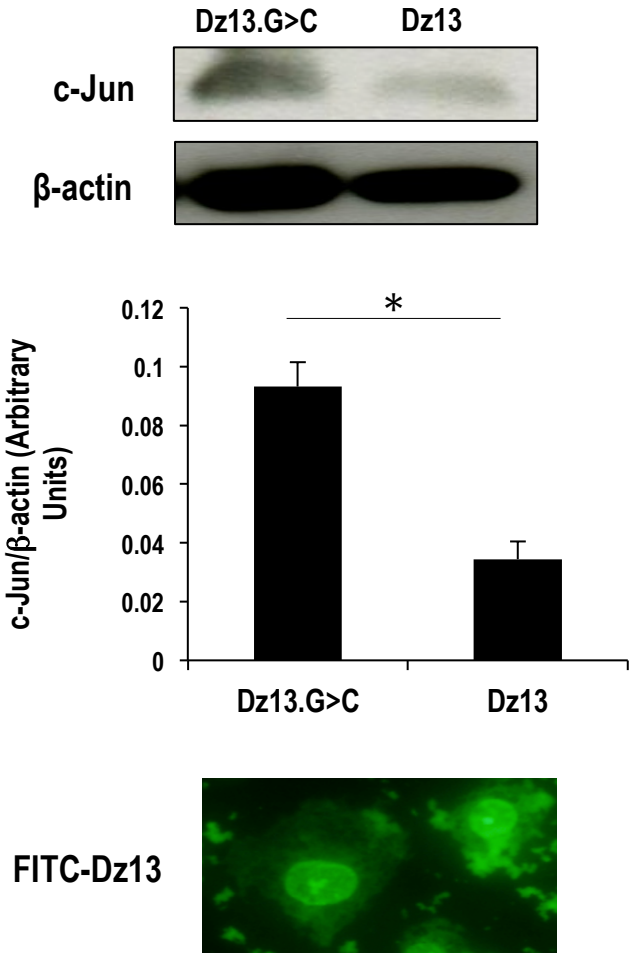


Figure 4.2 Dz13 in DOTAP/DOPE reduces c-Jun expression in vitro.

*SMC were grown in 100 mm petri-dishes and rendered growth quiescent in serum-free medium for 24 h, then treated with Dz13 or Dz13.G>C in DOTAP/DOPE at 37^{o}C for 24 h. The medium was then changed to 5% (v/v) FBS containing medium for 2 h. Western blot and densitometry shows that Dz13 in DOTAP/DOPE reduced c-Jun protein levels compared with Dz13.G>C. Differences between groups were tested for statistical significance using the Mann-Whitney U test, * indicates statistical significance P<0.05.*

4.2.3 *In vivo* delivery of Dz13 in DOTAP/DOPE

To demonstrate the *in vivo* delivery of Dz13, rabbit jugular veins were incubated with FITC-Dz13 in DOTAP/DOPE for 30 min at 37^{o}C. Visualisation of green fluorescence image against the blue DAPI-stained nuclei revealed that the DNAzyme was delivered to the tissue to be grafted within this time period, thus further experiments were performed using this protocol **(Figure 4.3).**

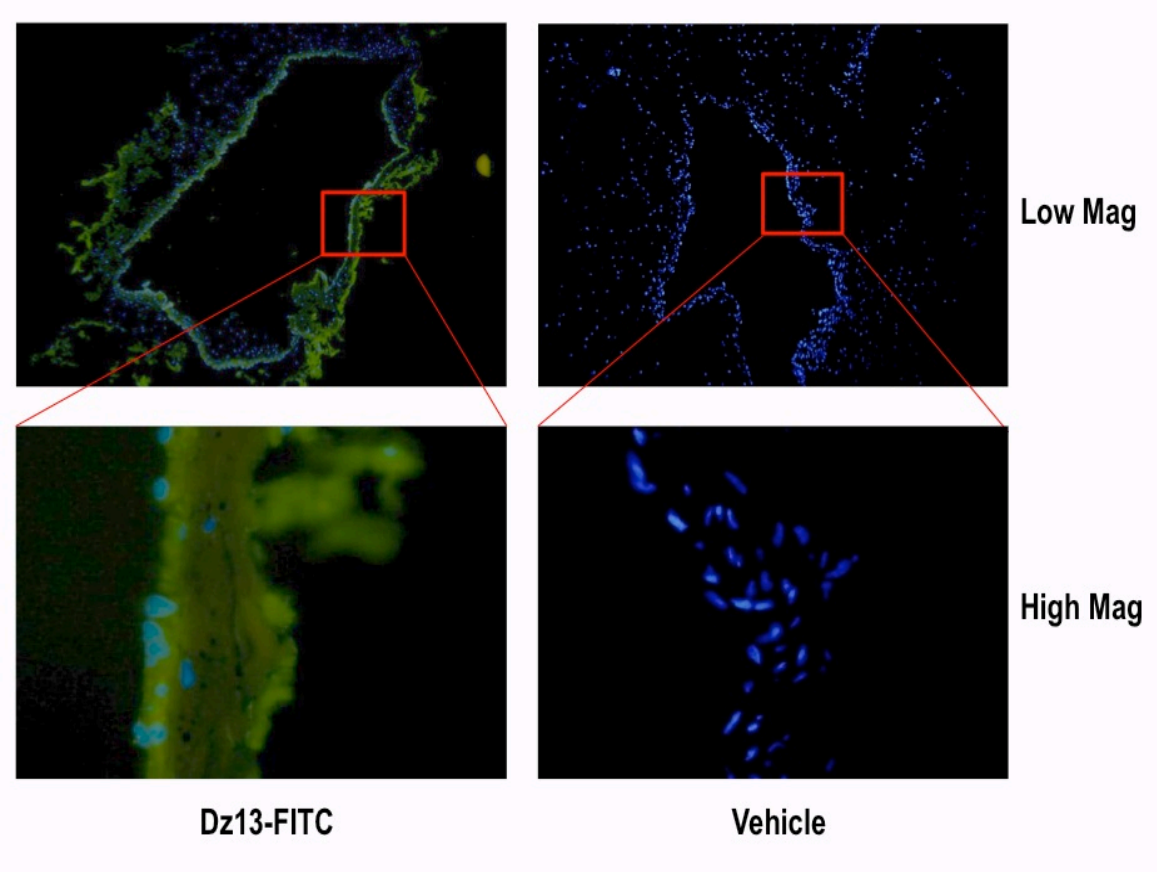


Figure 4.3 Transfection of Dz13 in DOTAP/DOPE in veins.

Rabbit jugular veins were incubated in 300 µL transfection solution containing 500 µg FITC-Dz13 (TriLink Biotechnologies) and 37.5 µL DOTAP/DOPE (Avanti Polar Lipids) at 37°C for 30 min, then rinsed in PBS and snap frozen with OCT. Blocks were sectioned, placed onto slides and fixed in 95% (v/v) ethanol. DAPI nuclear staining (blue) was imaged under fluorescence microscopy. Green fluorescence demonstrates that Dz13 was delivered into rabbit veins.

4.2.4 Effect of Dz13 in DOTAP/DOPE on intimal hyperplasia 28d after autologous vein bypass grafting

To investigate the effect of the Dz13 in DOTAP/DOPE on intimal hyperplasia in a rabbit model of bypass grafting, autologous end-to-side transplantation of the jugular vein to the carotid artery was performed. Vein grafts were collected 28 days after transplantation. Typically these grafts underwent intimal thickening as would occur in human bypass failure.

These vein grafts were subjected to an endovascular scan using OCT. The rate of patency (OCT catheter can go through the lumen of vein graft) was respectively 1/5, 5/5 and 2/5 in Vehicle, Dz13 and Dz13.G>C groups. This revealed that vessels treated with Dz13 in DOTAP/DOPE had a trend of reducing neointima formation compared with the Dz13.G>C treated group. The ratio of intima to total vessel area in the Dz13 group was approximately half that of the Dz13.G>C group **(Figure 4.4)**. More rabbits are needed in this study for statistic analysis as the vein grafts patency rate are quite low in Vehicle and Dz13.G>C groups.

Vein graft cross sections were stained with Verhoeff van Geison's elastin stain and examined for intima and total vessel areas, and the ratio of intimal area to total vessel area was obtained for each sample. Intimal thickening in the Dz13 treated group was greatly reduced by half comparing with the Dz13.G>C or vehicle groups **(Figure 4.5)**. Reduced proliferation within the vessel was confirmed by immunostaining for PCNA. Expression of this mitogenesis marker was significantly lower in the Dz13 group than the Dz13.G>C group (**Figure 4.6).**

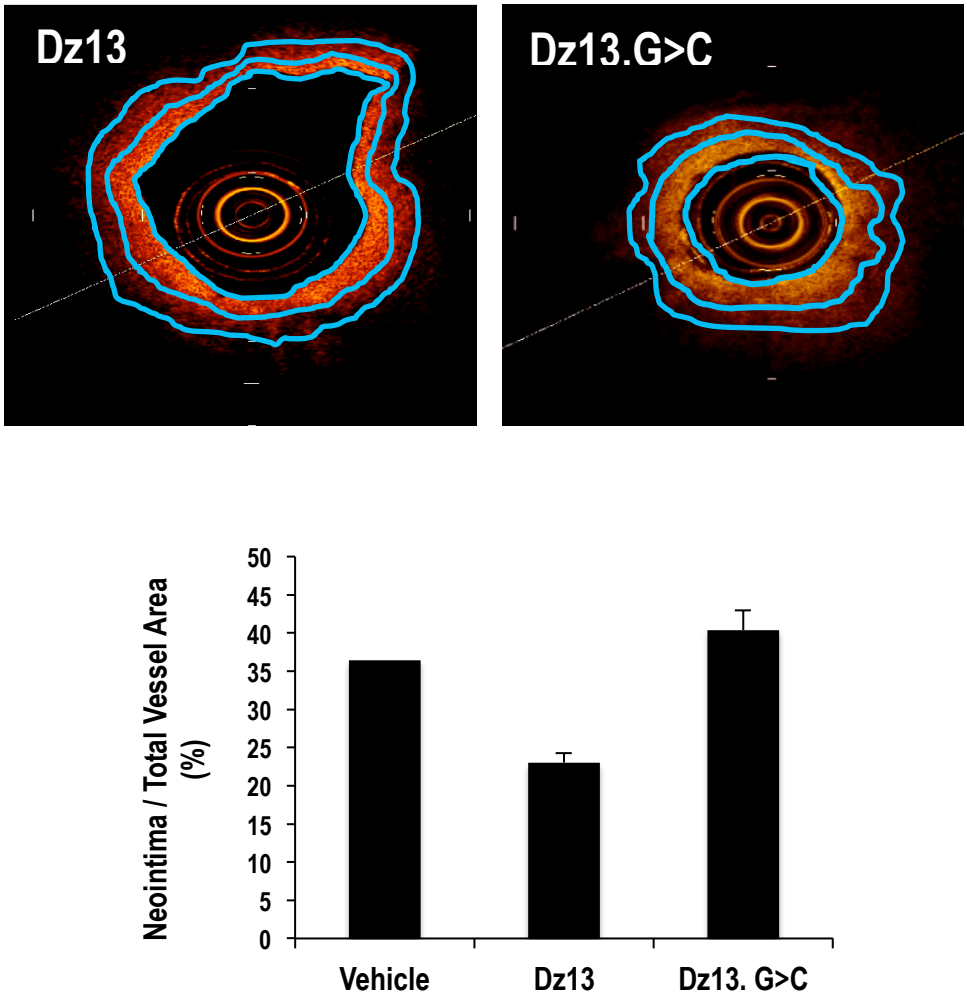


Figure 4.4 Effect of Dz13 with DOTAP/DOPE on intimal hyperplasia in rabbit vein bypass grafts using optical coherence tomography (OCT).

At twenty-eight days after transplantation, vein grafts were collected and subjected to endovascular scan using OCT. The Dz13 (n=5) showed a trend of reducing neointima formation compared with the Dz13.G>C group (n=2).

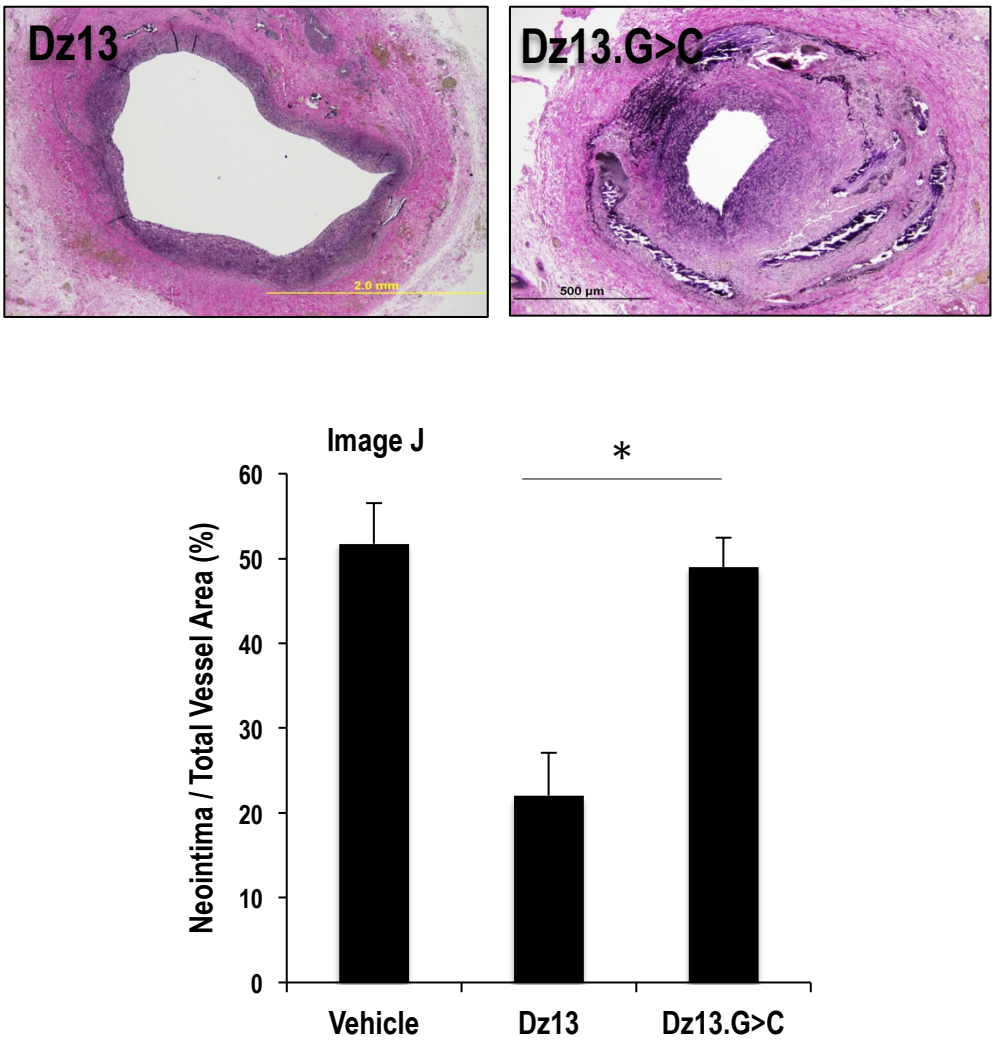


Figure 4.5 Effect of Dz13 in DOTAP/DOPE on intimal hyperplasia in rabbit vein bypass grafts by morphometric analysis.

*Twenty eight days after transplantation, vein grafts were collected and cross sections were stained with Verhoeff van Geison's elastin stain. The ratio of intimal area to total vessel area in the Dz13 treated group was significantly lower than in the Dz13.G>C or vehicle groups. Differences between groups were tested for statistical significance using Kruskal Wallis test (Dunn's Multiple Comparison test). * indicates statistical significance P<0.05.*

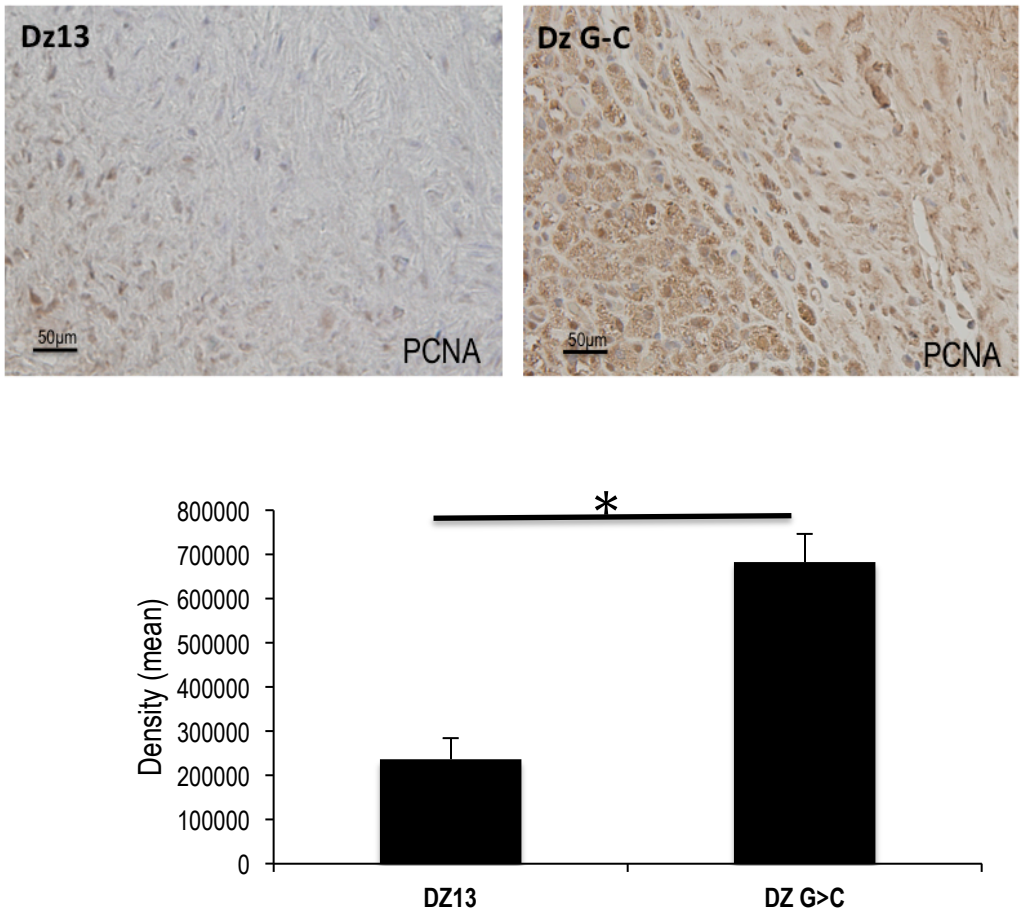


Figure 4.6 Dz13/DOPE/DOTAP decreases PCNA expression after bypass graft transplantation.

*Immunohistochemical analysis was performed for PCNA in consecutive paraffin-embedded sections of formalin-fixed rabbit vein bypass graft. At 28 days, PCNA staining in the Dz13 group was significantly lower than that in the Dz13.G>C. Differences between groups were tested for statistical significance using the Mann-Whitney U test, * indicates statistical significance P<0.05.*

4.2.5 Decreased c-Jun expression in vein grafts after transplantation

The effect of Dz13 in DOTAP/DOPE on the vessel wall *in vivo* was further examined through the expression of c-Jun. At 28 days, c-Jun staining in the Dz13 group was significantly lower than that in the Dz13.G>C and vehicle groups, with the magnitude of the reduction consistent with the extent of Dz13 inhibition of intimal thickening (**Figure 4.7**).

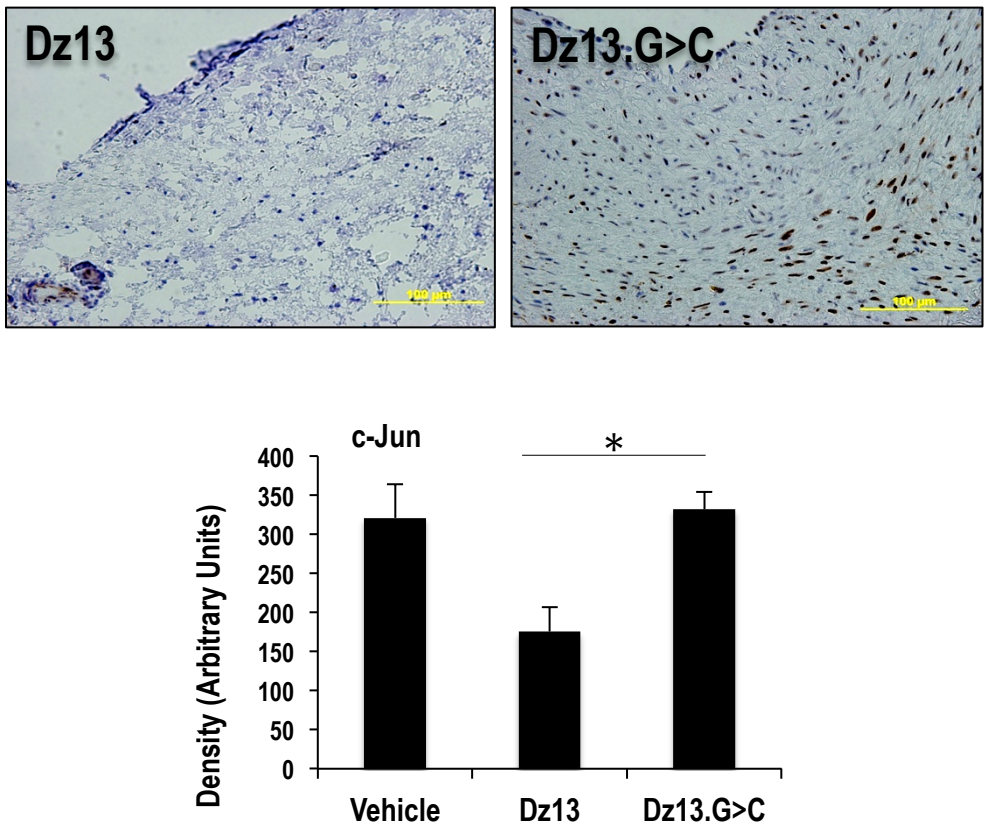


Figure 4.7 Dz13 in DOTAP/DOPE decreases c-Jun expression after bypass graft transplantation.

*Immunohistochemical analysis was performed for c-Jun in consecutive paraffin-embedded sections of formalin-fixed rabbit vein bypass graft. At 28 d, c-Jun staining in the Dz13 group was significantly lower than that in the Dz13.G>C or Vehicle groups. Data is represented mean +/- SEM (N=3-5). Differences between groups were tested for statistical significance using Kruskal Wallis test (Dunn's Multiple Comparison test). * indicates statistical significance P<0.05.*

4.3 Discussion

DNAzymes have demonstrated potential for use as inhibitory agents in the therapy of various experimental diseases. These synthetic agents are composed entirely of phosphodiester-linked DNA that bind and cleave their target mRNA, destabilising the mRNA and preventing c-Jun synthesis. Using this gene-silencing approach, the basic leucine zipper protein c-Jun have been targeted in a variety of disease settings such as cancer [298, 299], acute inflammation [203], rheumatoid arthritis [203], arterial thickening [71, 72] and ocular neovascularisation [203, 297]. Dz13 also inhibits intimal hyperplasia in human saphenous veins and vein bypass graft stenosis in rabbits using FuGENE6, a transfection agent that cannot be used in humans [148].

In this study, the biological activity of Dz13 in the context of SMC hyperplasia using a liposomal formulation comprising DOTAP/DOPE was investigated. Dz13 in DOTAP/DOPE inhibited SMC proliferation and c-Jun protein expression *in vitro.* In the rabbit bypass graft model, the jugular vein was transplanted end-to-side to the carotid artery. This triggered neointima formation within 28 d. The vein graft was exposed to Dz13 in DOTAP/DOPE prior to autologous transplantation, analogous to the local delivery approach employed previously for the *E2F decoy* oligodeoxynucleotide Edifoligide [148]. That Dz13 was delivered into the vein conduit before the anastomosis was confirmed by visualising FITC-labeled Dz13 under fluorescence. Morphometric and OCT analysis demonstrated that Dz13 in DOTAP/DOPE inhibited neointima formation and increased luminal size at the conclusion of the study. Immunohistochemical analysis further demonstrated that Dz13 inhibited the expression of its target c-Jun in the vessel wall. In all experiments, Dz13 was compared with its size-matched counterpart, Dz13.G>C,

which carries the same 9+9 hybridizing arms and 15nt “10-23” catalytic domain as Dz13 except for a single point mutation in the catalytic domain.

Different from our previous study in rabbits, this study evaluated Dz13 delivery and efficacy in a formulation containing DOTAP/DOPE, rather than FuGENE6. The former transfection agent DOTAP/DOPE has been tested clinically and is safe and well tolerated in a local delivery setting [300]. FuGENE6 is a proprietary multi-component lipid-based transfection reagent with its ingredients and concentrations unspecified, thus presenting potential regulatory challenges in clinical translation. Recently, our lab cooperated with Koch Institute for Integrative Cancer Research (Boston, USA) to show the Dz13 (500 μg)/DOTAP/DOPE mixture formed lipoplexes and were colloidally stable over the experimental procedure, with lipoplex formation prior to anastomosis. Furthermore, a catalytically-inactive DNAzyme was used here rather than a DNAzyme with scrambled hybridization arms to control for Dz13. These studies demonstrate the versatility of Dz13's inhibitory effect on intimal thickening in vein conduits in relation to lipid carrier and bring Dz13 closer to clinical application for the prevention of CABG failure.

DOTAP and DOPE have been widely studied. DOPE is a zwitterionic lipid, and DOTAP a cationic lipid. Dz13 in a formulation containing DOTAP/DOPE recently underwent preclinical toxicological testing in a range of species including mice, rats, minipigs and monkeys [298] and a Phase I clinical trial in skin cancer patients indicated that it was safe and well tolerated after local delivery [300]. Studies by our group using this formulation also showed that Dz13 reduced retinal microvascular density and improved forepaw reach in mice [297]. The present demonstration that Dz13 in DOTAP/DOPE can inhibit intimal thickening in larger blood vessels

suggests that this formulation may provide a safe and effective strategy in CABG and other human settings amenable to local delivery.

5 Chapter V

Role of MicroRNA191 in Neointima Formation

5.1 Introduction

The development of post angioplasty restenosis impedes the long-term success of PTCA, bypass graft, ischemia-reperfusion and leads to morbidity and mortality of patients. The main reason for restenosis is the proliferation and migration of VSMC induced by mechanical injury. In this process, the immediate-early gene Egr-1 plays an important role. Normally, the expression of Egr-1 in VSMC is at low or undetectable levels, however, a rapid and transient increase of Egr-1 expression in VSMC in the arterial wall occurs within minutes after injury. During this process, Egr-1 protein works as a master regulator of migration and proliferation via the control of an array of downstream genes by initiating transcription at promoters containing specific recognition elements [254, 255, 301, 302].

In previous studies performed in our laboratory, siRNA or DNAzyme targeting Egr-1 were administered to reduce Egr-1 protein levels, which resulted in a suppression of restenosis in a rat balloon-injury model [303]. These findings proved that inhibition of this pathway could be a potential clinical strategy to attenuate restenosis. However, both siRNAs and DNAzymes are artificial, unnatural molecules that are designed to target and destroy Egr-1. For this reason our group set out to investigate naturally present microRNAs in VSMC, attempting to identify regulatory effects on Egr-1 levels that may play a role in the control of restenosis after injury.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs that act as gene regulators by degrading or inhibiting the translation of mRNA [304-306]. In the nucleus, miR genes are transcribed by RNA polymerase II or III into primary-miR

(pri-miR), which are processed by Drosha-DGCR8 to generate precursor-miR (pre-miR). Pre-miR are exported by Exportin-5-Ran-GTP into the cytoplasm and further edited by a complex comprising Dicer-TRBP, creating a miR/miR* duplex. The passenger strand, miR*, is released and degraded. The mature guide miR is incorporated into a miRISC complex which binds to target mRNAs inducing mRNA degradation or translational repression [204]. MicroRNAs most commonly target the 3' untranslated (3'UTR) region of mRNAs. The role of miRNAs as regulators has been identified in many biological behaviors, including cell differentiation, proliferation, apoptosis and tissue development [307]. Recent studies have identified a reciprocal relationship between miR-191 and Egr-1 [308] [252]. In prion disease, Egr-1 is consistently down-regulated, whereas miR-191 is consistently up-regulated [253]. In the context of cardiovascular disease, microarray studies have indicated that miR-191 is down-regulated after injury, consistent with a reciprocal relationship with Egr-1 which is simultaneously up-regulated [248]. Recent *in vitro* studies in our laboratory performed by Ms Margaret Patrikakis have now indicated that overexpression of miR-191 in VSMC can suppress Egr-1 up-regulation after scraping injury and reduce proliferation and migration. These studies show a direct link between miR-191 and Egr-1 in VSMC *in vitro* and a potential role in the vascular system. Restenosis, however, is a complicated biological process involving the participation of many cell types which may affect the function of miR-191 *in vivo*. The aim of this work was therefore to investigate the effect of miR-191 on injury-induced intimal thickening in the rat carotid balloon injury model.

5.2 Results

5.2.1 miR-191 overexpression in VSMCs reduces Egr-1 protein levels after scraping injury. (This experiment was performed by Ms Margaret Patrikakis)

To investigate the effect of miR-191 on Egr-1 expression in VSMCs after scraping injury, Western blot analysis was performed by Ms Margaret Patrikakis. VSMCs were starved in serum free medium and then transfected with 50, 100 or 200nM of the miR-191 inhibitor (2'OMe-191), mimic (pre-191), pre-191control (pre-CTL) and miR-191 inhibitor control (anti-CTL). Transfected VSMCs were then scratch injured and 1 h later the total protein was extracted. Over-expression of miR-191 with pre-191 greatly suppressed Egr-1 protein expression in VSMCs after scraping injury at concentration of 100 nM and 200 nM. (**Figures 5.1**)

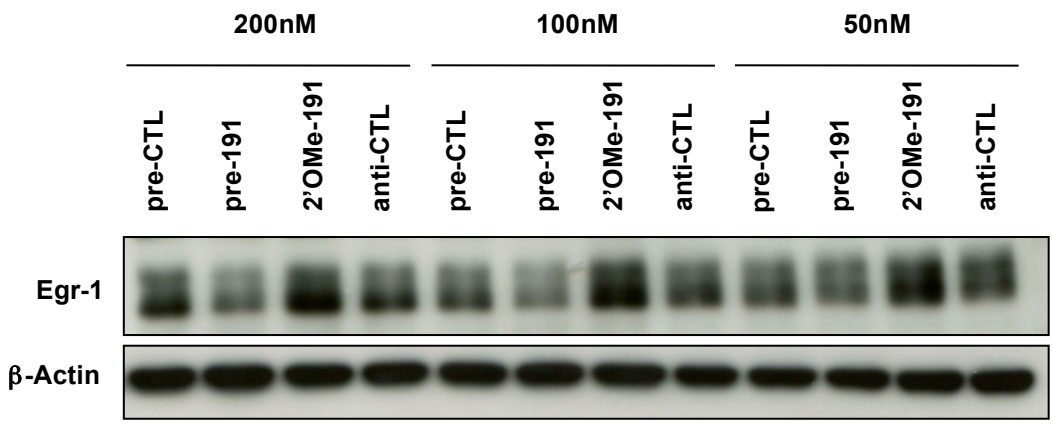
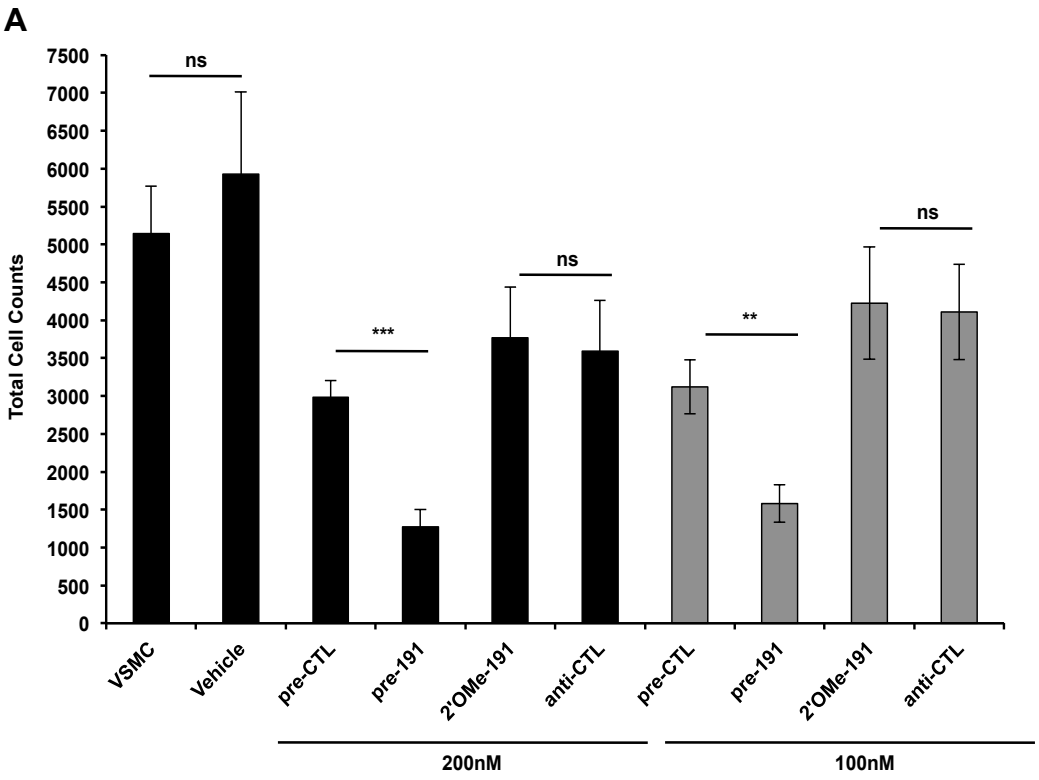


Figure 5.1 Over-expression of miR-191 inhibits Egr-1 protein levels after scraping injury in VSMCs. *(This experiment was performed by Ms Margaret Patrikakis)*

Egr-1 protein expression at 1 h after scratch injury in VSMCs which were priorly transfected with 50 nM, 100 nM and 200nM of pre-191, miR-191 antisense (2'OMe-191) and their respective controls (pre-CTL & anti-CTL). β-actin was used as the protein loading control. Pre-191 significantly suppressed Egr-1 protein expression in VSMCs after scraping injury at the concentration of 100 nM and 200 nM.

5.2.2 Over-expressing miR-191 inhibits serum inducible VSMCs proliferation and migration. (These experiments were performed by Ms Margaret Patrikakis)

To investigate both VSMCs proliferation and migration in relation to miR-191, Ms Margaret Patrikakis performed VSMCs proliferation and *in vitro* transwell migration assays. The results showed that over-expression of miR-191 with pre-191 suppressed serum-inducible VSMCs proliferation within 96 h in a dose-dependent manner (**Figure 5.2A**) and significantly reduced the ability of serum starved VSMCs to migrate toward 10% (v/v) serum chemoattractant (**Figure 5.2B**).


A
Total Cell Counts
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4000
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VSMC
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2'OMe-191
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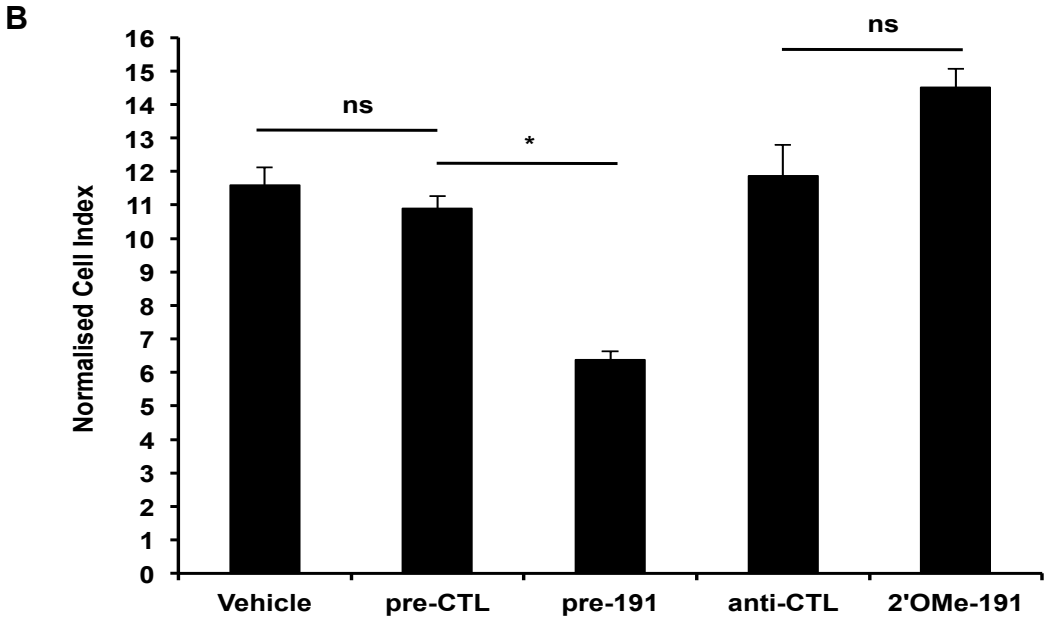
B
Normalised Cell Index
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ns
Vehicle
pre-CTL
pre-191
anti-CTL
2'OMe-191

Figure 5.2 Over-expressing miR-191 inhibits serum inducible VSMCs proliferation and migration. *(These experiments were performed by Ms Margaret Patrikakis)*

(A) *Over-expression of miR-191 with pre-191 significantly inhibited the proliferation of VSMCs. Sub-confluent populations were transfected with 100nM and 200nM pre-191, miR-191 antisense (2'OMe-191) and their corresponding controls (pre-CTL & anti-CTL) and then exposed to 5% (v/v) serum for 96 h. ***P=0.0005, **P=0.0097. ns, no significant difference.* ***(B)*** *Over-expression of miR-191 with pre-191 greatly suppressed the migration of VSMCs. Populations were starved with serum free medium, transfected with 200 nM of pre-191, miR-191 antisense (2'OMe-191) and their corresponding controls (pre-CTL & anti-CTL). An xCELLigence CIM-Plate dual-chamber transwell assay was used to monitor migration. Transfected VSMCs were seeded into the upper chamber, while the lower chamber was filled with Waymouth medium supplemented with the chemoattractant, 10% (v/v) FBS. xCELLigence cell detection system (Roche) allowed for real time monitoring of cell migration through the membrane. Values represent cells migrated after 5 h incubation. *p=0.05. ns, no significant difference.*

5.2.3 miR-191 inhibits neointima formation

Balloon catheter injury was performed on carotid arteries of Sprague-Dawley rats to determine the effect of the miR-191 on intimal thickening. The miRNA was delivered locally at the time of injury (see Methods section 2.2.2.1). The carotid arteries were removed for morphometric analysis at 14 days post-injury. Artery cross sections were stained for elastin and were examined for lumen, intima (I) and media (M) cross-sectional areas and I/M ratios were calculated for each sample. At 14 days, the intimal area and I/M ratios in the pre-191 treated group were significantly lower than that of the pre-CTL and vehicle treated group, while the lumen area in the pre-191 treated group was increased compared with that of the pre-CTL treated group. (**Figures 5.3A&B**).

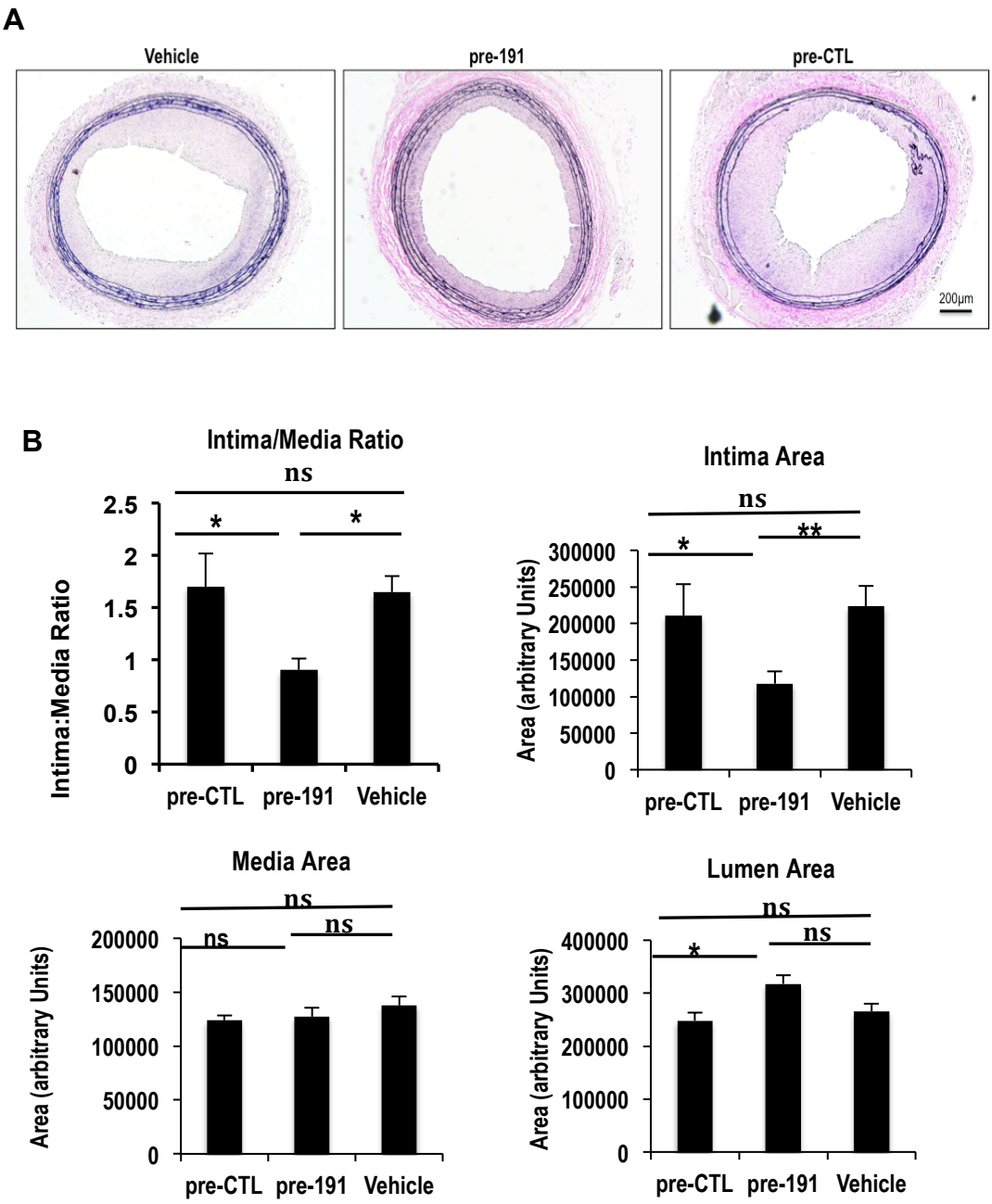


Figure 5.3 Over-expression of miR-191 inhibits neointima formation.

The left carotid arteries of Sprague-Dawley rats were subjected to balloon injury and treated locally with Pluronic gel containing vehicle (1% RNAiMax, n=5), pre-191 (30-60 µg; n=7) or pre-CTL (30-60 µg; n=4). Neointima formation was examined after 14 days. ***(A)*** *Representative photomicrographs of cross-sections of rat carotid arteries 14 days post injury, stained with Verhoeff van Geison stain for elastin. 100X magnification.* ***(B)*** *Intimal, medial and luminal areas were quantified using ImageProPlus software and mean ± SEM determined. Data analysed by Kruskal-Wallis test, *p<0.05, **p<0.01. ns=no significant difference.*

5.2.4 miR-191 suppresses injury-induced Egr-1 *in vivo*

Due to their small size, miRs do not target one specific gene but can regulate the mRNA of a multitude of genes. To assess whether miR-191 overexpression was targeting Egr-1 *in vivo*, immunohistochemical analysis was performed to examine Egr-1 protein expression. As Egr-1 is an immediate early gene, it reaches its maximal expression within the first few hours after injury [260], thus arteries were harvested 1.5 h post-injury. Egr-1 expression was significantly suppressed by miR-191 compared with pre-CTL control group 1.5 h post injury (**Figure 5.4).**

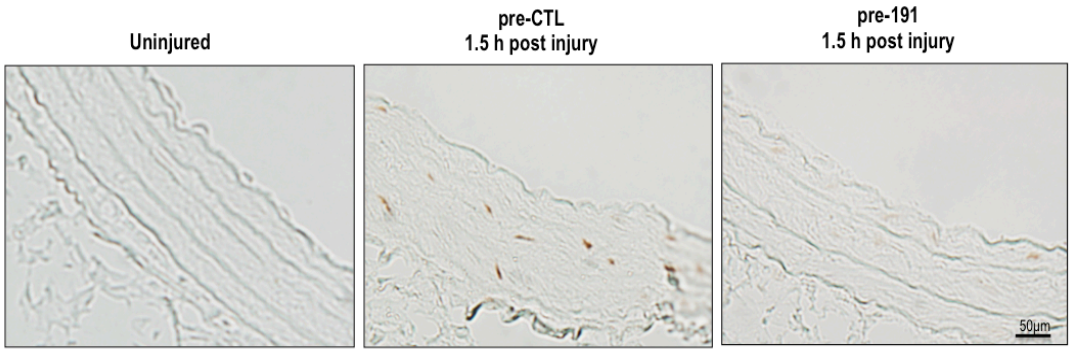


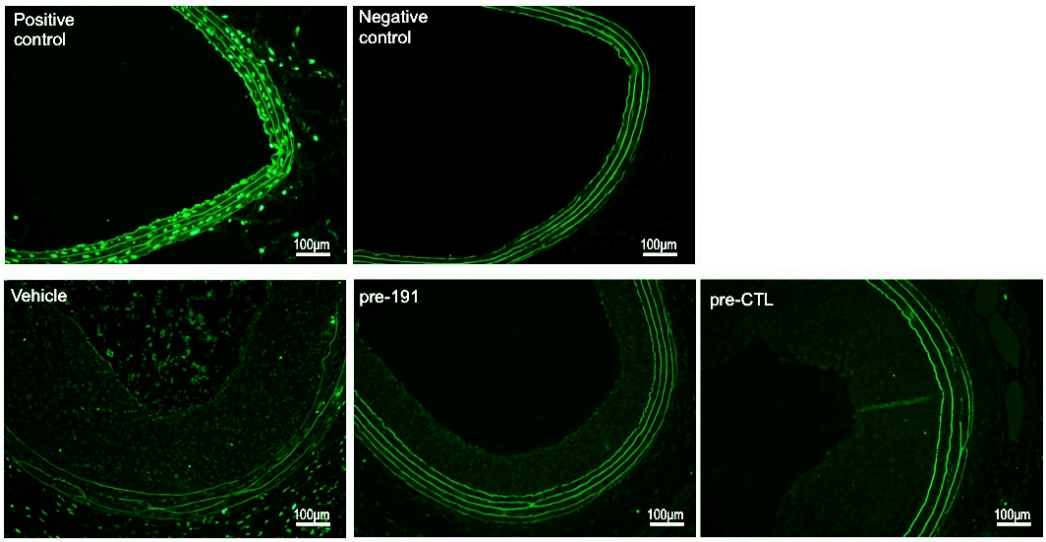
Figure 5.4 Over-expressing miR-191 abrogates injury-induced Egr-1 expression.

Representative photomicrographs of cross-sections of rat carotid arteries 1.5 h post injury, stained for Egr-1. Sections were not counterstained to allow improved visualisation of Egr-1 immunoreactivity. 400X magnification.

5.2.5 miR-191 does not increase cellular apoptosis *in vivo*

Preliminary *in vitro* studies using miR-191 overexpression and VSMC by our group (Ms Patrikakis) demonstrated a moderate increase in apoptosis, which may have contributed to the reduced level of SMC proliferation. To investigate whether cellular apoptosis may have contributed to the decrease in neointima formation, Tunel apoptosis assays were performed on 14 day injured arterial sections. No difference was observed between the treatment groups (**Figures 5.5A&B**). In contrast, expression of the Ki67 proliferation marker was markedly reduced in the presence of miR-191, consistent with an effect of the miR to attenuate SMC proliferation *in vitro* (Ms Patrikakis) (**Figures 5.6A&B**).

A



B

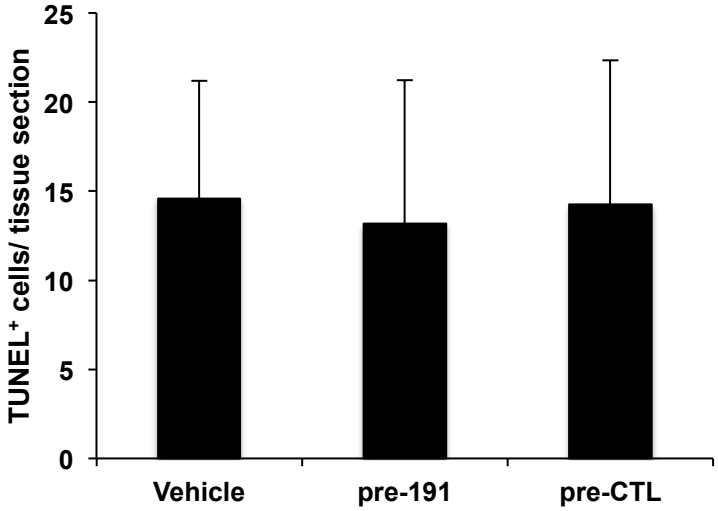


Figure 5.5 MiR-191 does not increase cellular apoptosis in vivo.

*(**A**) Representative photomicrographs of TUNEL stained cross-sections of rat carotid arteries 14 days post injury. 200X magnification. (**B**) TUNEL stained apoptotic cells were quantitated in cross-sections of rat carotid arteries taken 14 days after balloon injury. Stained cells were visualised under fluorescence microscopy (Olympus BX 53), n=4-6 per group ± SEM.*

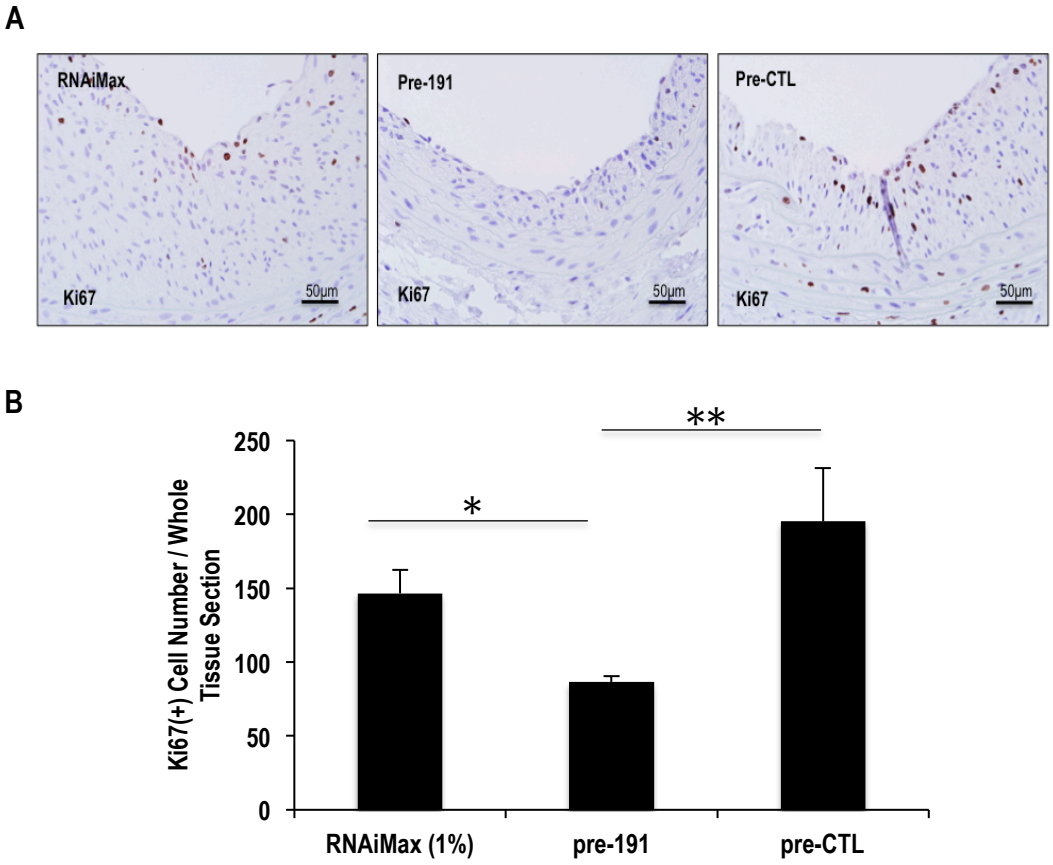


Figure 5.6 miR-191 reduces the expression of the Ki67 proliferation marker in balloon- injured rat carotid arteries.

Expression of the proliferation marker ki67 was examined by immunohistochemical staining of the Ki67 marker in fixed and embedded rat carotid artery sections taken 14 days after balloon injury and treatment with vehicle (1% RNAiMax, n=5), pre-191 (30-60 μg; n=7) or pre-CTL (30-60 μg; n=4). Protein labeling was visualised with DAB. Sections were counterstained with haematoxylin. ***(A)*** *Representative photomicrographs of cross-sections of rat carotid arteries 14 days post injury, stained for Ki67 proliferation marker. 400X magnification.* ***(B)*** *Quantitating Ki67 positive cells in cross-sections of rat carotid arteries and mean ± SEM determined. Data analysed by Kruskal-Wallis test, *p<0.05, **p<0.01.*

5.2.6 miR191 does not affect re-endothelialisation in balloon-injured rat carotid arteries.

To evaluate the effect of the miR-191 on re-endothelialisation *in vivo*, endothelial coverage of the neointima was assessed by immunostaining for CD31. At 14 days, re-endothelialisation in the miR-191 group was not different from that of the pre-CTL group and RNAiMax vehicle control group (**Figure 5.7**).

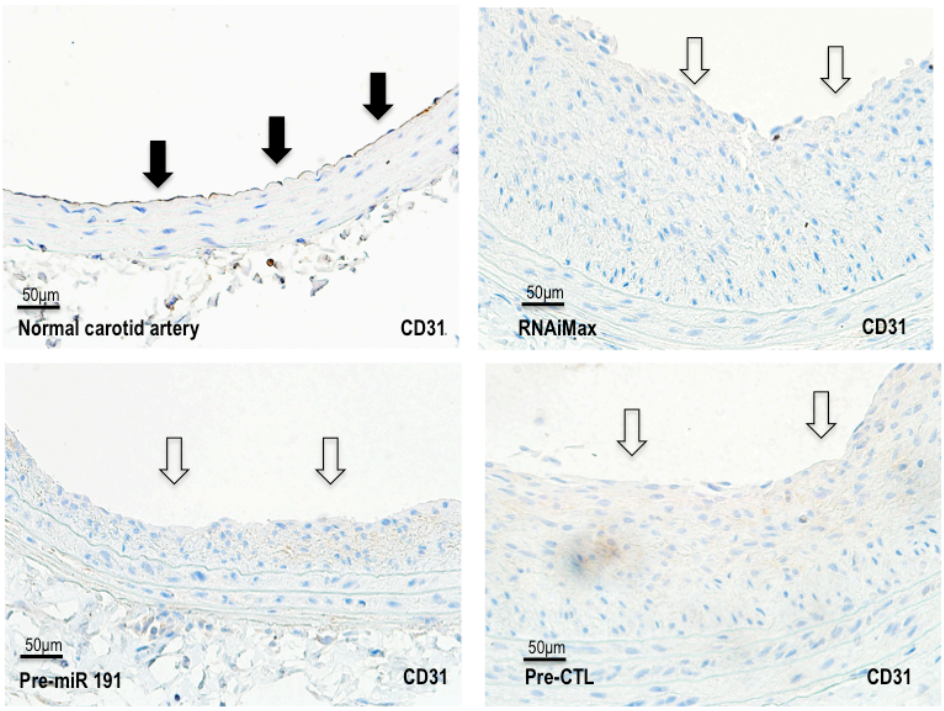


Figure 5.7 MiR-191 does not affect re-endothelialisation in balloon- injured rat carotid arteries.

Re-endothelialisation was examined by immunohistochemical staining of the CD31 marker in fixed and embedded rat carotid artery sections taken 14 days after balloon injury and treated with pre-191 (30-60mg, n=7), pre-CTL (30-60 mg, n=4) and RNAiMax (1%, w/v, n=5). Sections were counterstained with haematoxylin. Photomicrographs show representative sections of neointimal area at 400X magnification. Open arrows indicate lack of endothelial staining. Closed arrows indicate positive staining.

5.3 Discussion

In the present study, the rat carotid artery balloon injury model was adopted to examine the effects of miR-191 on neointima formation and injury induced Egr-1 expression. In this injury model, ECs were completely removed and VSMCs were activated by balloon catheter inflation, followed by the application of pre-miR-191 or controls to the adventitia at the time of injury. The data demonstrated that over expression of miR-191 significantly reduced neointima formation and abrogated injury induced Egr-1 expression.

Understanding the regulation of transcription factors such as Egr-1 and c-Jun, which are master regulators of VSMC proliferation and migration in restenosis and atherosclerosis [148, 309], is crucial to delineating the underlying mechanisms of vascular disease progression and hence to develop novel ways to target and treat these diseases. An earlier study by Wang *et al.* indicated that Egr-1 can be regulated by miR-191 [310] and other studies have also shown a reciprocal relationship between miR-191 and Egr-1 [252, 253]. Avraham *et al,* demonstrated that miR-191 has an inverse correlation with Egr-1 mRNA in mammary epithelial cells [252]. Saba *et al.* found an inverse correlation between miR-191 and Egr-1 during scrapie infection in the mouse brain [253]. Our group's preliminary *in vitro* experiments and the present *in vivo* studies of this thesis both indicated and confirmed the regulatory role of the microRNA miR-191 on Egr-1 transcription and translation. Further, this *in vivo* study has now established a subsequent effect of miR-191 on VSMC growth and activation, which has not been previously demonstrated.

Egr-1 can regulate a cascade of downstream genes involved in proliferation and migration and is crucial in intimal hyperplasia [256, 258, 259]. Thus the reduction in Egr-1 protein levels in response to over-expressed miR-191, highlights this microRNA as a factor in reducing cellular growth and intimal hyperplasia. MicroRNAs do not typically act on just one gene, but can bind to the 3'-UTR of multiple targets [311]. Thus the effect on Egr-1 expression and cellular growth may be direct or indirect. Our group's preliminary *in vitro* experiments have demonstrated that miR-191 suppresses the activity of a reporter construct linking the 3'UTR of Egr-1 to the firefly luciferase gene, indicating a direct relationship between miR-191 and the 3'UTR of Egr-1 (Ms Patrikakis). Further, other upstream activators of Egr-1 including c-Jun and FGF-2, were not affected by miR-191 overexpression or suppression in the same study. Thus, miR-191 effect on Egr-1 expression is not indirect via this pathway. Yet it cannot be discounted that miR-191 effects on VSMC growth both *in vitro* and *in vivo* are not solely the result of Egr-1 suppression. Other miR-191 regulated genes may contribute to some degree. Further experiments will need to be performed to determine whether over-expression of Egr-1 can completely rescue the phenotype of miR-191 expressing cells or to identify other possible miR-191 targets in the vasculature.

Whether miR-191 acts similarly through Egr-1 *in vitro* and *in vivo* is a question to be asked given that our earlier *in vitro* studies demonstrated some increase in VSMC apoptosis in response to miR-191 over-expression (Ms Patrikakis), yet no evidence for this was seen *in vivo*. Conditions within the vasculature are obviously far more complicated with the involvement of multiple cell types and this may alter the regulatory capabilities of miR-191 *in vivo* and hence account for the observed differences. Alternatively, the timepoints selected to study the expression of

apoptotic markers may not be optimal. Gene expression after injury is very cyclical; immediate early genes come up within a matter of hours but disappear relatively quickly, growth factor expression appears within a period of hours to days but tends to abate before the 14 day time point. Similarly, expression of proliferation markers is optimal around week one after injury, and although still detectable after 14 days expression is not as significant. Thus in this study, some increase in apoptosis may have occurred earlier in the process and may have been missed. Time points apart from 1.5 h and 14 days post-injury were not included in this study, but may prove useful in future work.

Re-endothelialisation is crucial for the prevention of late thrombosis after drug-eluting stent (DES) implantation. Unlike the cocktail study in Chapter 3, which significantly increased re-endothelialisation in balloon injured rat carotid arteries, our miR-191 *in vivo* experiment data showed that miR-191 had no impact on re-endothelialisation in the same animal model. Studies have shown a role of miR-126 in maintaining vascular endothelial cell function and miR-16 can promote the capacity of EPCs to repair injured tissues [224, 225], however, the role of miR-191 on EC and EPC has not been examined. It cannot be concluded that miR-191 has no effect on EC, due to limitations in the experimental protocols, such as the miR-191 application method (i.e. locally applied to the perivascular surface of the carotid artery) and the dose of miR (i.e. 30-60 µg). Further studies of miR-191 on EC *in vitro* and *in vivo* are needed.

Our study has conclusively indicated that local over-expression of miR-191 can significantly reduce Egr-1 levels and subsequently neointima formation in the arteries of balloon-injured rats. This finding, highlights the potential of microRNAs

to be used as therapies to treat abnormal induction of transcription factors in a variety of proliferative vascular diseases.

6 Chapter VI

Discussion and Future Directions

6.1 Discussion

As discussed, revascularisation, including PCI and CABG, is an important treatment of CAD. Nowadays more and more patients throughout the world undergo revascularisation treatment. Restenosis, late stent thrombosis and vein graft failures are the main reasons for the failure of PCI and CABG treatment. VSMC proliferation and migration is the primary driver of restenosis after PCI and vein graft failure after CABG. Endothelial dysfunction also plays an important role in both restenosis and late thrombosis following PCI.

The aim of work in this thesis was to seek more effective strategies to inhibit SMC hyperplasia and intimal thickening while promoting re-endothelialisation. The data presented in the above chapters demonstrate that localised delivery of Dz13, miR191 and a novel cocktail of VEGF-A, VEGF-D and cRGD can inhibit neointima formation in animal models, and also indicate that the cocktail significantly enhances re-endothelialisation in a balloon injured artery. Further, the mechanisms behind these effects were investigated in this thesis. It was shown that nitric oxide (NO) plays an important role in the mechanism mediating the effects of the cocktail, that Dz13 in DOTAP/DOPE decreases c-Jun expression and that miR191 suppresses the expression of injury-induced Egr-1. NO is vital for the maintenance of endothelial function and regeneration, and it also promotes EPC recruitment, mobilization, and differentiation, moreover, NO can reduce VSMC proliferation through suppressing VSMC mitogenesis and mitochondrial respiration. Both Egr-1 and c-Jun, act as transcription factors that are master regulators of VSMC proliferation and migration in restenosis and atherosclerosis [148, 309].

According to these studies, the cocktail, Dz13 and miR191 may be useful

approaches for reducing in-stent restenosis and late thrombosis. The ability of these biomolecules to be delivered at a local level makes them ideal for inclusion into coronary artery stents. Comparing with Sirolimus or Paclitaxel, the superiority of Cocktail, Dz13 and miR191 are: Cocktail can promote re-endothelialistion while inhibiting neointimal formation, Dz13 and miR191 are gene-specific “magic bullets” which are characterized by greater specificity, decreased toxicity, and fewer side effects [312]. To date, others have coated some of these biomolecules onto stents and achieved some inspiring results.. Blindt et al. showed that cRGD-coated stents reduced in-stent restenosis by accelerating endothelialization in a porcine coronary artery model [193]. Yang *et al.* showed that VEGF gene- and paclitaxel-coating stents promote re-endothelialisation while inhibiting neointima formation [163]. The future directions of this work will aim to further examine the potential of these molecules i.e. Cocktail, Dz13 and miR191 in this context, by coating them onto coronary artery stents using new biomolecule coating techniques and pre-clinical evaluation in the pig PCI model. As it has been shown that shear stress and injury induced Egr-1 expression in rabbit vein bypass graft model [148], miR191, which targets Egr-1, could also be expected to work in CABG. Besides, recent experiments in our lab indicated that the cocktail (VEGF-A/D and cRGD) reduced Egr-1 expression *in vitro* and *in vivo*, and this suggests that cocktail has the potentials to be applied in CABG to prevent vein graft failure.

These studies showed cocktail and miR191 significantly reduced neointimal formation, but did not obviously enlarge the artery lumen size. The reason is that changes in the artery wall can be compensated by arterial positive remodeling during restenosis development after balloon injury.

As mentioned in Chapter 1, there are limitations to the rat model of balloon angioplasty used in this study. Typically in humans, PCI or CABG is performed in the first instance because of vessel narrowing or occlusion resulting from atherosclerosis. Hence PCI or CABG in clinical practice is performed on a background of underlying atherosclerosis which would be expected to alter to some extent the pathogenesis and progress of restenosis. This is not apparent in the rat model used in this study. The in vivo experiments represent a fairly simple form of restenosis encompassing the basic arterial response to injury which primarily involves the migration and proliferation of VSMC. Certainly in diseased human arteries the situation is more complex, involving interactions between VSMC, macrophages, and T-cells. Further, there are some basic differences in the anatomy of the human and rat arteries, such as less medial wall elastin, a condensed subintimal layer, and a lack of vasa vasorum in the latter. Thus the rat model is more simplistic and not identical to the human situation but the point is to provide a basic proof-of-principle that the novel drug treatment can be effective in vivo on at least some aspects of disease pathogenesis, namely vascular cell proliferation and migration. The rat model is still one of the most highly used methods for establishing drug efficacy [130]. However, recent failures of clinical trials of drugs that were effective in this model suggest that the use of multiple pre-clinical models to establish potential efficacy in humans is appropriate [313, 314]. Thus using this model in this is an initial step to establish proof-of-principle efficacy of this drug treatment in vivo, however more pre-clinical studies would be appropriate. Further to this, the rat model of balloon injury is limited by the inability to use stents in the smaller vessels of this animal. Thus as suggested in the future directions, ongoing aims would involve coating stents with the cocktail and applying them to other larger animal models such as the pig. This would further the

development of the cocktail in terms of application potential but also reduce the risk that the drug is only active in limited species.

PCI and CABG are often associated with post-operative infection which may affect formation of the neointima. This is not particularly common in the “clean” rats used in these studies. While this is not a limitation of the study per se as this would profoundly confound the data if random infection was introduced, it certainly represents a difference between the controlled nature of animal experiments and the human situation.

The animal models for Aim 1 (cocktail), Aim 2 (Dz13 with Dope/Dotap) and Aim 3 (miR191) all focus on the VSMC proliferation and migration in vivo. But Aim 2 is an advanced study to the previous research of our laboratory. Our laboratory has previously demonstrated that Dz13 in the proprietary lipid blend FuGENE6 inhibited neointimal formation in a rabbit model of autologous end-to-side transplantation [148]. FuGENE6 is not authorised for clinical use. As an alternative, the transfection agent DOTAP/ DOPE has been used in human gene therapy trials and is safe. So in Aim 2, the transfection agent DOTAP/ DOPE should be evaluated in the same animal model.

6.2 New biomolecule coating techniques

6.2.1 The immobilisation of biomolecules on metals using a plasma-activated coating (PAC)

Metal alloys have been widely utilised in clinical medicine and the most common types are stainless steel, titanium, cobalt–chromium and nitinol [315]. Clinical therapy for CVD has seen the development of metallic implants, which are

represented by replacement of heart valves, implantation of pacemakers and coronary artery stents [316]. However, these metallic implants usually include surfaces that directly contact the blood flow and have poor endothelial cell interactions, thus thrombosis can be induced easily and profoundly [317]. In recent years, the improvement of biocompatibility has been focused in biomaterial science to further develop the application of metallic implants in medicine, which enables the acceptable co-existence of metallic devices and tissues, and has greatly increased the effects of the treatment.

The technique of immobilising proteins on metals has been a focus of attention. On one hand, the metallic vascular device surface has to have high capacity to bind the proteins while still keeping the activity of the proteins. On the other hand, the device surface is required to be robust enough to withstand exposure to blood flow [318].

Yin *et al.* have developed the PAC (plasma-activated coating) technology that can generate reliable and ready-to-use surfaces on stainless steel through a plasma deposition process and hence achieve the enhancing effect of chemical vapour deposition and immobilise biomolecules covalently [318]. Briefly, heating a gas causes the ionisation of its molecules or atoms, increases or decreases the number of electrons, and hence a plasma containing positive and negative ions is generated, which can promote chemical vapor deposition and forms organic interlayers to immobilise the biomolecules covalently [319].

As PAC technology has been identified to be substrate independent, dry and reliable, and can generate a strong, smooth and solid organic surface layer onto

the metallic surface through ion stitching [318], it becomes possible to coat appropriate biomolecules onto the coronary artery stents to further improve the efficacy of PCI treatment.

6.2.2 Convergence of biotechnology and nanotechnology

In the previous chapters the problems and development of DES have been discussed. To solve these problems, special nanocomposite polymers could be involved to coat the next generation of cardiovascular stents. For example, POSS-PCU (polyhedral oligomeric silsesquioxane poly (carbonate-urea) urethane) **(Figure 6.1)**, a proprietary nanocomposite polymer developed and patented by Tan *et al.* [320], and they have proved that this polymer has achieved the properties of ideal stent coatings, such as high biocompatibility, thrombogenicity resistance, provision of an interface for anti-proliferative drugs, optimal mechanical properties to prevent polymer fracture, as well as a favorable surface for EC growth.

Another important aspect in DES is sustained and controlled release of drugs. The layer-by-layer (LbL) self-assembly technique can be used to achieve this effect [321]. This LbL stent coating technique has been identified that the tunable drug release enables the match between drug delivery rate and healing time. Moreover, drugs with different effects such as anti-proliferative, anti-platelet and endothelialisation-enhancing agents can be coated onto coronary artery stents simultaneously to improve the efficacy of DES. Yang *et al.* have used LbL technique to coat VEGF gene and paclitaxel onto stent to promote re-endothelialisation while inhibiting neointima formation [163]. In the stent coating technique, polymers play a key role. As different polymers have their own specific

properties, they are used for different stent layer coating. With the assistance of the polymers, multiple drugs could be coated together onto the stents. For instance, as POSS-PCU is non-biodegradable, it could be utilised as the base coat to prevent blood flow contacting with bare metal stents directly. Furthermore, a layer of biodegradable POSS-PCL (polyhedral oligomeric silsesquioxane poly caprolactone) could be used for the layers to incorporate drugs and hence achieve the tunable drug release [320].

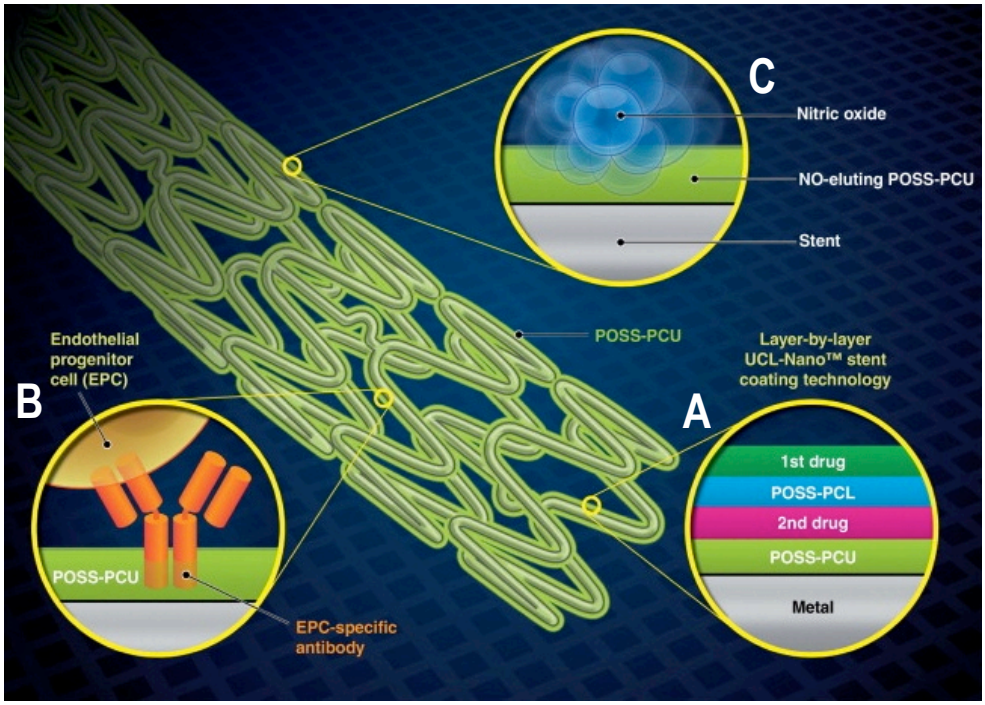


Figure 6.1 *Tunable layer-by-layer (LbL) drug release technique and application of specific polymers for next generation coronary stents. A. Layer-by-layer stent coating technology: POSS-PCU, non-biodegradable, can be utilised as the base coat thus preventing bare metal contacting with blood directly; POSS-PCL, biodegradable, can be used for the layers to incorporate drugs to achieve the tunable drug release. B. Endothelial progenitor cell capture technology to promote endothelialisation: EPC-specific antibodies are incorporated into the polymer POSS-PCU. C. Bio-functionalised nanocomposite polymers: NO is eluted into polymer POSS-PCU to maintain healthy endothelium and prevent thrombosis [320]. (The figure extracted from* **Trends Biotechnol.** *2012 Aug;30(8):406-9.)*

6.3 Cocktail-coated stents

According to our studies, the cocktail, Dz13 and miR191 have the potential to reduce in-stent restenosis and late thrombosis. The development of new coating techniques such as PAC, convergence of biotechnology and nanotechnology has made it possible to coat these biomolecules on stents and further investigate their biological effects.

In the cocktail research, It has been stepped forward to test at what temperature cocktail could still keep bioactivity when coating onto coronary stents. In the experiments, the cocktail and vehicle were separately coated onto the 12-well plates, which were respectively dried at 4 ^{o}C, 22 ^{o}C and 37 ^{o}C overnight. EC and VSMC were grown in these cocktail/vehicle-coated plates and the cells were counted at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h. The results showed that when the cocktail-coated plates were dried at 4 ^{o}C, cocktail still displayed its effects, i.e. enhancing EC growth and inhibiting VSMC proliferation, and these suggested that the best cocktail coating temperature is 4 ^{o}C, under which cocktail can still remain bioactivity, however, no such effects were found at 22 ^{o}C and 37 ^{o}C **(Figure 6.2)**, These studies should be helpful in future efforts to coat stents and evaluate their efficacy in cell culture and in animal models.

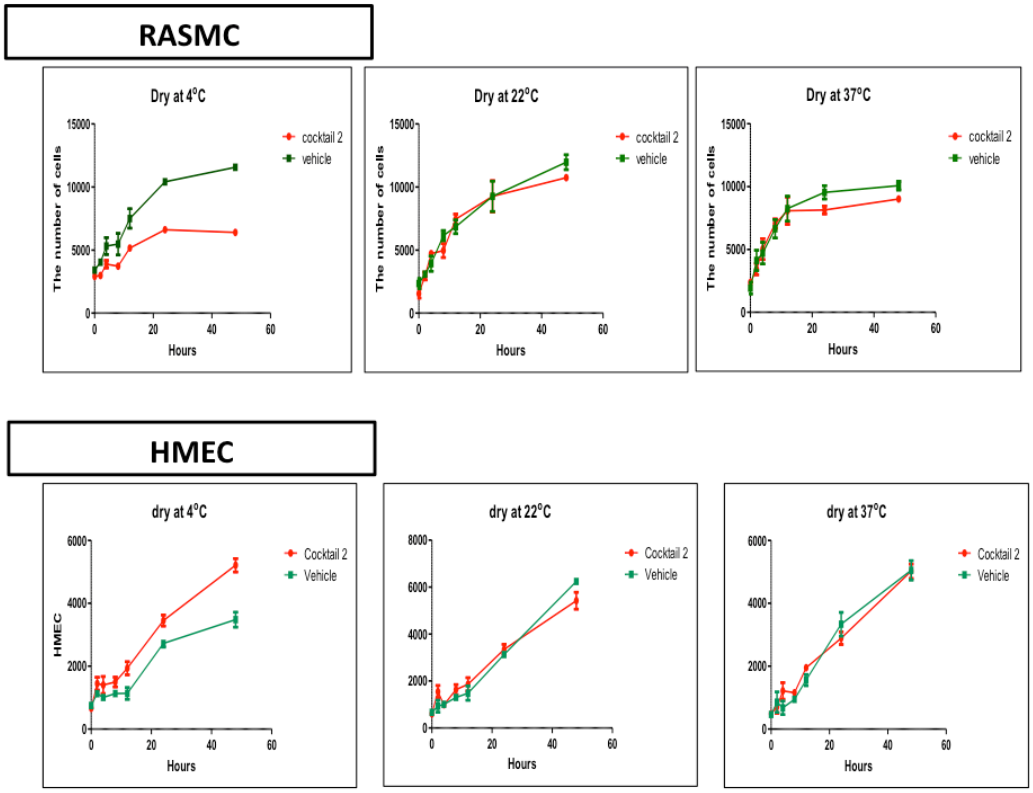


Figure 6.2 *Cocktail bioactivity at different temperature. The cocktail (VEGF-A/D 50 ng/mL plus cRGD 200 nM) and vehicle (PBS) were separately coated onto the 12-well plates and then dried respectively at 4 °C, 22 °C, 37 °C overnight. EC and VSMC were grown in these cocktail-coated plates and the cells were counted with a cell counter at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h.*

We (our group) are currently cooperating with the University of Sydney in the use of PAC technique and the University College London adopting nanocomposite polymer POSS-PCU and POSS-PCL to coat the cocktail onto coronary artery stents. Firstly, the cocktail will be coated on the stent steel using both new techniques. The cocktail-coated steels will be tested through cell proliferation experiments *in vitro*, to evaluate the bioactivity of cocktail when coated on stent steel. In the application of the nanocomposite polymer technique，three alternative methods will be utilised to coat the cocktail. One method is to dissolve the cocktail in water and spray it onto steel that has a polymer base coat. A second method is to dissolve the cocktail in polymer and spray it onto the steel. The third method is to employ non-biodegradable polymer POSS-PCU as the basecoat, cocktail in water as the middle layer, and biodegradable polymer POSS-PCL is used as topcoat to control the release of cocktail. If promising results are achieved with the cocktail-coated base metal *in vitro*, the cocktail will be coated on stents using the same techniques and further investigated *in vivo*.

While the initial studies will involve cocktail-coated stents, further work will be done for Dz13 and miR191 in a similar context. While these two molecules did not tend to promote re-endothelialisation in the in vivo studies, this work has demonstrated the efficacy of these novel methods that target immediate early genes such as c-Jun and Egr-1 in inhibiting neointimal formation and furthered our understanding of how these processes can be controlled. Future studies may incorporate these molecules into other cocktail-type formulations that can simultaneously promote re-endothelialisation. Preliminary experiments that were performed prior to deciding on the cocktail used in this study (but not shown in this thesis) demonstrated that

the effect of the combination of Dz13 and VEGF on EC proliferation was not satisfactory (Dz13 also inhibited EC proliferation). Therefore, use of such a Dz13-containing “cocktail” in a future study may not directly mix the biomaterials together, but adopt new reliable technology to bring each biomaterial into play at appropriate time points. For example, the layer-by-layer (LbL) self-assembly technique could be used. Dz13 and transfection agent would be put into the first releasing layer to inhibit VSMC proliferation in the early stages while VEGF would be put into the second releasing layer to increase re-endothelialisation.

miR191 is new in cardiology, and its effect on EC proliferation is still unclear. Therefore, miR191 needs to be further studied in this context and its effect will determine how miR191 may be incorporated into other cocktail-type formulations. It is hoped that in future clinical practice, biomolecule-eluting stents could provide another option in the coronary artery stent family, and together with BMS and DES, improve the effectiveness of CAD treatment.

Appendix

Authority to Conduct Animal Research Project

UNSW

ANIMAL CARE
AND ETHICS COMMITTEE
(ACEC)

Authority to Conduct Animal Research Project

ACEC Number:	**07/75A**
Title:	Determination of the role of c-Jun in intimal thickening using the rabbit model of vein bypass grafting
Start/Expiry Dates:	09 July 2007 to 09 July 2010
Name of the Researcher:	Professor Levon Khachigian
Address:	CTVR, School of Medical Science
Contact Phone Number:	9385 2537
Email Address:	l.khachigian@unsw.edu.au
Authorised Personnel	Dr Jun Ni, Mr Yue Li
Modification Approval Date:	2 December 2009, 25 November 2009

Species/Strain	Year 1	Year 2	Year 3	TOTAL
New Zealand Rabbit	40	40	20	100

Conditions of Approval Particular to this Project NIL

Conditions of Approval Applicable to All Projects

1. ***All cages holding animals for this project should be labelled with its ACE Number, Expiry Date, Chief Investigator, and Contact Phone Number, as listed above.***
2. ***Modifications to this project and the addition of new personnel must have prior written approval of the ACEC. Please send requests to (ethics.sec@unsw.edu.au).***
3. ***All projects are subject to annual review by the ACEC.***
4. ***Please have this letter of authority available during site inspection by ACEC members.***
5. ***Please distribute copies of this letter of authority to the other authorised personnel listed above (copies enclosed).***
6. ***In the event of an unforeseen adverse incident (e.g. unexpected deaths), the NHMRC Code of Practice (3.1.11) requires that investigators promptly notify the ACEC*** (m.wright@unsw.edu.au).
7. ***If a project involves surgery, the researchers are required to monitor animals daily for at least first week post operatively.***
8. ***Monitoring sheets must be kept with the animals, unless approved by the ACEC Committee.***

Authorised on behalf of the Vice-Chancellor on 9 July 2007

Professor Peter Gunning
Presiding Member - ACEC, Committee 'A'

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Authority to Conduct Animal Research Project

UNSW

ANIMAL CARE AND ETHICS COMMITTEE (ACEC)

ACEC Number: **10/115B**

Title: Effect of a Cocktail of VEGFA, VEGFD and cRGD on Intimal Thickening After Carotid Injury

Start/Expiry Dates: 08 September 2010 to 07 September 2013

Please note that under Section 27 of the NSW Animal Research Act. 1985, the Animal Research Authority is current for a period of 12 months from the date of issue unless cancelled sooner. Renewal of the authority is conditional upon submission of a satisfactory annual report to the ACEC.

Name of the Researcher: Prof Levon Michael Khachigian

Address: Centre for Vascular Research

Contact Phone Number: 52537,51293

Email Address: z8571226@unsw.edu.au

Authorised Personnel Mr Yue Li, Ms Kristine Paz Malabanan

Species & Strain (Common name)	Year 1	Year 2	Year 3	Total	Supplier
Rats (Sprague Dawley)	50	20	0	70	BRC - Animal Resources Centre, (ARC, Perth, WA)

Conditions of Approval Particular to this Project

Nil

Please also download the Adverse or Unexpected Report Form from the following site:
http://www.gmo.unsw.edu.au/Ethics/AnimalEthics/AnimalEthics_index.html

Conditions of Approval Applicable to All Projects

1. *All cages holding animals for this project should be labelled with its ACEC Number, Expiry Date, Chief Investigator, and Contact Phone Number, as listed above.*
2. *Modifications to this project and the addition of new personnel must have prior written approval of the ACEC. Please send requests to (ethicsonline.gmo@unsw.edu.au).*
3. *All projects are subject to annual review by the ACEC.*
4. *Please have this letter of authority available during site inspection by ACEC members*
5. *Please distribute copies of this letter of authority to the other authorised personnel listed above (copies enclosed).*
6. *In the event of an unforeseen adverse incident (e.g. unexpected deaths), the NHMRC Code of Practice (3.1.12) requires that investigators promptly notify the ACEC (m.wright@unsw.edu.au).*
7. *If a project involves surgery, the researchers are required to monitor animals daily for at least first week post operatively.*

The ACEC considered the above project and is pleased to advise that this project meets the requirements as set out in the Australian code of practice for the care and use of animals for scientific purposes* The committee recommends approval.

* (http://www.nhmrc.gov.au/guidelines/publications/ea16)

Having taken into account the advice of the Committee, the Deputy Vice-Chancellor (Research) has approved this project to proceed.

Authorised on behalf of the Vice-Chancellor on 08 September 2010

Professor Richard Bryan Lock

Presiding Member, Committee 'B'

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Authority to Conduct Animal Research Project

UNSW

ANIMAL CARE AND ETHICS COMMITTEE (ACEC)

ACEC Number: **12/52B**

Title: Role of MicroRNA in Neointima Formation

Start/Expiry Dates: 01 May 2012 to 30 April 2015

Please note that under Section 27 of the NSW Animal Research Act. 1985, the Animal Research Authority is current for a period of 12 months from the date of issue unless cancelled sooner. Renewal of the authority is conditional upon submission of a satisfactory annual report to the ACEC.

Name of the Researcher: Prof Levon Michael Khachigian

Address: Centre for Vascular Research

Contact Phone Number: 93852537

Email Address: s8571226@unsw.edu.au

Authorised Personnel Mr Yue Li, Dr Lucinda McRobb

Species & Strain (Common name)	Year 1	Year 2	Year 3	Total	Supplier
Rats (Sprague Dawley)	60	0	0	60	BRC - Animal Resources Centre, (ARC, Perth, WA)

Conditions of Approval Particular to this Project

NIL

Please also download the Adverse or Unexpected Report Form from the following site:
http://www.gmo.unsw.edu.au/Ethics/AnimalEthics/AnimalEthics_index.html

Conditions of Approval Applicable to All Projects

1. *All cages holding animals for this project should be labelled with its ACEC Number, Expiry Date, Chief Investigator, and Contact Phone Number, as listed above.*
2. *Modifications to this project and the addition of new personnel must have prior written approval of the ACEC. Please send requests to (ethicsonline.gmo@unsw.edu.au).*
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7. *If a project involves surgery, the researchers are required to monitor animals daily for at least first week post operatively.*

The ACEC considered the above project and is pleased to advise that this project meets the requirements as set out in the Australian code of practice for the care and use of animals for scientific purposes* The committee recommends approval.

* (http://www.nhmrc.gov.au/guidelines/publications/ea16)

Having taken into account the advice of the Committee, the Deputy Vice-Chancellor (Research) has approved this project to proceed.

Authorised on behalf of the Vice-Chancellor on 01 May 2012

Professor Richard Bryan Lock

Presiding Member, Committee 'B'

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