

**Role of microRNA-21 in the regulation of human
saphenous vein smooth muscle cell function and
vascular remodelling**

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Intellectual Property and Publication Statements

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors of this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Figure 3.4A in Chapter 3 of the thesis has appeared as a Supplementary Figure 1. Differential cytoskeletal morphology and proliferation rate of SV-SMC from non-diabetic and diabetic patients, in publication as follows: Elevated expression levels of miR-143/5 in saphenous vein smooth muscle cells from patients with Type 2 diabetes drive persistent changes in phenotype and function, 2014. Riches, K., Alshanwani, A. R., Warburton, P., O'Regan, D. J., Ball, S. G., Wood, I. C., Turner, N. A. and Porter, K. E. *Journal of Molecular and Cellular Cardiology*, 74, 240-50. I performed 7-day proliferation comparison between ND- and T2DM-SV-SMC. KR conducted all of the other experimental work and drafted the article, PW isolated primary cells from tissue, DOR provided samples of saphenous vein for SMC culture, SGB provided some of the funding and helped revised the article, NAT, ICW and KEP were responsible for study conception and design, critical revision and approval; KEP had overall responsibility and led the project.

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Abstract

Cardiovascular diseases including coronary artery disease are the major cause of death worldwide. Saphenous vein (SV) bypass grafting is widely used to revascularise atherosclerotic coronary arteries, although failure rates are problematic. Adverse remodelling and intimal hyperplasia (IH) underlie graft failure primarily driven by aberrant smooth muscle cell (SMC) function.

Whilst altered SMC function is pivotal to the adaptation of SV grafts, in the longer term excessive cell proliferation and migration lead to intimal hyperplasia that compromises patency rates. Whilst there are no pharmacological agents to effectively prevent IH, accumulating evidence has implicated microRNAs (miRs), short non-coding RNAs that negatively regulate gene expression, in SMC phenotypic changes and vascular remodelling. In particular, miR-21 is highly expressed in the cardiovascular system and is reportedly dysregulated in pathological conditions. This thesis confirmed a distinct phenotype in SV-SMC from T2DM patients, relative to those from patients without diabetes (ND), although this was not associated with differential miR-21 expression.

Artificial overexpression of miR-21 in ND SV-SMC increased total cell area and cell proliferation and its knockdown was able to reverse the increase in cell area. In addition, treating SV-SMC with PDGF-BB, commonly associated with vascular remodelling, led to increased miR-21 expression through activation of both ERK and Akt; inhibition of either pathway abrogated miR-21 expression. Exploring the downstream target genes of miR-21 using a vascular remodelling specific array and follow-up RT-PCR yielded two genes affected by miR-21 overexpression; increased MMP-1 mRNA and decreased IL-1A mRNA. Surprisingly, SV-SMC migration in cells overexpressing miR-21 did not, however, modulate migration through collagen-1 or -3 coated membranes. Potential downstream target genes of miR-21 mediating functional changes related to SMC proliferation and migration were tested. Whilst there were no clear effects on “classical” targets (PDCD4, PTEN and Spry), a significant downregulation of RECK mRNA and protein in miR-21 overexpressing SV-SMC was apparent. Further studies are required to explore in detail how RECK is involved in miR-21 mediated cellular functions and whether targeting miR-21 in SV-SMC would provide a promising therapeutic strategy to improve outcomes in patients following SV grafting.

Publications and Presentations Arising From This Study

Joint author publication

“Elevated expression levels of miR-143/5 in saphenous vein smooth muscle cells from patients with Type 2 diabetes drive persistent changes in phenotype and function”. Riches, K., **Alshanwani, A. R.**, Warburton, P., O'Regan, D. J., Ball, S. G., Wood, I. C., Turner, N. A. and Porter, K. E. *Journal of Molecular and Cellular Cardiology*; 74, 240-50 (2014).

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List of Abbreviations

| | |
|-------------------|-----------------------------------------------------------------------------------------------------------|
| ACCORD | Action to Control Cardiovascular Risk in Diabetes |
| ADVANCE | Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation |
| AGEs | Advanced Glycation End Products |
| Ago2 | Argonaute 2 |
| α -SMA | Alpha Smooth Muscle Actin |
| ANG | Angiotensin II |
| AMV RT | Avian Myeloblastosis Virus Reverse Transcriptase |
| AP-1 | Activation Protein-1 |
| APMA | p-Aminophenylmercuric Acetate |
| APS | Ammonium Persulphate |
| BCA | Bicinchoninic Acid |
| BCL2 | B-Cell Lymphoma 2 |
| BMP | Bone Morphogenic Protein |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine Serum Albumin |
| CABG | Coronary Artery Bypass Grafting |
| CAD | Coronary Artery Disease |
| Ct | Threshold Cycle |
| CVD | Cardiovascular Disease |
| CVS | Cardiovascular System |
| DAPI | 4', 6-Diamidino-2-Phenylindole |
| DCCT | Diabetic Control and Complications Trial |
| dH ₂ O | Distilled Water |
| DMEM | Dulbecco's Modified Eagle Media |
| DMSO | Dimethyl Sulphoxide |
| EC | Endothelial Cells |
| ECM | Extracellular Matrix |
| EDIC | Epidemiology of Diabetes Interventions and Complications |
| ERK | Extracellular Regulated Kinase |
| FACS | Fluorescence-Activated Cell Sorting |
| FCS | Foetal Calf Serum |
| FFAs | Free Fatty Acids |
| FGM | Full Growth Media |

| | |
|---------------|------------------------------------------------------------|
| FITC | Fluorescein Isothiocyanate |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| h | Hour |
| HbA1c | Glycated Haemoglobin |
| IGF | Insulin-Like Growth Factor |
| IH | Intimal Hyperplasia |
| IL | Interleukin |
| IL-1 α | Interleukin-1 Alpha |
| IL-1 β | Interleukin-1 Beta |
| IMA | Internal Mammary Artery |
| LCA | Left Coronary Artery |
| LDL | Low-Density Lipoprotein |
| LPs | Lipoproteins |
| MAPK | Mitogen-Activated Protein Kinase |
| MGM | Minimal Growth Media |
| Min | Minute |
| miRNAs, miRs | MicroRNAs |
| MMPs | Matrix Metalloproteinases |
| mRNA | Messenger RNA |
| ND | Non-Diabetic |
| NO | Nitric Oxide |
| PBS | Phosphate Buffered Saline |
| PCI | Percutaneous Coronary Intervention |
| PCNA | Proliferation Cell Nuclear Antigen |
| PDCD4 | Programmed Cell Death 4 |
| PDGF | Platelet-Derived Growth Factor |
| PI3K | Phosphoinositide-3-Kinase |
| PKB (Akt 1) | Protein Kinase B |
| PMSF | Phenylmethylsulphonyl Fluoride |
| Pri-miR | Primary miRNA |
| PTEN | Phosphatase and Tensin Homolog |
| PVDF | Polyvinylidene Difluoride |
| RCA | Right Coronary Artery |
| REC | Local Research Ethics Committee Approval |
| RECK | Reversion-Inducing-Cysteine-Rich Protein with Kazal motifs |

| | |
|---------------|--------------------------------------------------------------|
| RISC | RNA-Induced Silencing Complex |
| ROS | Reactive Oxygen Species |
| RT | Room Temperature |
| RTK | Receptor Tyrosine Kinase |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| S | Seconds |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis |
| SERCA | Sarco/Endoplasmic Reticulum Calcium ATPase |
| SFM | Serum Free Medium |
| SKP2 | S-phase Kinase-Associated Protein 2 |
| SMC | Smooth Muscles Cells |
| SM-MHC | Smooth Muscle Myosin Heavy Chains |
| Spry | Sprouty |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| SV | Saphenous Vein |
| T1DM | Type 1 Diabetes Mellitus |
| T2DM | Type 2 Diabetes Mellitus |
| TEMED | N,N, N',N' Tetramethyl-Ethylene Diamine |
| TGF- β | Transforming Growth Factor β |
| TIMPs | Tissue Inhibitors of Metalloproteinases |
| TNF- α | Tumour Necrosis Factor-Alpha |
| UK | United Kingdom |
| UKPDS | United Kingdom Prospective Diabetes Study |
| UNG | Uracil-N-Glycosylase |
| 3'-UTRs | 3'-Untranslated Regions |
| VADT | Veterans Affairs Diabetes Trial |
| VEGF | Vascular Endothelial Growth Factor |
| WHO | World Health Organisation |

Chapter 1
INTRODUCTION

Chapter 1

Introduction

1.1. Coronary heart disease

According to the World Health Organisation, cardiovascular disease (CVD) is the number one cause of death globally. Although the survival rate of patients with coronary artery disease (CAD) has been progressively improving, approximately 17.5 million people died from CVD in 2012, 7.4 million of the death cases resulted from CAD and 6.7 million were due to stroke. CVD accounts for 31% of universal deaths and is expected to rise to more than 23.6 million by 2030 (Laslett et al., 2012). In addition to its mortality burden, the non-fatal cases following myocardial infarction and stroke result in significant morbidity with ensuing social and economic consequences. Therefore, CAD has become a universal public health problem which deserves more attention. Risk factors can be classified into modifiable biological factors such as hypertension, diabetes, elevated plasma low-density lipoprotein (LDL) and triglycerides, smoking, physical inactivity, and obesity. Whereas, the non-modifiable risk factors are age, ethnicity and family history of early heart disease or stroke (Thayer et al., 2010, Saran et al., 2010).

The myocardium is supplied by two main arteries; the right coronary artery (RCA) and left coronary artery (LCA). RCA branches into the right marginal artery and posterior descending artery while the LCA branches into circumflex and left anterior descending artery (Figure 1.1). The right and left coronary arteries originate from the aorta and provide a source of blood supply to the most vital muscle structure of the body, the heart. The RCA supplies the SA and AV nodes, the myocardium and external wall of the right atrium and right ventricle. Both the RCA and circumflex branch of the LCA supply the atria via small branches. The LCA typically supplies the interventricular septum, the anterior wall of the right ventricle and the external wall of the left ventricle (Moore et al., 2013). The coronary circulation is the network that supplies oxygenated blood to the heart and collects deoxygenated blood from the heart muscle (Kern et al., 2006). Obstruction and interruption of the coronary circulation by atherosclerotic plaque in one or more of the coronary arteries retards the supply of oxygen and other essential nutrients to the myocardium (Figure 1.1). As this plaque lesion progresses, unstable coronary syndromes, myocardial infarction

and stroke may then occur, which thus result in high mortality rates (Marieb and Hoehn, 2007).

Angiography is the diagnostic procedure utilised to detect whether a plaque is blocking a coronary artery and the extent of coronary lesion. A small catheter is inserted into the patient's wrist radial artery or groin femoral artery and when it reaches the heart a dye is injected into the coronary circulation. This is accompanied by a special X-ray to image the atherosclerotic lesion of the coronary arteries. Angiographic images shown in Figure 1.2 demonstrate the normal architecture of coronary arteries from patients without CAD (A), and patients with CAD in the presence (C) and absence of diabetes (B).

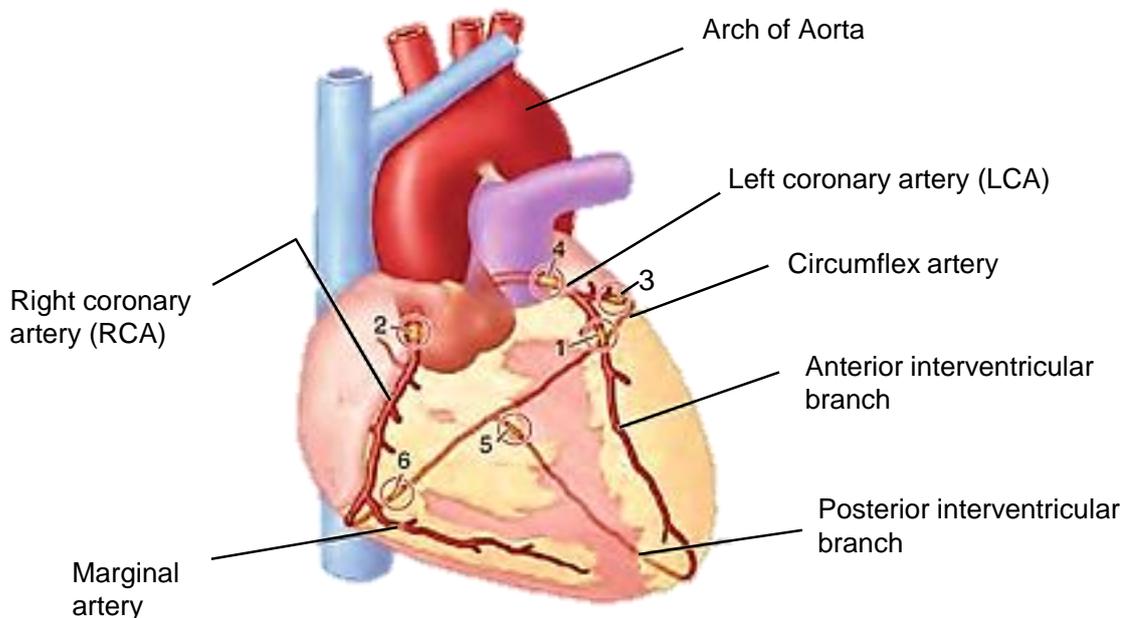


Figure 1.1: Schematic diagram of coronary artery and common sites of coronary artery occlusion.

Sites 1-3 account for 85% of all occlusions. 1- Anterior interventricular branch of LCA (40-50%), 2- RCA (30-40%), 3- Circumflex branch of the LCA. The remaining 4-6 account for 15% of total occlusions. 4- Left coronary artery, 5- Posterior interventricular branch, 6- Posterior descending artery. Adapted from Moore et al. (2013).

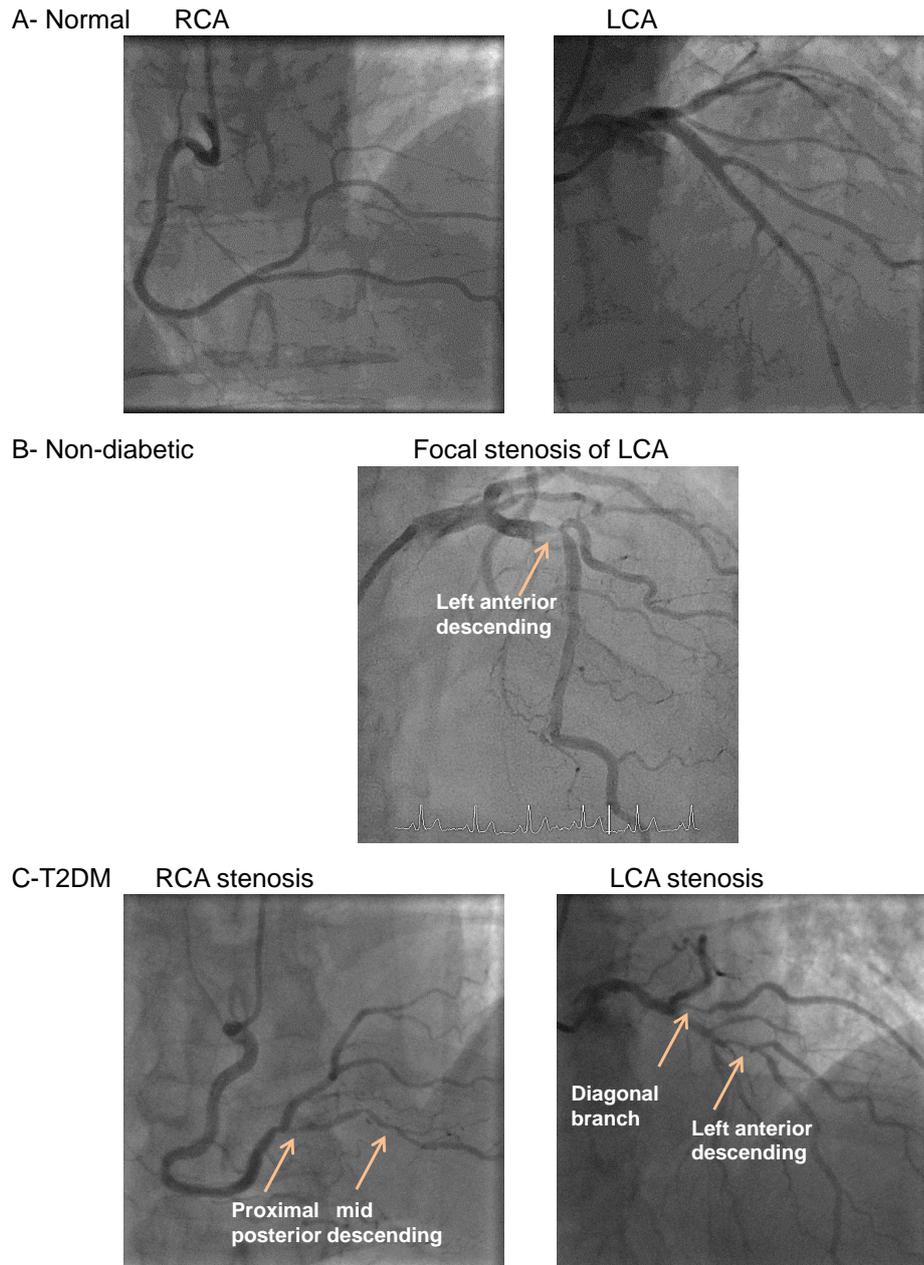


Figure 1.2: Angiographic images of coronary artery disease.

A- The coronary arteries of the patient without CAD have a smooth outline with no angiographic filling defects within the vessel lumen to indicate atherosclerosis or stenosis. B- A discrete, focal stenosis of the left anterior descending branch of the LCA in non-diabetic patient. C- The RCA of patient with type 2 diabetes (T2DM) has diffuse narrowing in the proximal and mid segments of its posterior descending branch. The vessels of T2DM patient appear smaller, consistent with diffuse atherosclerosis with multiple stenosis. There is a long stenosis affecting the proximal segment of the first diagonal branch with a further stenosis in the mid segment of the left anterior descending branch of the LCA. Images kindly provided by Dr Stephen Wheatcroft, Leeds General Infirmary, UK.

1.2. Blood vessel wall

The blood vessel walls of both arteries and veins consists of three main layers: the intima, media, and adventitia (Figure 1.3).

1.2.1. Basement membrane endothelium (intima)

The intimal layer underlying the lumen is a continuous monolayer of endothelial cells (EC). A proteoglycan-rich layer lies directly beneath the endothelial layer together with, few vascular smooth muscle cells (SMC), and macrophages (Newby and Zaltsman, 2000). This layer acts as a barrier between the blood and vessel wall, controls the transport of ions and fluids across the vascular space and also controls homeostasis and vascular tone (Stevens et al., 2000). The basement membrane or the extracellular matrix (ECM) is comprised of type IV collagen, which provides the structural support to form a fibrillar network of basal lamina, and also can modulate the adhesion, proliferation, migration, and survival of EC (Sell et al., 2009). Additional components include laminin, elastin, and heparan sulphate proteoglycans (Califano and Reinhart-King, 2013).

1.2.2. Vascular smooth muscle cells (media)

Underlying the intima is the media which contains mainly vascular SMC and these compose the majority of blood vessel wall (Califano and Reinhart-King, 2013). Vascular SMC in the medial layer are tightly packed into their interstitial matrix, which comprises type I and III collagen, fibronectin, and proteoglycans. Collagen I followed by collagen III constitute the major structural components of the ECM structure comprising 80% and 11% respectively (Newby and Zaltsman, 2000). The principal role of vascular SMC is to maintain prominent plasticity and to regulate blood pressure and blood flow distribution. The presence of the well-defined organisation of the vascular SMC and ECM in the media provide the structural foundation of the physiological property of the blood vessels. This will allow the differentiation of SMC that constitutes a critical part of vascular development to perform biosynthesis, proliferation, and contraction of the vasculature (Kawai-Kowase and Owens, 2007). On the other hand, in response to pathophysiological stimuli such as cytokines and growth factors in the circulation, disorganisation of the media structure together with other secreted mediators, provoke SMC and ECM to proliferate and migrate into the intima, thus initiating the development of atherosclerosis and restenosis (Alexander and Owens, 2012).

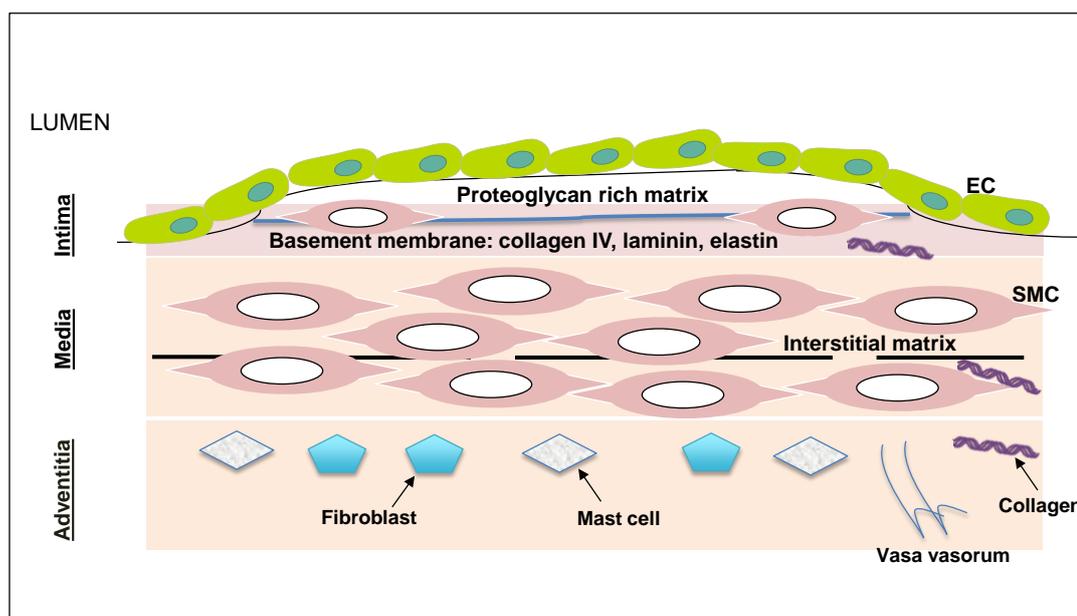


Figure 1.3: Normal blood vessel structure.

Normal blood vessel is composed of 3 layers. The innermost is the intima which is composed of endothelial cells (EC), lying on the basement membrane. The middle layer is the media which mainly contains smooth muscle cells (SMC). The outermost layer is the adventitia which contains fibroblasts, mast cells, and the vasa vasorum.

1.2.3. Adventitia

The adventitia is the outermost connective tissue surrounding the vessel and normally contains collagen, vasa vasorum (comprising arterioles, capillaries, and veins), nerve endings, fat, quiescent inflammatory cells and fibroblasts (Manon-Jensen et al., 2016). The main functions of the adventitia are to support the outer layer of the blood vessel, molecular exchange, storage and secretion of key regulators for the vessel wall function (Chen, 2011). The activated adventitia cells also generate reactive oxygen species (ROS), cytokines, growth factors and matrix metalloproteinases (MMPs) that collectively activate the medial cells for phenotypic modulation (Gareri et al., 2016). In severe arterial injury, stretching of the adventitia is accompanied by increased adventitial thickness, myofibroblast proliferation and collagen formation (Shi et al., 1996). The adventitial fibrotic response to vascular intervention plays a crucial role in restenosis by either circumferential compression of the vessel wall or by inhibiting external elastic lamina expansion (Lafont et al., 1995).

1.3. Atherosclerosis

Despite the continued advances in therapeutic treatment, atherosclerosis remains the leading cause of CVD and death around the world (Libby et al., 2011). Atherosclerosis is a chronic, progressive inflammatory disorder and interactions of different vascular cell types that are the underlying cause of angina, CAD and stroke (Libby, 2002). When coronary atherosclerosis progresses, the collateral vessels expand as a compensatory mechanism to the reduction of the perfusion rate in the resting state (Baykan et al., 2015). However, when there is increased activity, the myocardium requires an additional blood supply to cover the high oxygen demand. The insufficiency of blood supply to the heart (ischaemia) may then lead to myocardial infarction.

The endothelial layer is an interface between the blood stream and blood vessel wall. It normally promotes vasodilatation and protective antithrombotic properties by secreting prostacyclin, nitric oxide (NO) and adenosine (Holt et al., 1993). Disturbance of blood flow by a reduction in shear stress or endothelial injury permits white blood cell (leukocyte) adhesion onto the endothelial layer and provokes proatherogenic responses (Chatzizisis et al., 2007). Leukocyte adhesion can also be activated by the cell surface receptors, integrins (Luo et al., 2007). Subsequently, this can trigger the sub-endothelial layer to retain LDL cholesterol in focal areas of arteries through electrostatic binding to proteoglycan in the intima (Tabas et al., 2015). The subendothelial accumulation of LDL particles become oxidised and modified by enzymes into proinflammatory particles which lead to activation of EC and chronic inflammation (Insull Jr, 2009). Once resident in the arterial intima, leukocytes and T-lymphocytes communicate and recruit several cell types and mediators including EC, monocyte-derived macrophages, mitogens, chemoattractants, and chemokines (Figure 1.4A). Since the antithrombotic property of the lumen is lost by the disturbed inner endothelial layer of the lumen wall together with proteoglycan, this will further facilitate platelet adhesion.

Additionally, both activated macrophages and SMC promote the secretion of enzymes such as MMPs which degrade ECM components (George et al., 2000). Upon the influence of mechanical stress, growth factors and MMP secretion, vascular SMC become phenotypically modulated and thus proliferate and migrate into the damaged intimal layer (Zemskov et al., 2012, Owens et al., 2004). Accordingly, this results in the formation of necrotic lesions, apoptosis and narrowing of the interior lumen (Figure 1.4B).

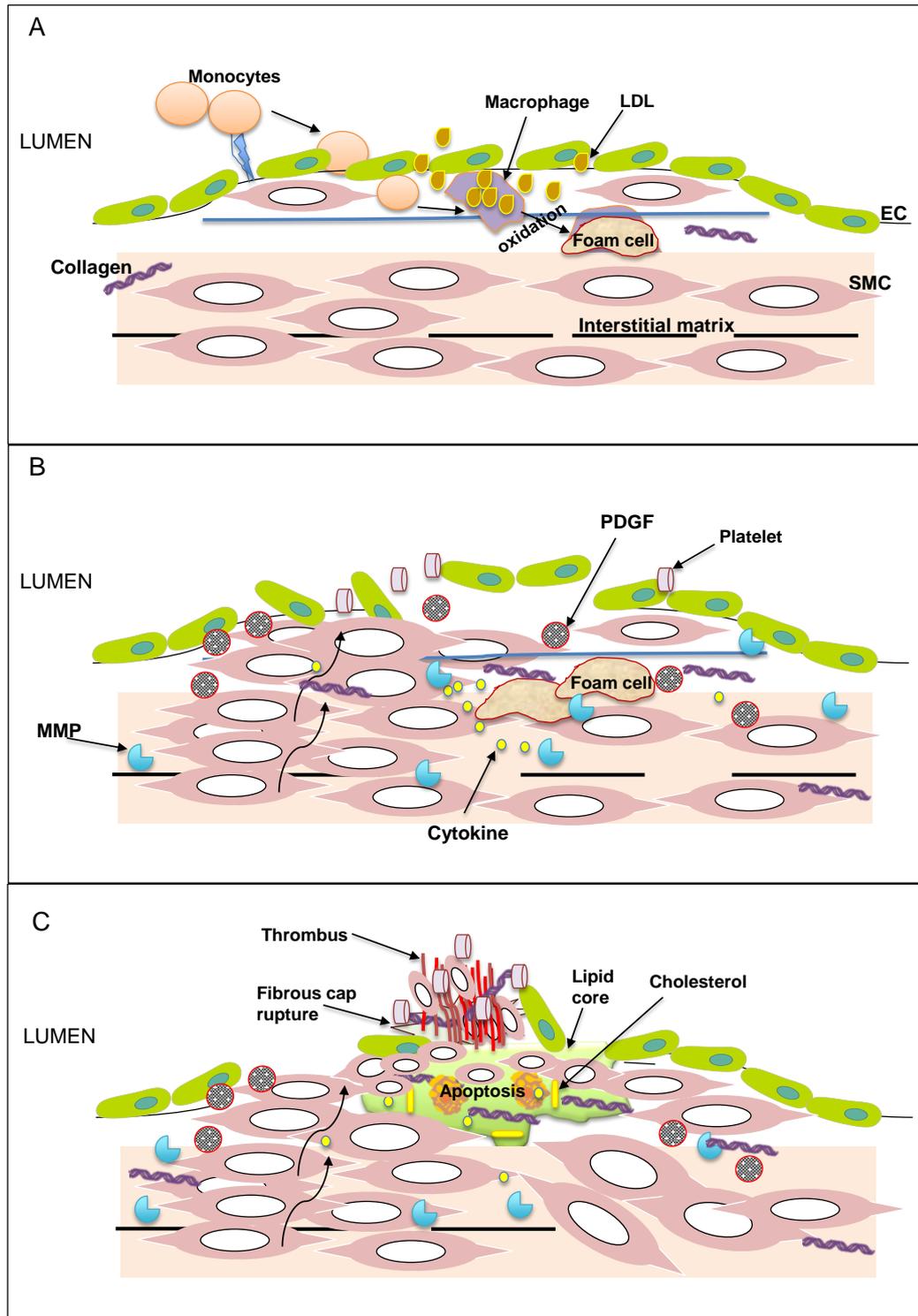


Figure 1.4: Stages of atherosclerosis development.

A- Initial step of atherosclerosis begins by adhesion of leukocytes to the endothelial cell (EC) monolayer, maturation of monocytes into macrophages yielding foam cells and activated EC layer. **B-** Lesion progression involves migration and proliferation of SMC, platelet adhesion and synthesis of ECM macromolecules. **C-** Thrombosis, disruption of atherosclerotic plaque and blood coagulation all can impede blood flow.

Vascular inflammation is orchestrated by a cascade of inflammatory and growth mediators. For example, ROS generation and secretion of inflammatory molecules by activated macrophages such as cytokines including interleukins (IL-1, IL-6 and IL-8), tumour necrosis factor alpha (TNF- α), and interferons (McDonald et al., 2012). Important signalling proteins and growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β) are also attracted to the atherosclerotic lesion in response to injury and contribute to ECM modulation (Hansson et al., 2015, Usman et al., 2015).

Atherosclerosis is a gradual progression from lipid particles into a complicated fibrotic plaque inside the inner wall of the artery. The atherosclerotic plaque consists of a lipid-rich core which is found in the centre of the luminal part. On the luminal side of the lipid core is a fibrous cap, which consists of lipid particles with macrophages, connective tissues and foam cells. In the initial atherosclerotic stages, the balance between SMC proliferation, matrix synthesis and cellular senescence in the intima can help in mediating plaque repair, resulting in plaque stability (Bennett et al., 2016). The stability of the atherosclerotic cap relies also on the thickness of fibrous cap and the level of cap inflammation. However, the core is highly thrombogenic and in the advanced lesion of atherosclerosis, the plaque can then grow large enough until it harmfully occludes the artery lumen and eventually reduces its blood flow (Hansson et al., 2015, Plutzky, 1999). The increased synthesis of SMC and a parallel increase in ECM production by SMC can stabilise the atherosclerotic plaque (Hao et al., 2003).

As a major consequence to inflammatory mediators' secretion, macrophages increase lipid content and foam cells are stabilised and SMC together with collagen are activated to migrate into the intimal layer inducing atherosclerotic scar. Another serious drawback is when the unstable fibrous plaque becomes fragile and may eventually rupture, causing thrombotic mass and occlusion of the artery (Figure 1.4C). Plaque rupture is enhanced by cap thinning stimulated by the accelerated death of SMC and degradation of collagen and ECM (Bennett et al., 2016, Lo and Plutzky, 2012). This can further trigger vascular thrombosis, ischaemia, myocardial infarction, stroke and limb ischemia (Jamaluddin et al., 2011).

Patients with T2DM have an increased prevalence of coronary atherosclerosis and account for a significant proportion of coronary interventions compared with ND individuals (Rong et al., 2016). T2DM patients have a 2-4 fold higher incidence of CVD and more dramatic rates of morbidity and mortality than patients without diabetes (Wang et al., 2015b). Moreover, several studies reported the high incidence of

restenosis in T2DM patients that reached up to 70% following coronary intervention (Gao et al., 2015, Kip et al., 1996).

Whilst available drug therapies for CAD such as lipid-lowering drugs, vasodilators, and coagulation and platelet aggregation inhibitors are helpful in mild-moderate stable conditions (Yusuf et al., 2011, Antiplatelet Trialists, 1994). These pharmacological therapies help to control the symptoms and slow disease progression rather than preventing the actual cause of the disease. In severe cases and inappropriate treatment, this can lead to complete vessel occlusion, acute myocardial infarction, stroke, angina or sudden cardiac death (Busing et al., 2005). Owing to the high prevalence of CHD, interventional surgical treatment including percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) are widespread procedures to rescue the patients with acute and severe coronary occlusion (Pugh et al., 2001).

1.3.1. Percutaneous coronary intervention

An emergency treatment for acute coronary syndrome, PCI or angioplasty involves non-surgical widening of the occluded coronary artery. This treatment involves inserting a catheter with an attached deflated balloon through the skin into radial (arm) or femoral (groin) artery of the patient, guided up into the obstructed coronary artery. The balloon is then inflated inside the artery at the site of obstruction, dilating the vessel. Alternatively, this can also be accompanied by a subsequent stent placement to provide mechanical support and keep the artery open, together with antiplatelet medications to prevent blood clotting (Busing et al., 2005, Dangas and Kuepper, 2002).

The most common PCI available for treating patients with symptomatic CAD are either bare-metal stents (commonly stainless steel) or drug-eluting stents which release immunosuppressive or anti-proliferative reagents (Joner et al., 2006, Celik et al., 2016). These procedures improve the haemodynamic flow rate and restore normal blood flow. PCI can also lead to blood vessel damage by the catheter and endothelial disruption, an important problem following endovascular treatment that can eventually cause vascular restenosis (Kimura et al., 2002, Moore et al., 2013). Therefore, thrombotic risk and atherosclerotic plaque formation accompanying stent placement are the major pathological outcomes. This eventually leads to atherosclerosis and serious occlusion, with an incidence rate of 30-40% within 3-6 months following stent implantation (Chen et al., 2012).

1.3.2. Coronary artery bypass graft surgery

Coronary artery bypass grafting remains a mainstay therapy for patients with multivessel occluded coronary arteries and multiple risk factors, and for patients with left main CAD and low ejection fraction (Yahagi et al., 2016). Reports have shown that 20-25% of patients undergoing CABG have diabetes (Flaherty and Davidson, 2005). Additionally, diabetic patients are at higher risk of perioperative morbidity, poor outcome and lower survival rate following CABG in comparison to ND counterparts (Weintraub et al., 1993, Kappetein et al., 2013). The commonly used bypass conduits for the occluded artery in CABG surgery are the autologous internal mammary artery (IMA) which is re-routed to the heart circulation, or the saphenous vein (SV) as illustrated in Figure 1.5., a suitable piece of the graft is attached to the ascending aorta and to the coronary artery above the occluded segment of the artery. CABG is highly effective in relieving chest pain and restoring the normal blood flow into the heart. The bypass can be performed to one occluded artery, but it is more common to perform double, triple or up to quadruple bypasses of the coronary arteries. Early CABG surgery provides a significant increase in survival rate compared with initial medical therapy (Yusuf et al., 1994, Sako et al., 2014).

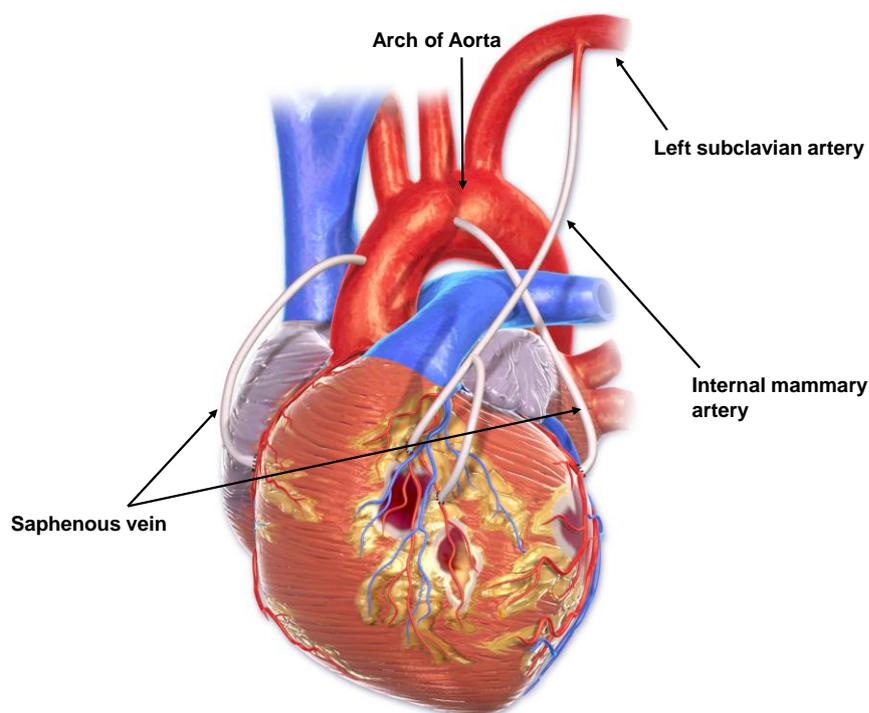


Figure 1.5: Schematic shows SV and IMA grafts in CABG surgery.

The IMA and SV are utilised to re-route the occluded coronary artery circulation. SV harvested from patient's leg is grafted to the aorta and the thoracic IMA is attached to left subclavian artery (https://en.wikipedia.org/wiki/File:Blausen_0154_CABG).

1.3.3. Comparison between IMA and SV (differential patency)

The use of autologous IMA was first utilised as a bypass conduit for CABG over 50 years ago. It is still the favoured graft with superior patency and stronger resistance to thrombosis than the SV. As it is re-routed from the arterial circulation, this has a higher survival advantage over the use of SV grafts (Cameron et al., 1996). However, most CHD cases are associated with multivessel coronary artery occlusion which necessitates the use of more than one graft. Thus, because of the limited number of arterial grafts available to cover the multivessel occlusion, the use of SV graft is both necessary and indispensable.

The underlying pathological lesion of SV graft stenosis is the characteristic development of intimal hyperplasia (IH) following CABG. IH often starts approximately 4-6 weeks following SV implantation (Dietrich et al., 2000) and this can be followed by an atherosclerotic lesion in the vein graft which starts within one year following vein implantation (Wang et al., 2013b). During the first year after CABG, up to 15% of SV grafts fail with an expected 4-5% increase each year with severe disease of the partially functioning graft (Nwasokwa, 1995). Therefore, in 10 years SV graft failure rates are close to 50% and approximately 31% of all CABG surgeries in the United Kingdom (UK) necessitate reintervention (Deb et al., 2012). Conversely, 80-95% of IMA grafts remain patent 10 years following CABG (Wilson, 2015).

Compared with IMA counterparts, SV has a greater diameter but always shows a lower patency rate and higher mortality rate following CABG surgery (Taggart et al., 2010). The aetiology of IH and graft failure is a complex multifactorial procedure and the specific mechanisms are still unclear. Initial considerable damage of the endothelial layer during harvesting and implantation of the SV graft almost certainly triggers initiation of the subsequent processes. Therefore, vasodilatory mediators (for example, NO and prostacyclin) are often reduced and instead there is increased secretion of vasoconstrictor agents such as endothelin-1 (Verrier and Boyle, 1996). The secretion of NO and prostacyclin is intrinsically lower in the SV, thus, the inherent antithrombotic property of SV is weaker than IMA, conferring greater susceptibility of the SV to thrombosis and occlusion (Mitra et al., 2006). Relocating the SV from the patient's leg into the higher pressure and flow rate of the new coronary arterial circulation can also cause graft trauma, loss of intimal integrity and aggravate the development of IH (Bryan and Angelini, 1994, Davies and Hagen, 1995).

The SV and IMA grafts have different gene expression patterns (Zhu et al., 2013) and react differently to the factors secreted by the graft's surface, including platelets, complement, coagulation and growth factors (Jia et al., 2006). Exposure of the

subendothelial matrix activates leukocyte deposition, platelet adhesion, and inflammatory and immune cells migration (Tanaka et al., 1993). Additionally, the excessive production of MMPs and cytokines promotes SMC phenotypic changes into proliferative and migratory phenotypes towards the lesion. This leads to the obstructive neointimal formation of the new graft resulting in graft remodelling and re-occlusion following CABG (Motwani and Topol, 1998, Sabik et al., 2005).

Furthermore, a number of studies compared human paired cultured SMC of SV and IMA, SV-SMC showed a consistently higher proliferation rate, invasive capacity and MAPK activation compared to cells obtained from IMA (Turner et al., 2007, Yang et al., 1998). These inherent functional differences between SV and IMA-SMC provide a further explanation for higher failure rates of SV grafts, by conferring susceptibility to accelerated IH and subsequent re-occlusion.

The high propensity for the failure of vein grafts is a major limitation of bypass surgery as it causes recurrent ischaemic events, with more than 20% of the patients requiring further revascularisation procedures within 6 months (Rutanen et al., 2002). Lastly, cardiovascular risk factors such as patient age, smoking, hyperlipidaemia, hypertension, and T2DM can all facilitate and aggravate graft failure. Owing to a large number of patients living with the risk of late vein graft failure, and the high morbidity and mortality rates (Wijns and Kolh, 2010), further investigation to uncover the exact biological mediators involved in IH is an increasing challenge to improve vein graft patency.

1.4. Alteration of vascular SMC phenotype

1.4.1. SMC plasticity and phenotypic switching

SMC exhibit two distinct phenotypes and they play an important physiological role during normal vascular development and growth (Figure 1.6). In the early stages of blood vessel formation, SMC are characterised by a relatively high proliferation rate and cellular synthesis of ECM components. Whereas in the adult blood vessel wall, the structure of vascular SMC remain in the mature differentiated phenotype (Hungerford and Little, 1999). The differentiated SMC are highly specialised cells, and exhibit a mainly spindle-shaped contractile phenotype with very low turnover and migratory actions.

Vascular SMC lack terminal differentiation and exhibit remarkable plasticity enabling them to switch between the two different phenotypes in response to local environmental milieu (Figure 1.6) (Owens, 2010). This phenotypic modulation of SMC

results in a rhomboidal shape that is characterised by a migratory and proliferative phenotype, lack of selective SMC differentiation marker genes (Chaabane et al., 2013), and increased production of ECM components (Horita et al., 2011, Zhang, 2009). The presence of intact ECM usually maintains the contractile phenotype of SMC from being switched into the proliferative and migratory phenotype and reduced susceptibility to mitogens. The phenotypic modulation from the quiescent contractile into active synthetic phenotype confers a survival advantage during vascular development, repair and adaptation. However, this alteration in differentiation state can be disadvantageous and might contribute to critical pathological changes in the blood vessels in vascular pathologies such as atherosclerosis, aortic aneurysm (Ailawadi et al., 2009, Shi and Chen, 2016), and restenosis, after vascular interventions such as coronary angioplasty and SV bypass grafting (Owens et al., 2004, Albinsson and Sessa, 2011). The precise mechanism underlying the high degree of plasticity and the precise stimulus involved in this modulation are not well identified. Therefore, intensive efforts are being made to identify various stimuli and understand the molecular mechanisms underlying phenotypic modulation in SMC.

Cytoskeletal proteins contributing to cellular structure and organisation have also been documented as valuable tools to identify SMC differentiation (Sherwood, 2015). Specific marker genes for differentiated SMC are α -smooth muscle actin (ACTA2) which is the predominant isoform in vascular SMC (Kawasaki et al., 2008), smooth muscle myosin heavy chains (SM-MHC), smooth muscle myosin light chains, h1-calponin, smoothelin, and smooth muscle α -tropomyosin (Alexander and Owens, 2012, Gomez and Owens, 2012). Actin filaments together with other actin-binding proteins form a complex network at the cortical region of the cell. This network supports the cell, acts as tracks for movement of intracellular molecules and a barrier for other molecules to diffuse into the cell (Cherng et al., 2008). Furthermore, the F-actin cytoskeleton plays an important role in the maintenance of cellular integrity, determining cell shape and function and driving cell movement. Actin together with myosin motor protein undergoes dynamic alterations to force cell movement in response to physiological and pathological abnormalities such as changes in shear stress or vascular injury (Searles et al., 2004, Pollard and Cooper, 2009). Studies have shown that F-actin bundling into fibres is related to SMC differentiation, and therefore, phenotypic modulation of vascular SMC is accompanied by changes in actin cytoskeleton alignment (Han et al., 2009).

1.4.2. Role of SMC in adaptation and remodelling

The environmental changes caused by various disease states or upon tissue injury result in the exposure of SMC to several factors that are involved in and facilitate phenotypic switching of SMC into the dedifferentiated phenotype (Figure 1.6). Following vascular wall damage, the controlled phenotypic modulation of SMC into the dedifferentiated phenotype is essential for effective adaptation to withstand arterial circulation, however, the imbalance and excessive SMC proliferation and migration signals can contribute to coronary re-occlusion in vascular pathological conditions (Southerland et al., 2013).

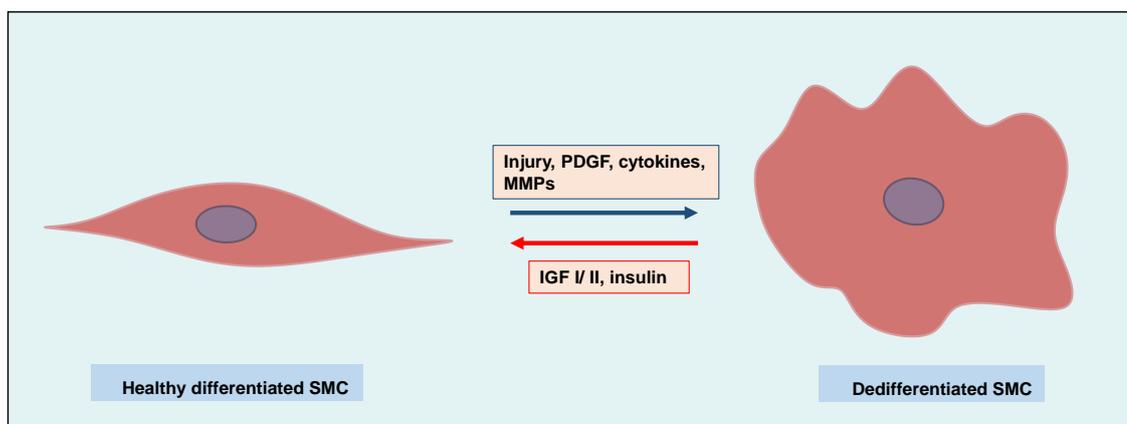


Figure 1.6: Vascular smooth muscle cell differentiated and dedifferentiated phenotype.

Differentiated SMC have spindle elongated morphology, high contractile gene expression (α -SMA), aligned actin fibres, lower cell area, and decreased ECM production and migration (Owens et al., 2004). In contrast, dedifferentiated SMC appear rhomboid, have low contractile gene expression, disorganised actin fibres, larger cell area, increased ECM and high proliferative and migratory rate (Riches et al., 2014). The differentiated phenotype can be protected by insulin-like growth factor (IGF) I and II or by insulin, whereas the dedifferentiated phenotype can be stimulated by platelet growth factor (PDGF), cytokines and matrix metalloproteinases (MMPs) (Metz et al., 2012, Hayashi et al., 1998).

1.4.3. Intimal hyperplasia

IH is the thickening process of the vessel intima as a result of the accumulation of SMC and ECM components into the intimal compartment following endothelial injury. Physiologically, IH occurs during the closure of the ductus arteriosus and uterus after birth (Newby and Zaltsman, 2000). Pathologically and upon vascular injury, IH occurs as a result of endothelial injury following balloon catheter denudation, angioplasty, autologous SV bypass grafting, vascular aneurysm, atherosclerosis and in hypertensive pulmonary arteries (Willis et al., 2004, Rzuclidlo, 2009). Normally, most grafted SV develop an adaptive minor intimal thickening to resist the new stress of arterial haemodynamics within 4-6 weeks of CABG (Motwani and Topol, 1998). The dedifferentiated SMC phenotype together with the excessive secretion of ECM components form the bulk of extensive IH lesion that eventually lead to restenosis and graft failure.

Effective wound repair is tightly regulated by the balance between ECM synthesis and degradation. However, the excessive rate of ECM production can lead to different pathological diseases, including atherosclerosis, neointima formation and vascular remodelling (Tomasek et al., 2002). Furthermore, the lack of an intact endothelial layer at the site of the implanted graft activates monocytes and promotes platelet adherence to the intima, inflammatory cells and mitogens are subsequently recruited, together with increased deposition of ECM components (Wan et al., 2012). The pathogenesis of vascular abnormalities has been postulated to be induced by phenotypic modulation in vascular SMC, which is accompanied by aberrant proliferation and migration of SMC (Figure 1.7) (Parmacek, 2009). Following injury of the vascular wall, the secreted collagen can in turn bind to the platelet receptors, enhancing platelet adhesion to the injured vascular wall (Manon-Jensen et al., 2016). Osteopontin is a matricellular protein which is activated in response to vascular injury and is stimulated by inflammatory cytokines and growth factors (Denhardt et al., 2001). A study using murine aortic SMC showed that osteopontin was implicated in recruiting and enhancing SMC migration into the intima which can further trigger IH and vascular remodelling (Leali et al., 2007).

1.4.4. Restenosis

Restenosis is the generation of the neointima and development of intimal narrowing of the coronary artery following PCI or in grafted blood vessel after CABG as a compensatory vascular response to injury (Lingman et al., 2011). Restenosis occurs within months following PCI and this will subsequently lead to occlusion. Multiple factors contribute to restenosis, in particular, patients with diabetes (Bauters et al., 2003) and hypertension (Suurkula et al., 1994) account for the high-risk group of negative outcome and restenosis following coronary intervention.

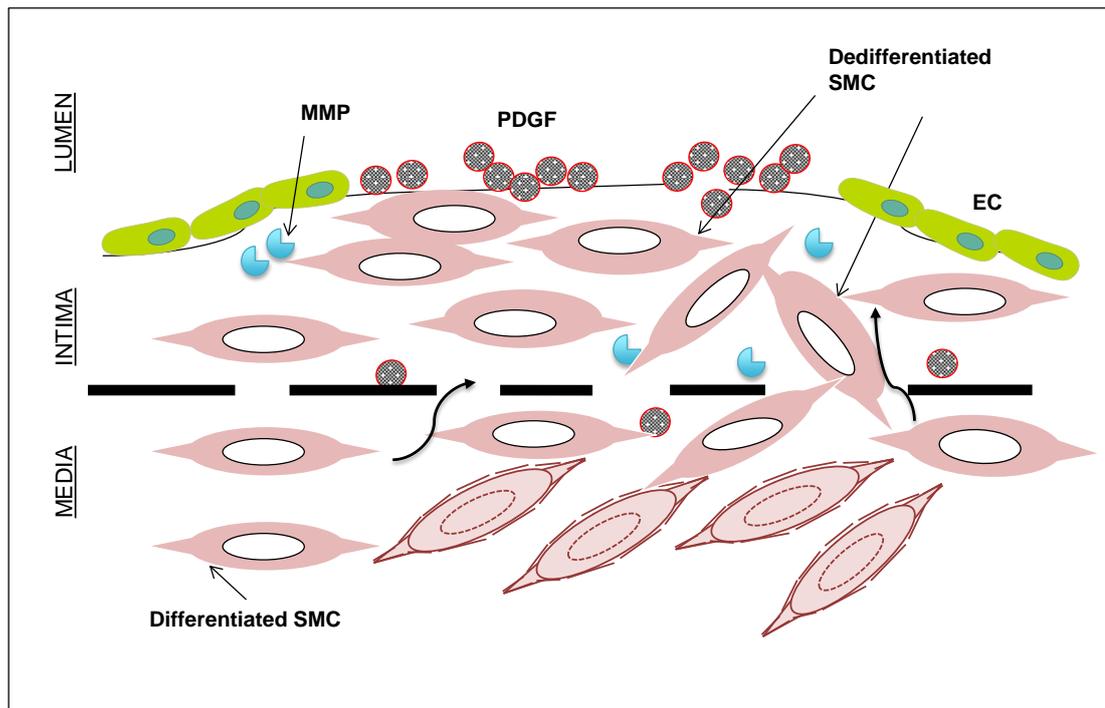


Figure 1.7: SMC response to vascular injury.

Following vascular injury, PDGF and MMPs are upregulated and secreted into the damaged endothelial layer, this will stimulate the phenotypic switching of SMC into the dedifferentiated phenotype which have proliferative and migratory characteristics.

1.4.5. Mediators and inflammatory factors involved in vascular remodelling

Impaired control of the differentiated state of vascular SMC is accompanied by increased SMC proliferation and migration towards the site of injury as a consequence of the secretion of various mediators involved in vascular remodelling (Ip et al., 1990) as illustrated in Figure 1.7 and listed in Table 1.1. These mainly include growth factors, inflammatory cytokines and MMPs. On the other hand, insulin and closely related peptide insulin-like growth factor (IGF) are known to inhibit phenotypic switching of SMC into the dedifferentiated phenotype (Bitto et al., 2010).

Table 1.1 Common mediators involved during vascular remodelling and restenosis

| Mediators secreted | Source | Action | Reference |
|---------------------------|----------------------------------|-------------------------------------|-------------------------|
| PDGF | Platelets, SMC, EC | SMC proliferation and migration | Salabei et al. (2013) |
| VEGF | EC | EC proliferation and migration | Wang et al. (2004) |
| TGF- β | SMC, EC | SMC proliferation | Massagué et al. (2000) |
| Cytokines (IL1, IL6, IL8) | Leukocytes, macrophages, SMC, EC | Neutrophil and monocyte recruitment | Pasi et al. (2015) |
| MMPs (MMP- 1,2,3, 9) | SMC, EC | ECM degradation, SMC migration | Amin et al. (2016) |
| TIMPs | SMC, EC | Inhibit MMPs | Visse and Nagase (2003) |

PDGF: Platelet-derived growth factor, VEGF: vascular endothelial growth factor, TGF- β : Transforming growth factor- β , IL: interleukin, MMPs: Matrix metalloproteinases, TIMPs: Tissue inhibitors of metalloproteinases.

A. Growth factors

Various types of growth factors appear to regulate SMC phenotypic modulation. These include vascular endothelial growth factor (VEGF) and PDGF which have been reported to be released from matrix stores in response to endothelial injury (Wang et al., 2004).

PDGF is the most potent chemoattractant and mitogen for vascular SMC, thus, it is known to promote the rapid transition of SMC into synthetic and migratory phenotypes (Salabei et al., 2013). It has 2 disulphide-linked polypeptide chains (A and B) that form 3 isoforms denoted PDGF-AA, PDGF-BB, and PDGF-AB. PDGF initiates its intracellular signalling by binding to 2 subtypes of receptors; PDGF-R α or PDGF-R β (Adachi et al., 2004). The A chain binds to PDGF-R α only, whilst the B chain can bind to both receptors. PDGF has an important physiological role in wound healing and is synthesised by platelets and other cell types such as macrophages, EC and SMC (Hassoun et al., 2009). Under pathological conditions, PDGF has been shown to mediate SMC proliferation and migration (from the media to intima) as in the case of angiogenesis, atherosclerosis, and restenosis (Clunn et al., 1997). PDGF regulates SMC dedifferentiation through stimulation of the mitogen-activated protein kinase extracellular regulated kinase 1/2 (MAPK/ERK1/2) and Phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways (Gan et al., 2013). The action of PDGF on SV would be a challenging area of research as PDGF is a major growth factor associated with IH and increased SMC migration and proliferation constitute the cornerstone of SV graft failure (Xu et al., 2015a).

B. Cytokines

Cytokines are small, soluble secreted proteins that have pro- or anti-inflammatory actions. They include interleukins (ILs), TGF- β , and TNF- α , all of which are associated with metabolic syndrome and involved in mediating SMC phenotypic changes (Salabei and Hill, 2013).

TNF- α is found in atheromas and can be secreted by inflammatory cells and vascular SMC (Newby and Zaltsman, 1999). TGF- β is a powerful profibrotic agent released from EC and SMC in a latent form in response to inflammation. Its activation requires extracellular enzymes such as plasmin, urokinase and MMPs. TGF- β 1 is the most frequently upregulated isoform in response to endothelial injury and vascular remodelling (Massagué et al., 2000). It has an important role in promoting SMC migration, increased ECM production and plaque stabilisation (Hishikawa et al., 1999, Ito et al., 2009).

IL-1 comprises two related subtypes, IL-1 α and IL-1 β . Both IL-1 subtypes stimulate IL-1R and are involved in the different immunological response, pro-inflammatory reactions and hematopoiesis (Pasi et al., 2015). IL-1 α is an intracellular cytokine, constitutively available in most cell types, synthesised by monocytes and macrophages as an inactive proprotein (Clarke et al., 2010, Turner, 2014). IL-1 α is released upon tissue damage or necrosis, can activate other cytokines and neutrophil recruitment, and controls physiological cell proliferation, growth, and apoptosis (Clarke et al., 2010). Furthermore, IL-1 α plays an important role in ECM degradation by activating MMP-1, MMP-3, MMP-9 and MMP-10 expression (Turner et al., 2010). Subsequently, IL-1 α is proteolytically processed and activated by calpain then secreted in response to viral infections, autoimmune diseases, tissue necrosis and vascular injury (Dinarello and van der Meer, 2013, Clarke et al., 2010). Unlike IL-1 α precursor, IL-1 β is inactive and needs to be cleaved by caspase-1, to release the active cytokine into the extracellular space (Garlanda et al., 2013). IL-1 β is synthesised by blood monocytes, tissue macrophages and dendritic cells, and activated by caspase-1 in response to inflammation or immune reaction.

C. Matrix metalloproteinases

Cell-matrix structure is controlled by a dynamic balance between matrix synthesis and breakdown and a disturbance in that balance is a major contributor to vascular pathological conditions (Stetler-Stevenson, 1996). MMPs play a crucial role in the degradation of collagen and elastin; the major components of ECM (Newby et al., 2009) and regulate matrix turnover rate during morphogenesis, angiogenesis and tissue repair (Park et al., 2004). On the other hand, excessive proteolysis and an imbalance between MMP activation and degradation contributes to tissue destruction and various CVDs including, fibrosis, atherosclerosis and vascular remodelling (Amin et al., 2016).

MMPs are a family of more than 25 genetically-related proteinases (Gill and Parks, 2011). These are classified into 5 major groups according to their structure or substrate specificity into collagenases, gelatinases, stromelysins, matrilysins, membrane-bound MMP, and other unclassified subgroups. The main classes of MMPs are summarised in Table 1.2.

Table 1.2 Types of matrix metalloproteinases (MMPs)

| Enzyme | MMP | Substrate | Reference |
|-------------------------------------------------|--------|---------------------------------------------------------------------|--------------------------|
| Collagenases: Interstitial collagenase 1 | MMP-1 | Collagen type I, II and III, gelatin | Visse and Nagase (2003) |
| Neutrophil collagenase 2 | MMP-8 | Collagen type I, III, aggrecan | Kim et al. (2016) |
| Collagenase 3 | MMP-13 | Collagen type I,II, III, IV, X, XIV, gelatin, fibronectin, aggrecan | Nagel et al. (2005) |
| Collagenase 4 | MMP-18 | Collagen type I, III | Sang and Shi (2013) |
| Gelatinases: Gelatinase A | MMP-2 | Gelatin, collagen IV, V, VII and X | Halper and Kjaer (2014) |
| Gelatinase B | MMP-9 | Gelatin, fibronectin | Turner and Porter (2012) |
| Stromelysins: Stromelysin 1 | MMP-3 | Gelatin, fibronectin, laminin, collagen type III, IV, V, IX, X | Patterson et al. (2001) |
| Stromelysin 2 | MMP-10 | Fibronectin, aggrecan, collagen type I, IV, V | Rohani et al. (2015) |
| Stromelysin 3 | MMP-11 | Collagen VI, laminin, insulin-like growth factor protein 1 | Lin et al. (2015) |

| | | | | |
|----------------------------|--------------|--------|---------------------------------------------------------------------------------------------------|----------------------------|
| Matrilysins: | Matrilysin 1 | MMP-7 | Collagen type I, IV, gelatin, fibronectin, laminin, elastin, insulin-like growth factor protein 1 | Klein and Bischoff (2011) |
| | Matrilysin 2 | MMP-26 | Collagen type IV, gelatin, fibronectin, pro-MMP-9 activation | Gruber et al. (2012) |
| Membrane-type (MT): | MT1 | MMP-14 | Collagen III, laminin, gelatin, Pro-enzyme MMP-2 activation | Itoh and Seiki (2006) |
| | MT2 | MMP-15 | Pro-enzyme MMP-2 activation | Raffetto and Khalil (2008) |
| | MT3 | MMP-16 | Pro-enzyme MMP-2 activation | Zhao et al. (2004) |
| | MT4 | MMP-17 | Gelatin, fibrin | Truong et al. (2016) |
| | MT5 | MMP-24 | Pro-enzyme MMP-2 activation | Wang et al. (1999) |
| | MT6 | MMP-25 | Fibronectin, type IV collagen, proteoglycans | Kang et al. (2001) |

MMP activity is usually controlled at 3 different levels starting from gene expression, followed by zymogen activation and proteolytic inhibition (Raffetto and Khalil, 2008). MMP secretion from macrophages and vascular SMC is transcriptionally stimulated by different factors including PDGF, cytokines and inflammatory mediators (Kong et al., 2013, Sheu et al., 2004). The MMPs all share similar properties of having a Zn²⁺-containing catalytic site and being generated as pro-enzymes (zymogens) that necessitate proteolytic activation. Activation is catalysed both at the cell surface and in the extracellular space. Intracellularly, activation of pro-MMPs is initiated by cellular endoproteases or by phosphorylation (Cha et al., 1996). At the extracellular level, proteolytic activation of MMPs is mediated by thrombin, fibrinolytic protease plasmin, serine protease tissue kallikrein, trypsin and other enzymes (Page-McCaw et al., 2007). MMPs play key roles in cellular migration and tissue remodelling by together digesting all ECM components of the vascular wall (MacColl and Khalil, 2015). Normally their excess activation is inhibited by either α 2-macroglobulin in plasma or by one or more of tissue inhibitors of metalloproteinases (TIMPs) (Nagase et al., 2006).

TIMPs are endogenous tissue inhibitors of metalloproteinases and there are four main types (TIMPs 1-4) which can inhibit all active forms of MMPs with variable preference. TIMP-1 inhibits MMP-1, MMP-3, MMP-7, and MMP-9, whereas TIMP-2 mainly inhibits MMP-2. TIMP-3 can inhibit MMP-1, MMP-2, MMP-3, and MMP-9 (Lei et al., 2007), whereas TIMP-4 inhibits MT1-MMP and MMP-2 catalytic effects (Bourboulia and Stetler-Stevenson, 2010).

Increasing evidence suggests that the proteolytic effects of MMPs play a crucial role in cellular migration, vascular formation and remodelling (Galis and Khatri, 2002). Normally, the ECM components interact with vascular SMC through cell-to-matrix contacts via integrins. SMC in the medial layer are embedded in the basement membrane and integrins retard SMC phenotypic modulation (Newby, 2005). Studies have shown that the increased secretion of MMPs digest integrins and thus facilitate the dedifferentiation of SMC (Newby and Zaltsman, 2000). Additionally, the balance between MMP synthesis, TIMPs and matrix production by SMC plays a significant role in the stability of the atherosclerotic plaque (Lemaitre et al., 2003). Since vascular SMC are principal elements of the vasculature and MMPs contribute to SMC migration and proliferation, this seems to provide a viable therapeutic strategy in IH.

1.4.6. Cardiovascular therapy for vascular remodelling

Some of the routine cardiovascular therapies utilised for CVD patients show additional beneficial effects on SMC phenotype modulation. For example, a further function for the lipid-lowering drugs, statins, is a protective effect against vein graft failure by retarding SMC dedifferentiation into the synthetic and proliferative phenotype and antagonising inflammatory cytokines (Kiyon et al., 2007, Margaritis et al., 2012). Angiotensin II (ANG) was reported to activate monocyte chemoattractant protein-1 expression and induce vascular inflammation through activation of TGF- β and the oxidative factor NADPH oxidase (Niu and Kolattukudy, 2009, Wylie-Sears et al., 2014). Thus, this can trigger damage of the vascular endothelial layer and initiation of atherosclerosis (Münzel et al., 2008). Additionally, using cultured rat aortic SMC, ANG increased vascular SMC proliferation and migration and ECM deposition (Nagayama et al., 2015). Therefore, antihypertensive drugs such as ANG receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEIs) would be expected to reduce the incidence of atherosclerosis and vascular remodelling. Moreover, ANG blockers reduce ROS generation and inhibit calcium-activated potassium channel and therefore reduced neointimal formation in rats following arterial injury (Tharp et al., 2008, Patel et al., 2014). Carvedilol is another antihypertensive agent, acts as a β -adrenoceptor antagonist and showed an inhibitory effect against the chemoattractant and mitogenic action of PDGF on cultured rat aortic SMC (Ohlstein et al., 1993). Aspirin irreversibly inhibits cyclooxygenase and suppresses the production of prostaglandins and thromboxane, therefore, mediating beneficial antiplatelet and anti-inflammatory effects (Toth et al., 2013).

1.5. Diabetes Mellitus

Diabetes is the most common metabolic disorder and is considered as one of the most important cardiovascular risk factors, health burden and threats of our time (Berry et al., 2007). There are two types of diabetes. Type 1 diabetes mellitus (T1DM) is an autoimmune condition characterised by a lack of insulin secretion and high susceptibility to ketoacidosis (Morris et al., 2015). T1DM results secondary to pancreatic β -cell destruction by the immune system. Genetic factors and certain viral infections may also participate; however, the molecular mechanisms of β -cell death and its regulation are poorly understood (Ilonen and Akerblom, 1999). It usually presents in children and young adults but it can be diagnosed at any age. T1DM accounts for 5-10% of total diabetes and its treatment requires insulin injection (Jun and Yoon, 2004). Plasma levels of glycated haemoglobin (HbA1c) provide a measurement of blood glucose for the past 8-12 weeks, which is 4-6% in healthy individuals (Brown et al., 2010).

Type 2 DM (T2DM) accounts for 90-95% of adult diabetic cases, and results when target tissues (adipose tissue, liver, skeletal muscle) fail to respond adequately to circulating insulin. This activates the pancreas to produce more insulin as a compensatory mechanism for insulin resistance until it cannot make enough insulin to keep up with the increased demand (Baker et al., 2011). Accordingly, diagnosis of T2DM is often made following the insufficient pancreatic insulin secretion failed to maintain normoglycemia and symptoms of hyperglycaemia become overt. Subsequently, this results in hyperglycaemia and increased free fatty acid levels, along with raised insulin levels and insulin resistance; hallmarks of the metabolic syndrome (Goossens, 2008). Since 1980, the global number of people living with T2DM is dramatically growing all over the world. WHO reported until April 2016, the number of people with diabetes has quadrupled to 422 million adults, with most living in developing countries (<http://www.who.int/mediacentre/news>). In the UK, there were 3.5 million patients diagnosed with T2DM in 2015 and approximately more than half a million more people with undiagnosed diabetes (<https://www.diabetes.org.uk>). T2DM has a strong genetic tendency, although physical inactivity, eating an energy-dense diet and obesity accelerate its prevalence. T2DM patients are usually treated orally by metformin as an initial therapy, and in patients with persistent high HbA1c, a second oral agent can be added and insulin injection is recommended if the response to oral therapy is insufficient (Morris et al., 2015).

1.5.1. T2DM and cardiovascular complications

Owing to the higher tendency of obesity, sedentary lifestyles and increasing longevity, the epidemic of diabetes is an escalating public health problem that causes a reduction in life expectancy and raises morbidity due to its cardiovascular complications.

T2DM is associated with debilitating cardiovascular complications (Guay et al., 2011). Diabetes can confer up to 4-fold increase in the risk of ischemic heart disease with poor prognosis and more than double the mortality rate compared to non-diabetic (ND) patients (Miettinen et al., 1998, Cordero et al., 2016). Diabetic patients are highly susceptible to vascular complications; being either macrovascular (CAD and stroke) and/or microvascular (including retinopathy, nephropathy, and neuropathy) (Winer and Sowers, 2004). The major risk factors related to CVDs for diabetic patients are smoking, hyperglycaemia, hyperinsulinemia, hyperlipidaemia, obesity, hypertension, inflammation, hypercoagulability and atherosclerosis (Alexandru et al., 2016). These co-existing multiple risk factors of diabetic patients usually produce a hazardous response before surgery (Lawrie et al., 1986). Hyperinsulinemia can induce SMC proliferation and migration and increase ECM production (Aronson et al., 1996, Kannel et al., 1991). Additionally, hyperinsulinemia can produce coronary vasospasm and thrombus formation and reduction of fibrinolytic activity (Suzuki et al., 1996, Sobel et al., 1998). Following treatment of CAD with both stent and CABG, the reduction in long-term survival outcome and unfavourable consequences and complications after SV revascularisation remain higher in T2DM patients than ND counterparts (Berry et al., 2007, Kubal et al., 2005). Increased arterial wall stiffness (Henry et al., 2003), exaggerated neointimal formation, restenosis and inefficient early adaptation to arterial conditions in T2DM can limit the graft patency outcome (Owens, 2010). In addition to the higher rate of in-hospital mortality, insulin-dependent diabetic patients were associated with higher incidence of acute renal failure, deep sternal wound infection, and prolonged post CABG surgery hospital stay (Kubal et al., 2005). In a cohort study with 4118 participants from 1950-1995, a 50% decline in the incidence of CVD was observed in both diabetic and ND patients, however, the absolute risk of CVD was still 2-fold higher in diabetic patients (Fox et al., 2004).

In diabetic patients, the endothelial layer is exposed to the diabetic environment, therefore, hyperglycaemia and oxidative stress in T2DM have been documented to contribute to endothelial membrane stiffness (Calles-Escandon and Cipolla, 2001). In the early stages, and even before the diagnosis of diabetes, the irregular glucose levels in patients or obese individuals is usually accompanied by insulin resistance,

ROS secretion and consequently all can mediate vascular changes, particularly SMC and EC abnormalities (Alexandru et al., 2016). Both constant and intermittent high glucose levels play a crucial role in endothelial dysfunction by impairing NO production and metabolism and inducing changes in F-actin expression and arrangement (Chen et al., 2013a). Endothelial impairment and apoptosis in diabetes, in addition to the high prothrombin activation, would further increase diabetic patients' susceptibility to atherosclerosis and abnormal blood clotting factors (Dokken, 2008, Risso et al., 2001). Moreover, diabetic patients have greater susceptibility to myocardial infarction, severe CAD, and coronary thrombosis than ND individuals (Hakala et al., 2005). Therefore, the underlying mechanisms accelerating CVD in patients with T2DM are variable and need further investigation.

1.5.2. Glycaemia and metabolic memory

Normalisation of glucose levels, according to clinical studies published in the Diabetic Control and Complications Trial (DCCT) and UK Prospective Diabetes Study (UKPDS), is highly effective in retarding microvascular complications in diabetic patients (Skyler et al., 2009, Brown et al., 2010). On the other hand, results obtained from ACCORD, ADVANCE, and VADT trials have shown that macrovascular complications were persistent, at least in the medium-term, especially when the cardiovascular complications were well established (Skyler et al., 2009). The UKPDS and DCCT studies have revealed that intensive glucose control and intervention in the early stages of the disease can minimise cardiovascular events in the long-term. However, early diagnosis of T2DM is difficult and more than 50% of patients when diagnosed with the disease, already have evidence of cardiovascular complications (Stolar, 2010). This was shown by Epidemiology of Diabetes Interventions and Complications (EDIC) trial, in which 1441 T1DM individuals were assigned into intensive or conventional diabetic therapy and they monitored the incidence of cardiovascular complications (myocardial infarction, stroke, death from CVD, angina, or the need for CABG) for an average of 17 years follow-up. It was reported that 52 patients treated with a conventional glucose-lowering therapy developed 98 CVD events whereas intensive therapy reduced the incidence to 46 CVDs events in 31 patients (Nathan et al., 2005). This was also supported by a recent DCCT trial that showed intensive diabetic therapy reduced the incidence of CVD by 30% in 30 years follow-up study for T1DM patients (Gubitosi-Klug et al., 2016).

It is suggested that in T2DM, early and long exposure to abnormal environmental cues (hyperglycaemia, hyperinsulinaemia, and various inflammatory mediators) can

lead to persistent alteration of vascular gene expression and adverse phenotypic changes, a phenomenon called “metabolic memory” (Cooper, 2009). Short-term exposure of aortic EC to hyperglycaemia induced pro-inflammatory gene expression in ND mice *in vivo* and *in vitro*, and this was maintained even under subsequent normal glycaemic conditions (El-Osta et al., 2008). Moreover, vascular SMC cultured from diabetic *db/db* mouse aortas displayed an increased inflammatory gene expression compared to ND cells and this was maintained throughout multiple cell culture passages (Li et al., 2006).

Apparently, high glucose levels seem to be the main culprit in pathogenesis of diabetes, but other mediators such as pro-inflammatory cytokines, advanced glycation end products (AGEs), hyperinsulinaemia and free fatty acids (FFAs), all seem to be involved in the diabetes phenotype (Ceriello et al., 2009, Yan et al., 2008). Studies have shown that the interaction between AGEs and SMC can stimulate SMC migration, cytokine synthesis and ECM deposition (Yan et al., 2008). Collectively, these will cause overproduction of oxidative stress that persists despite normalising glycaemia. Thus, if a new therapeutic agent could erase this long-term memory phenomenon, several diabetic cardiovascular complications could potentially be resolved.

1.5.3. Can T2DM influence SMC phenotype?

Distinct phenotypes have been observed between human SV-SMC cultured from diabetic patients and ND counterparts. SMC cultured *in-vitro* from T2DM SV exhibited rhomboid morphology, F-actin fragmentation and reduced proliferation rate (Madi et al., 2009). Additionally, cell area was increased and higher gene expression of ACTA2 (the gene encoding α -SMA) was observed by our group (Riches et al., 2014); characteristics that might contribute to adverse outcome after CABG and poor adaptation in diabetes. Conversely, ND cells were spindle in shape, had higher proliferation rates and normal alignment of F-actin fibres (Madi et al., 2009). At variance to these previous studies, Faries et al. (2001) reported a significant increase in SMC proliferation of SV and infragenicular arterial cells of T2DM relative to ND counterparts. In addition, T2DM cells had abnormal morphology such as the loss of normal hill and valley shape and increased cell migration. Vascular SMC proliferation and migration, especially after stent implantation, play an important role in neointimal hyperplasia through cellular expansion and ECM aggregation (Lorusso et al., 2003). Another study performed by our group also showed that SV-EC cultured from T2DM

patients were aberrant; exhibiting 30% lower migration and 40% reduction in angiogenic capacity relative to ND EC (Roberts et al., 2015).

Experimental animal studies have shown that short-term exposure to hyperglycaemia induced persistent phenotypic alterations and even after normalisation of blood glucose levels, atherosclerosis progressed in diabetic mouse models (Brasacchio et al., 2009). The complexities in the pathogenesis of T2DM make it challenging to uncover the culprit factors mediating phenotypic modulation in SMC and thus further investigations regarding dysfunctional characteristics such as coronary revascularisation and restenosis complications in diabetic SMC phenotypic changes are critically important. The UKPDS trial termed the persistent phenotypic changes a “legacy” effect as these phenotypes were retained even following tight metabolic control (Cooper, 2009), which potentially supports the idea of epigenetic changes that would induce vascular dysfunction.

1.6. Current therapy for intimal hyperplasia and vascular remodelling

The pathophysiology of IH is complex and multiple culprit factors have been implicated. Although many studies have been conducted to treat vascular diseases, IH and vascular restenosis remain a limitation and a remarkable challenge for the field of vascular biology following vascular injury. A number of studies are ongoing and a newer field of research has emerged for identifying the key component(s) and treating neointimal hyperplasia.

1.6.1. Endothelial layer regeneration

Due to the EC layer’s pivotal role of being the first line to be damaged during surgery, and its ability to trigger the subsequent steps of IH, new research has focused on the regeneration of EC in the injured vessel. For example, endothelial progenitor cells isolated from bone-marrow or umbilical cord blood have been transplanted into injured vessels to replace the damaged EC and repair vascular injury (Asahara et al., 1999). However, these progenitor cells are limited in number and cannot match the clinical requirements, are not totally specific and can affect different areas, and can only be used for autologous transplantation due to high immunogenicity (Xu et al., 2015c).

1.6.2. Gene therapy for SV grafting

Despite the increasing number of molecular and clinical trials, all of the currently available pharmacological therapies have limited impact on early vein graft remodelling, long-term patency rate and the accelerated risk of atherosclerosis. Therefore, novel therapeutic targets remain a clear clinical requirement for the prevention of SV graft failure. A few new gene delivery approaches have been tested clinically, however, the creation of efficient gene delivery to the target human tissue remain an obstacle (Seo et al., 2013). Moreover, all of the mediators involved in neointimal formation and SV graft failure represent potential targets for molecular and genetic intervention for prolonging vein graft patency.

Inflammation is one of the important factors in neointima formation and vein graft failure and TGF- β is one of the important mediators involved in atherosclerosis and IH (Nikol et al., 1992). Blocking TGF- β 1 signalling suppressed restenosis and IH following porcine coronary artery angioplasty (Kingston et al., 2001) and silencing TGF- β 1 in a rat jugular vein model led to decreased MMP-1 and increased TIMP-1 expression and subsequently reduced vein IH (Sun et al., 2012a). In addition, an adenoviral vector was widely tested pre-clinically and showed effective blockage of vein neointima formation in animal models. For example overexpression of TIMP-3 using adenovirus delivery therapy was effectively protected ECM and mediated 50% reduction in neointima formation via preventing MMP activity *in vivo* in porcine vein graft (George et al., 2011). However, a high concentration of adenovirus is required to achieve efficient uptake and therapeutic benefit (White et al., 2013). Additionally, adenoviral overexpression of TIMP3 reduced cell migration in human *in vitro* and *ex vivo* models and porcine SV graft neointima formation (Wan et al., 2012, George et al., 2000). Although preclinical trials revealed promising results obtained with gene therapy in treating CVD, phase II and III clinical trials did not show a successful result similar to those obtained in animal trials. Furthermore, in one animal study adenovirus gene therapy mediated vascular inflammation and proliferation using a rabbit model of vascular disease (Newman et al., 1995). The results of other animal adenovirus gene therapy targeting chemokine receptors (Eefting et al., 2009) and TGF- β (Ranjzad et al., 2009) are promising. However, none of these studies reached clinical therapeutic approval in retarding CABG complications. Therefore, designing effective gene vectors and choosing an appropriate target gene(s) would provide a novel therapy for the treatment of CVDs and neointimal hyperplasia following graft surgery.

Delivery of gene therapy can potentially occur during CABG surgery. Isolation of the SV provides a great advantage of the selective gene therapy to reach its target in

the harvested vein during the clinical window. Therefore, the gene vector must be efficient to achieve target gene modulation *ex vivo* and before vein implantation into the arterial circulation. The local and direct administration of gene therapy into the vein would also minimise the risk of the therapy reaching the systemic circulation (Southerland et al., 2013). Recently, targeting gene expression to ameliorate vascular remodelling has emerged as a new specific and non-invasive therapy for vascular diseases. For example the use of non-viral antisense therapy including short interfering RNA (siRNA) to induce specific gene silencing. These siRNA molecules comprise a short double-stranded DNA sequence which is specific to a particular target gene, inducing posttranscriptional gene silencing (Kanasty et al., 2013). They bind to the RNA interference-induced silencing complex (RISC) which then breaks down the target mRNA (Cullen, 2004). Using Edifoligide, an E2F transcription factor decoy siRNA, was effective in delaying vein graft IH in rabbits (Mann et al., 1999) however, Edifoligide failed to improve patient outcome one year after CABG surgery (Lopes et al., 2012). Therefore, identification of suitable targets of IH and in depth analysis of the cell signalling of the cells of the injured wall remain important areas for research.

1.6.3. Epigenetic therapeutics

Whilst SMC proliferation and migration from the media into the intima play a pivotal role in the development of vascular occlusion, finding new targets to oppose these phenomena is mandatory. The stable and heritable changes that occur in gene function without changes in the nucleotide sequence are called epigenetic regulation. Epigenetic changes include histone modifications (acetylation or methylation), DNA methylation and miRNA (miR)-induced gene silencing (Cheng et al., 2010), which have been detected in animal and cell models of diabetes as a result of hyperglycaemia (Ling and Groop, 2009, El-Osta et al., 2008). This explains how cells with matching DNA sequences can differentiate into different cell types with different phenotypes. Long-lasting epigenetic modification causes persistent changes in smooth muscle phenotype and altered gene expression of miR in the vasculature (Alexander and Owens, 2012).

As a new strategy of epigenetic regulation, the discovery of miRs in the treatment of vascular remodelling has emerged. Recent studies suggest that the modulation of SMC phenotype and function during vascular remodelling is controlled by miRs (Albinsson and Sessa, 2011). They have the capacity to post-transcriptionally regulate the expression of genes responsible for diverse physiological cellular

functions, such as differentiation, growth, proliferation and apoptosis (Ambros, 2004, Kawai-Kowase and Owens, 2007). However, dysregulation or aberrant expression of miRs is often associated with various types of human diseases (Kim et al., 2009). Thus, numerous studies have proposed that miRs might play a fundamental role in vascular SMC development, differentiation and proliferation (Albinsson et al., 2010).

1.7. MicroRNAs (miRs)

MiRs are a recently discovered class of endogenous, small (18-25 nucleotides), single-stranded and non-coding sequences of RNA (Virtue et al., 2012). MiRs bind through base pairing and the residues 2-8 at the 5' region of miR have a major role in miR specificity to interact with its target mRNAs. This region is called the seed and it binds to 3' untranslated regions (3'-UTRs) of the target mRNA (Jazbutyte and Thum, 2010). MiRs negatively regulate 100s of genes which depend on the sequence complementarity. They repress target gene expression and protein synthesis by promoting either translational inhibition and/or degradation of their target mRNAs, acting either alone or in combination (Chavali et al., 2013). Whilst a single miR can control the fate of multiple genes, targeting one or more miRs has potential to provide important new therapeutic strategies.

1.7.1. MiR biosynthesis and action

The mechanism of miR biosynthesis is a multistep event catalysed by specific RNA polymerases (Figure 1.8). Inside the nucleus, miR genes are transcribed from the genome by RNA polymerase II (more commonly) or RNA polymerase III into primary miR transcripts (pri-miR). Pri-miRs are then processed by sequential endonucleolytic cleavages induced by two RNase III enzymes, Drosha (nuclear) and Dicer (cytoplasmic) (Kwang Loong and Mishra, 2007). Pri-miR transcripts are transcribed by Drosha into precursor pre-miRs (70 nucleotides long) (Kim, 2005). Pre-miRs are then exported from the nucleus by Exportin 5 to the cytoplasm. In the cytoplasm pre-miR undergoes additional processing by Dicer which removes the hairpin loop and trims the ends, generating an approximate 22-nucleotide double-stranded mature miR-duplex. This mature double-stranded molecule has one guide strand that binds and forms a complex with Argonaute 2 (Ago2) (Lee et al., 2002). The other passenger strand is usually degraded or sometimes becomes active miR and binds to RISC (Haas et al., 2016, Hammond, 2015).

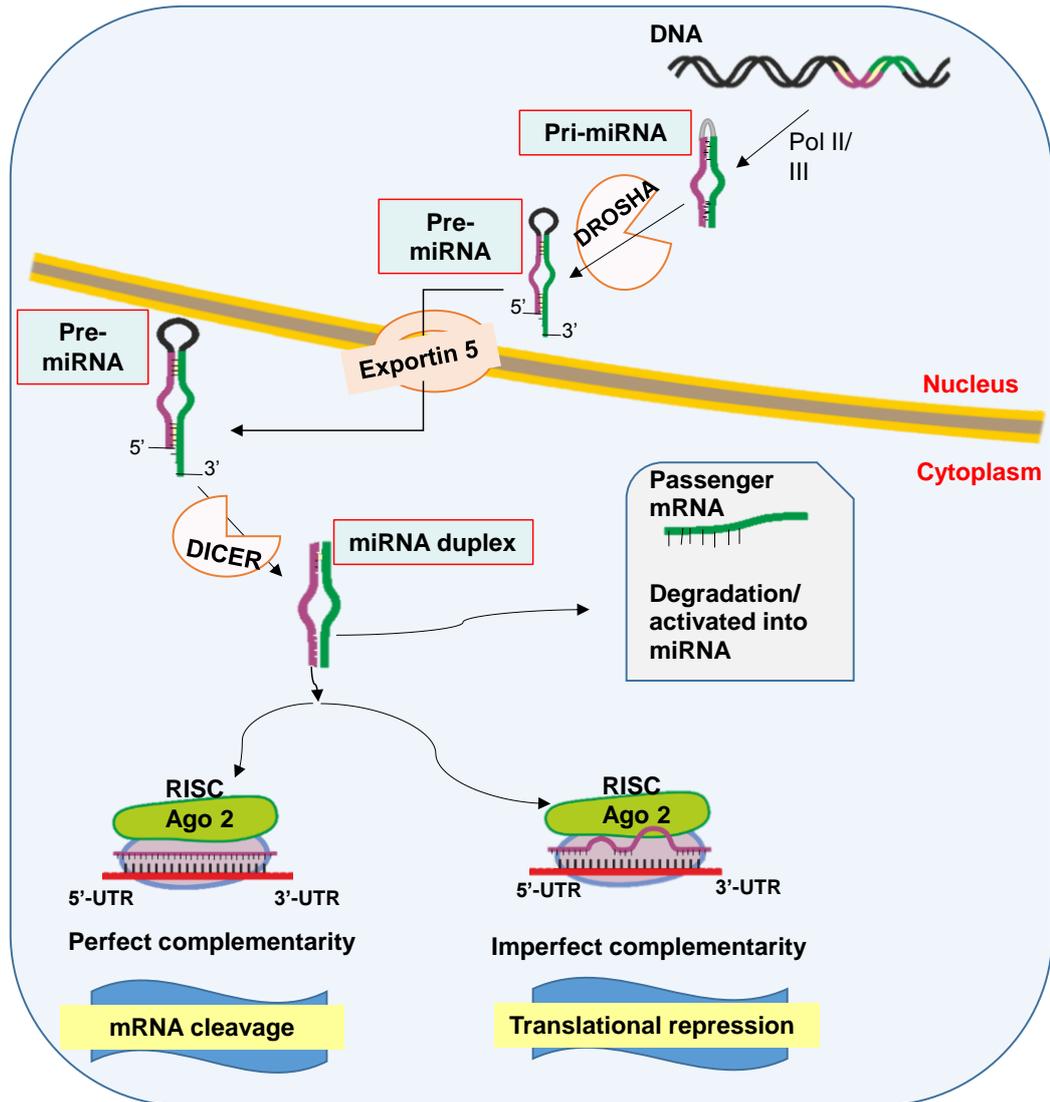


Figure 1.8: Biogenesis pathway of miRNAs.

MiRNA genes are transcribed by RNA polymerase II/ III to form primary-miRNA (pri-miRNA). Pri-miRNA is processed into precursor miRNA (pre-miRNA, 70 nucleotides in length) by Drosha in the nucleus. Pre-miRNA then exposed into the cytoplasm through Exportin 5 to be processed by Dicer into mature miRNA (double stranded 20-22 nucleotides). The mature strand then associates with a complex called RNA Induced Silencing Complex (RISC) and Ago2 protein by either perfect binding to the target mRNA causing mRNA degradation or imperfect binding causing translational repression. The passenger mRNA degraded rapidly or activated into miRNA.

The association between miR and Ago2 is known as the RISC (Bartel, 2009). The guide strand of the miR directs RISC to the complementary sites in the 3'UTR of mRNA by sequence-specific target recognition (Rana, 2007). The perfect binding that occurs between miR and its target mRNA cause the latter to be degraded through cleavage. However, if the complementarity is imperfect, this will cause translational inhibition which stops at the initiation step (Khvorova et al., 2003).

MiRs can be released from cells either by passive leakage from necrotic or apoptotic cells or through active secretion inside microvesicles such as microparticles and exosomes or in RNA/lipid-protein complexes (Ramachandran and Palanisamy, 2012). Being inside the microvesicles or binding of miRs into the Ago2 transporting protein, enable miRs to be stable in the blood and protect them from cleavage by ribonucleases (Busch and Zerneck, 2012). Importantly, miR expression is tissue specific (Baer et al., 2013). This will assign the potential role of particular miR according to cell type or pathophysiological condition. It is documented that one miR can control the expression of multiple target genes and regulate genes according to different environmental conditions (Bartel, 2009). On the other hand, one target gene can be regulated by multiple different miRs. The human genome encodes an estimated 1000 miRs that may directly regulate more than 30% of human protein-encoding genes (Lewis et al., 2005). These miRs are involved in the regulation of major cell functions, suggesting new management strategies for CVDs that may rely on targeting miRs in the vasculature.

1.7.2. MiRs in T2DM development and complications

MiRs are highly conserved and their expression is tissue-specific and affected by the severity of diseases (Chen and Stallings, 2007). Indeed, one miR may be highly expressed in a particular tissue but have low or no expression in other tissues (Lagos-Quintana et al., 2002). Recent studies have implicated miRs in the pathogenesis of diabetes and its related CVD. Several miRs have been shown to play an important part in the normal physiological development of the pancreas, insulin signalling in target tissues, and apoptosis (Guay et al., 2011, Shantikumar et al., 2012). The most commonly known miRs that have a role in diabetes are miR-375, miR-124a, miR-21, miR-34a, miR-146a and miR-133, detailed in Table 1.3.

Current trials are being performed to measure the relevant circulating miRs as biomarkers for T2DM. This can help to predict T2DM in early stages of diabetes-related CVDs. A study performed on 80 diabetic patients showed that circulating levels of miR-126 were reduced in individuals with impaired glucose tolerance compared to a glucose tolerant group, and the levels in patients with diagnosed T2DM were reduced further still (Zampetaki et al., 2010). This suggests that low levels of miR-126 in ND individuals might be a helpful guide to predicting early diabetes.

Table 1.3 Common miRs mediated changes in diabetes

| MiR | Target/species | Action | Reference |
|----------------------------------------------------|---------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|-----------------------|
| MiR-375 overexpression | Pancreatic islets of <i>ob/ob</i> mice | Inhibited insulin secretion in response to high glucose | Poy et al. (2004) |
| MiR-375 overexpression | T2DM patients | Pancreatic islet amyloid and cellular damage | Zhao et al. (2010) |
| MiR-124a overexpression | Forkhead box protein A2 (Foxa2)-a transcriptional factor for glucose metabolism and insulin homeostasis | β -cell development, glucose metabolism and insulin secretion | Baroukh et al. (2007) |
| MiR-21 overexpression | <i>db/db</i> diabetic mouse kidney | Renal fibrosis and inflammation | Zhong et al. (2013) |
| MiR-21 induced by hyperglycaemia | Renal cortices from OVE26 T1DM mice | Reduced PTEN and increased Akt phosphorylation | Dey et al. (2011) |
| MiR-21 downregulated | <i>in vivo</i> and <i>in vitro</i> using <i>db/db</i> mice | Inhibited glomerular hypertrophy and mesangial expansion, proteinuria | Zhang et al. (2009) |
| MiR-34a overexpression | Islets of T2DM <i>db/db</i> mice | Fatty acid induced β -cell dysfunction | Lovis et al. (2008) |
| MiR-21, miR-34a, and miR-146a induced by cytokines | Mouse insulin-secreting cell line (MIN6) | β -cell dysfunction | Roggli et al. (2010) |
| MiR-1 and miR-133 overexpressed | Alloxan-induced DM model in rabbit | Abnormal QT elongation and heart failure | Xiao et al. (2011) |

1.7.3. MiRs in CVD and vascular remodelling

While miRs have been well investigated in the cancer field, more recently, the relationship between miRs and CVDs have been explored (Small et al., 2010). Growing evidence suggests that miRs are highly expressed in CVDs and their aberrant expression profiles play an important role in CVDs, including angiogenesis, atherosclerosis, arterial remodelling, vascular injury and restenosis (Wang and Wen, 2015, Urbich et al., 2008). Because neointimal lesion generation is characterised by increased SMC proliferation and migration that is also similar to the mechanism of cancer cell spreading, thus miRs could reasonably be expected to be involved in the regulation of neointimal lesion formation after revascularisation (Ji et al., 2007). However, relatively few studies have directly revealed the exact role of miRs in vascular remodelling.

Numerous studies have implicated miRs in vascular SMC phenotypic changes, development and regulations. MiR-143/145 were deregulated following mechanical balloon-injured rat carotid artery *in vivo* and in cultured dedifferentiated vascular SMC (Cheng et al., 2009a). MiR-21, miR-210 and miR-34a were reported to be the most significantly upregulated in atherosclerotic plaque obtained from patients after CABG surgery (Raitoharju et al., 2011). MiR-1 has been reported to be elevated in human CAD and its overexpression resulted in rat heart induced arrhythmias (Yang et al., 2007). MiR-29 was reported to be downregulated in infarcted rat heart and this was accompanied by increased collagen expression (van Rooij et al., 2008). MiR-208 was found also to induce cardiomyocyte hypertrophy and fibrosis in mice (van Rooij et al., 2007). Other miRs including miR-143/145 and miR-221 have received great attention (Albinsson and Sessa, 2011), but intensive research is required to clarify their role in proliferative vascular diseases, such as increased rate of proliferation and loss of contractile property. Neointimal progression and vascular diseases may therefore potentially be resolved by normalising miR expression and preventing detrimental phenotypic modulation.

Additionally, studies have shown that miRs can affect ECM molecules in different types of tissues and are thus implicated in pathological diseases. For example, miR-21 overexpression increased collagen 1 deposition in human lung cell lines and mammary gland causing fibrogenesis (Li et al., 2011). MiR-301 was found to increase human breast cancer cell migration and proliferation through targeting collagen type II (Shi et al., 2011). Changes in collagen integrity can affect the differentiation and survival of surrounding cells and therefore, miRs can robustly regulate phenotypic changes of vascular SMC (Rutnam et al., 2013).

Recent clinical studies have demonstrated that some circulating miRs secreted from cells into the plasma can serve as sensitive biomarkers for tissue injuries or CVDs. For example, an increased plasma level of miR-423 was detected in heart failure patients, miR-208 and miR-499 were elevated in myocardial ischemia in rats and human samples; respectively (Corsten et al., 2010, Fukushima et al., 2011).

1.7.4. MiRs and SMC phenotype

The profound ability of vascular SMC to modulate their phenotype in physiological processes and wound healing has further drawbacks, mediating vascular diseases. Uncontrolled phenotypic changes in SMC can evoke accelerated migration, proliferation, and production of ECM products. Changes in the ECM components and their arrangement within the vascular wall contribute also to changes in SMC phenotypes. Using a mouse model of carotid artery ligation, miR-145/143 were found to be significantly downregulated, resulting in phenotypic modulation of SMC into a proliferative phenotype that was accompanied by the reduction of α -SMA marker gene (Cordes et al., 2009). Conversely, overexpression of miR-145/143 in human ND SV-SMC modulated the SMC phenotype by increasing spread cell area and driving the appearance of truncated F-actin fibres similar to those observed in native T2DM-SMC (Riches et al., 2014).

Recent studies have shown that phenotypic switching of SMC can play an important role during atherosclerotic plaque formation and in plaque stability and that a beneficial therapeutic outcome could be achieved by targeting SMC phenotypic switching (Chaabane et al., 2014, Gomez and Owens, 2012). Several studies have shown that miRs are highly expressed in vascular SMC and have a crucial role in proliferation and differentiation of SMC, thus, aberrant miR expression can induce pathological abnormalities (Zhang, 2009, Ji et al., 2007). In rat carotid arteries, miRs have been found to be differentially expressed and to mediate neointimal growth after angioplasty (Cheng et al., 2010). Downregulating miR-21 and miR-125 reduced expression of SMC marker genes; including α -SMA and calponin, suggesting the important role of these miRs in SMC-specific gene expression (Davis et al., 2008). Furthermore, both TGF- β and bone morphogenic protein (BMP) through increasing miR-21 and miR-199 levels, were able to induce contractile human pulmonary SMC phenotypes (Davis et al., 2008).

1.7.5. MiR-21

Unlike some other selectively expressed miRs, of particular interest, miR-21 is generally expressed in most mammalian organ systems including the heart, vasculature, lung, pancreas, spleen, intestine, colon and liver (Lagos-Quintana et al., 2002). MiR-21 is a well-known onco-miR due to its ability to repress the actions of various tumour suppressor genes (Bonci, 2010). In addition to its high expression profile in cancers, miR-21 has been associated with a variety of CVDs and interestingly, following vascular injury, miR-21 is aberrantly expressed in most types of cardiovascular cells, including vascular SMC (Ji et al., 2007), EC (Suarez et al., 2007), cardiomyocytes (Cheng et al., 2007) and cardiac fibroblasts (Roy et al., 2009).

1.7.6. MiR-21 in the cardiovascular system

MiR-21 was found to be upregulated in cardiac fibroblasts promoting cardiac fibrosis by activation of the MAPK/ERK signalling pathway (Thum et al., 2008) and knocking down miR-21 reversed the levels of interstitial fibrosis and cardiac remodelling. This was also regulated by TGF- β inducing miR-21 upregulation which repressed sprouty-1 (SPRY)-1 gene expression (Kumarswamy et al., 2012). By activation of the MAPK/ERK pathway, miR-21 was also able to induce oxidative stress and ROS formation (Sen and Roy, 2012). This resulted in inhibition of SPRY gene in human angiogenic progenitor cells (Fleissner et al., 2010) and thus, miR-21 upregulation appears to play a crucial role in vascular homeostasis and endothelial dysfunction.

Both in rat carotid arteries (Zhang, 2008) and human atherosclerotic plaques (Raitoharju et al., 2011), miR-21 has been found to be abundantly overexpressed in neointimal lesions of the vascular wall. In another *in vivo* study using a rat balloon injured carotid artery, miR-21 was found to be more than 5-fold overexpressed, while miR-145 expression was downregulated, relative to uninjured control (Ji et al., 2007). MiR-21 has been shown to be overexpressed in cardiac hypertrophy mediated by aortic banding while downregulation of miR-21 inhibited cardiomyocyte hypertrophy (Cheng et al., 2007).

1.7.7. MiR-21 in SMC

MiR-21 increased SMC proliferation and inhibited apoptosis after vessel injury through repressing phosphatase and tensin homolog (PTEN) and upregulating B-cell lymphoma 2 (BCL2) (Ji et al., 2007). Thereafter, using human pulmonary artery SMC, miR-21 was also shown to regulate SMC differentiation in response to TGF- β 1 and BMP via repressing another target; programmed cell death 4 (PDCD4) expression (Davis et al., 2008). In keeping with its role in regulating vascular SMC, knocking down miR-21 in balloon-injured rat IMA reduced SMC proliferation and protected the artery from neointimal lesion formation compared with controls (Wang et al., 2015a), indicating the important role of miR-21 in inducing dedifferentiation changes in vascular SMC. Furthermore, miR-21 induced cell proliferation and decreased apoptosis of porcine vascular wall SMC during abdominal aortic aneurysm (Maegdefessel et al., 2012). This was mediated by the suppressing effect of miR-21 on PTEN and subsequent Akt activation.

These various studies highlighted the major mechanisms by which miR-21 is implicated in CVDs. Using cultured SMC *in vitro* and in rat carotid arteries *in vivo*, the anti-apoptotic property and proliferative effect on SMC were the major target of miR-21 that mediated vascular remodelling. Despite these advanced studies, comprehensive knowledge of miR-21's exact role in SV-SMC and downstream targets are not completely known. Manipulation of miRs might be one of the new therapeutic strategies to prolong the patency rate of SV graft surgery and retards diabetic vascular complications. Therefore, miR-21, which has been shown to be involved in SMC differentiation in several studies, might be a possible target for therapeutic intervention in vascular remodelling since its expression can be manipulated using genetic approaches. These studies revealed a promising value of miR-21 as a new therapeutic target for CVDs however, with regard to vascular remodelling, identifying the exact role of miR-21 and networks of target genes mediating vascular remodelling would provide a novel therapeutic target for prevention or reversal of pathological SMC switching in the context of SV graft failure. Additionally, developing a highly efficient delivery method and manipulation of miR-21 at the time of vein harvesting, and before engrafting, could be promising. This would minimise expression of the target genes involved in SMC proliferation and migration and therefore, theoretically protect the patient from the risk of neointima formation in the new graft.

1.7.8. Signalling mechanism of miR-21

MiR-21 is aberrantly expressed in different types of human tissues and diseases, acting through numerous targets and signalling mechanisms that are listed in Table 1.4. Both PTEN and PDCD4 were the two target genes most commonly regulated by miR-21 in the literature (Lin et al., 2009, Ji et al., 2007). MiR-21 was initially found to induce cell migration, proliferation and anti-apoptosis in various carcinomas through targeting tumour suppressor genes including PDCD4 (Lu et al., 2008a), and tropomyosin 1 (Zhu et al., 2008). MiR-21 was also documented to control vascular SMC growth and survival through repressing PTEN protein and BCL2 gene expression (Kumarswamy et al., 2012, Joshi et al., 2012). By posttranscriptional inhibition of PTEN expression, miR-21 induced Akt activation and promoted proliferation and suppression of apoptosis of rat carotid artery SMC (Ji et al., 2007), hepatocellular carcinoma (Meng et al., 2007b), human glomerular mesangial cells (Dey et al., 2012), and breast cancer cells (Haverty et al., 2008).

Additional mechanisms of proliferation and migration were predicted for miR-21 action in human pulmonary artery SMC through targeting TGF- β 1, BMP, Smads and PDCD4 (Davis et al., 2008, Sarkar et al., 2010, Yao et al., 2011). MiR-21 mediated the protective effect of vascular SMC apoptosis and death (Lin et al., 2009) and has been shown to protect cardiac cell apoptosis following ischaemic reperfusion (Cheng et al., 2010) through the transcriptional inhibition of PDCD4. An experimental study in mice demonstrated the use of antimiR-21 to reduce interstitial cardiac fibrosis and enhance cardiac functions by activation of Spry-1, a negative regulator of MAPK/ERK signalling (Thum et al., 2008). Both TIMP3 and Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) are negative regulators of MMPs and they are additional targets of repression by miR-21 (Selaru et al., 2009). MiR-21 increased lung cancer cell line proliferation and invasion through direct inhibition of RECK, PTEN and BCL-2 (Xu et al., 2014). Finally, miR-21 has also been involved in the induction of oxidative stress by stimulating the MAPK/ERK pathway (Fleissner et al., 2010). These targets must be tested in specific cell types because the effect of miRs on gene expression and cellular functions are cell-type specific (Jazbutyte and Thum, 2010). Based on these reports, it is evident that miR-21 mediates phenotypic changes in SMC and may also play a central role in angiogenesis through regulation of various targets. Therefore, further studies need to be conducted to determine miR-21's potential biological function specifically in SV-SMC.

Emerging evidence has suggested that miRs are involved in many physiological and pathological processes in humans. MiRs have a potential causative role in T2DM

and its related cardiovascular complications, as the aberrant miR expression is observed in numerous human diseases (e.g diabetes, CVDs, cancer, and tissue fibrosis). The accumulating information about the role of miR-21 suggests that it plays a crucial role in many biological processes and pathological diseases. However, to the best of our knowledge, studies regarding its role in vascular complications and in particular human SV graft failure are lacking. Therefore, much additional work is needed to identify the precise role of miR-21 in vascular remodelling and to ascertain the underlying mechanisms driving its upregulation.

Table 1.4 Effect of miR-21 overexpression in different cell types and diseases, mediators involved and signalling mechanisms:

| Disease | Cell type | Mediators involved | Target mRNA | Cellular function/ Relevant pathology | Reference | |
|----------------|--------------------------------------------|----------------------------|---------------------------------|----------------------------------------------|------------------------|----------------------------------------------|
| Cancer | Hepatic cell carcinoma | Unknown | PTEN ↓ PDCD4 ↓ RECK | Increased cell migration and invasion | Zhou et al. (2013) | |
| | Myeloma and hepatocellular carcinoma cells | Unknown | ↓ STAT3 | Multiple myeloma | Loeffler et al. (2007) | |
| | Breast tumour cells | Unknown | ↓ SMAD ↓ PDCD4 | Breast cancer | Qian et al. (2009) | |
| | Activated fibroblast | Unknown | ↓ PDCD4 | Stroma, skin cancer | Yao et al. (2011) | |
| | Keratinocytes epithelial cells | BMP | ↓ PTEN ↓ PDCD4 | Skin cancer | Ahmed et al. (2011) | |
| | Glioblastoma | Unknown | ↓ RECK | Cancer invasion, glioma | Gabriely et al (2008) | |
| | Lung squamous cell | Unknown | ↓ RECK | Cancer cell proliferation | Xu et al. (2014) | |
| | Kidney | Glomerular mesangial cells | Unknown | ↓ PTEN | Mesengial hypertrophy | Dey et al. (2011) |
| | | Heart | Cardiac fibroblasts | Unknown | ↓ PTEN | Left ventricular pressure and heart fibrosis |
| | Cardiac fibroblasts | | Unknown | ↓ Spry-1 | Heart failure | Thum et al. (2008) |

| | | | | | |
|-----------------|-----------------------|------------------------|---------------------|---------------------------------------------------------------------------------|---------------------------|
| Lung | Fibrotic lung disease | TGF- β 1 | ↓ Smad7 | Lung fibrosis | Liu et al. (2010) |
| Pancreas | Islets β cells | Unknown | ↓ PDCD4 | Diabetic nephropathy | Bravo-Egana et al. (2012) |
| | β -cells | Nuclear factor-kB | ↓ PDCD4 | T1DM | Ruan et al. (2011) |
| SMC | Pulmonary artery SMC | TGF- β 1 BMP4 | ↓ PDCD4 | Cell migration and tumour metastasis | Davis et al. (2008) |
| | Airway SMC | PremiR-21 transfection | ↓ PTEN | Increased SMC proliferation and migration, asthma | Liu et al. (2015) |
| | Pulmonary artery SMC | Unknown | ↓ PDCD4 ↓ Spry-2 | Pulmonary hypertension | Sarkar et al. (2010) |
| | Pulmonary SMC | Hypoxia | ↓ BMP Receptor 2 | Chronic hypoxia-induced pulmonary vascular remodelling | Yang et al. (2012) |
| | Femoral artery SMC | Unknown | Tropomyosin 1 ↓ | Increased SMC proliferation and migration, atherosclerosis of lower extremities | Wang et al. (2011) |
| | Carotid artery SMC | Angioplasty | ↓ PTEN ↑ BCL2 | Proliferation, apoptosis Neointima formation | Ji et al. (2007) |

1.7.9. Manipulation of miR-21 expression in SV-SMC

Owing to the ability of individual miRs to manipulate multiple target genes, modulating the expression of a single miR can affect an entire gene network involved in a particular disease state. Because of the involvement of miR-21 in different types of CVDs, manipulation of the level of miR-21 in SMC represents a new promise as a novel approach to therapy. To determine its role in vascular remodelling, manipulation of miR-21 expression can be achieved by either overexpression of miR-21 to mimic its action, or knock down to repress its action in SMC (Patel and Nouredine, 2012).

A. Overexpression of miR-21

PremiR-21 is a duplex oligonucleotide that mimics endogenous precursor miRs. One strand has an identical nucleotide sequence to the mature miR-21 (Figure 1.9A). This can bind RISC to form a complex that posttranscriptionally inhibits the target mRNA. The other strand is the guide/passenger strand that facilitates cellular uptake (Martinez et al., 2002).

B. Knocking down of miR-21

In order to inhibit miR expression antimiRs were utilised. They usually have a reverse complementary nucleotide sequence to the mature miR of interest (Figure 1.9B). AntimiR-21 binds to the mature miR-21 and inhibits its action. Therefore, antimiR-21 eventually prevents the repression of the target mRNA (Krutzfeldt et al., 2005).

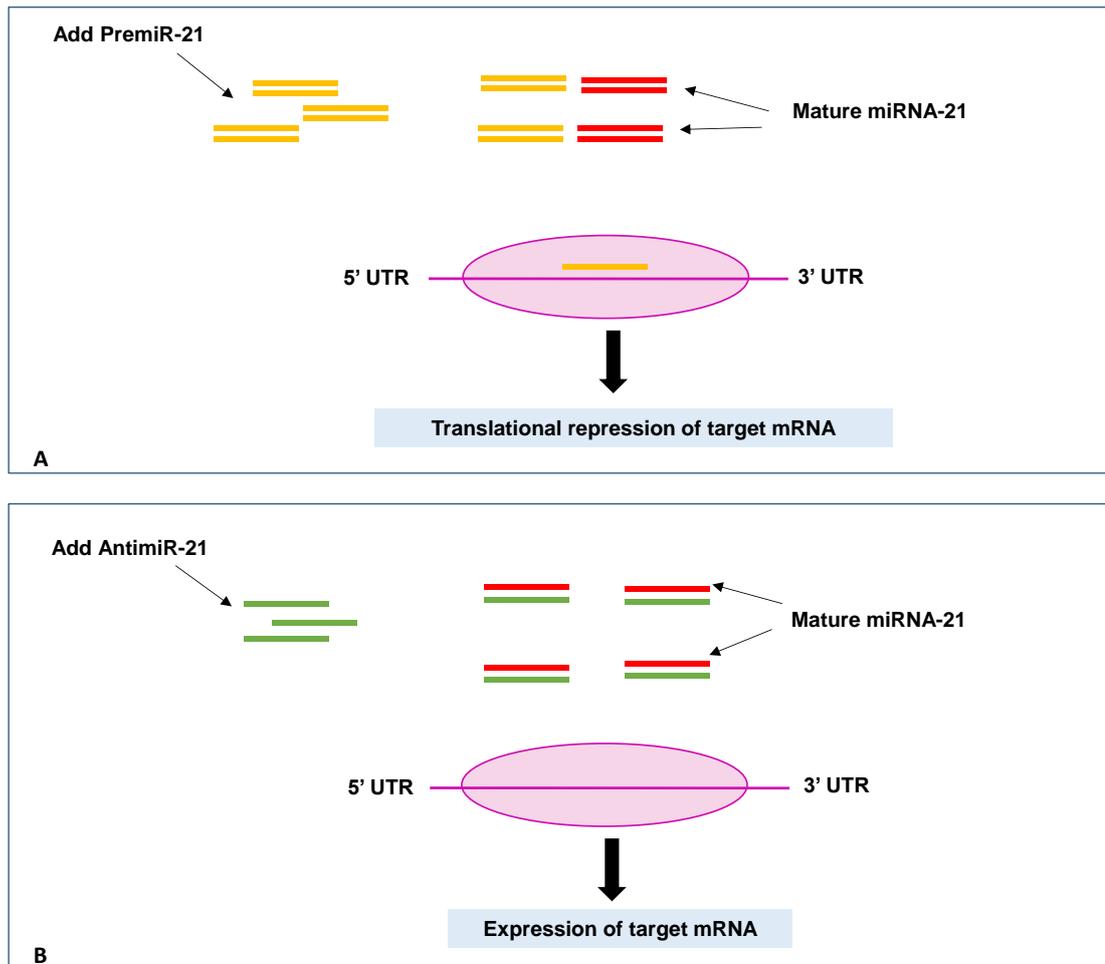


Figure 1.9: Mechanism of modulation of miR-21 expression and function by premiR-21 and antimiR-21.

PremiR-21 is a duplex oligonucleotide with an identical sequence to mature miRNA-21 and posttranscriptionally inhibits the target mRNAs (A). Conversely, antimiR-21 has a complementary sequence to the mature miRNA-21 and relieves the repression on the target mRNA (B). Modified version from (Patel and Nouredine, 2012).

1.8. Study Hypothesis

While a large amount of research is directed to targeting IH and prolonging graft patency, none of the available therapies treat the original factor(s) involved in this process and robust therapeutics are lacking. Recent research highlighted the important role of miRs in epigenetic regulation of the vasculature (Alexander and Owens, 2012) and in particular, vascular remodelling. MiR-21 is one of the most highly expressed miRs in the vasculature and it is aberrantly expressed in vascular remodelling. We hypothesise that miR-21 is differentially expressed in SV-SMC from patients with and without a diagnosis of diabetes. Secondly, aberrant expression of miR-21 plays a key role in human SV-SMC phenotype and function in a manner that is detrimental to saphenous vein (SV) bypass graft failure.

1.9. Aims of the study

- 1- To determine whether miR-21 is differentially expressed in ND and T2DM SV-SMC and to investigate whether manipulation of miR-21 levels in SV-SMC play a role in phenotypic changes and impaired cell function (chapter 3).
- 2- To explore which of the diabetic and inflammatory mediator(s) induce miR-21 upregulation in SV-SMC and to investigate the downstream CVD-related target genes affected by miR-21 upregulation in SV-SMC using atherosclerosis microarray (chapter 4).
- 3- To investigate the effect of miR-21 overexpression on SV-SMC invasion using Boyden chamber assay (chapter 5).
- 4- To investigate the effect of miR-21 overexpression on SV-SMC proliferation and identify the major targets of miR-21 and signalling pathways mediating its function in SV-SMC phenotypic changes (chapter 6).

Chapter 2
MATERIALS AND METHODS

Chapter 2

Materials and methods

2.1. Materials

2.1.1. Cell culture

DMEM, PBS, L-glutamine 200 mM (100X), antibiotic-antimycotic (penicillin, Streptomycin and Fungizone), 0.05% Trypsin-EDTA (1X), and Opti-MEM® (1X) were all purchased from Life Technologies (Paisley, UK). Foetal calf serum (FCS) was from Biosera Ltd (Ringmer, East Sussex, UK). Trypan blue solution (0.4%), cell culture grade BSA, DMSO and glycerol/gelatin were from Sigma-Aldrich (Poole, Dorset, UK). PD98059 was obtained from Calbiochem and LY294002 was from Alexis Biochemicals (Nottingham, UK). Human recombinant TGF- β 1 was from Bio-Techne (Abingdon, UK).

Cell culture chamber inserts and Falcon cell companion plates were purchased from Scientific Laboratory Supplies (Hessle, UK). Human collagen solutions type I Vitrocol (Cat# 5008) and type III (Cat# 5021) were from Advanced BioMatrix (San Diego, California, USA). Haematoxylin was from BDH (Lutterworth, Leicestershire, UK) and Eosin 0.5% was from Merck (Nottingham, UK).

Tissue culture flasks (25 cm² and 75 cm²) and tissue culture plates (6 and 24 well) were from Corning Incorporated (Corning, New York (NY; USA). 96-well plates for protein assays were from Nunc (Rochester, NY). Microcentrifuge and centrifuge Falcon tubes were from STARLAB (Milton Keynes, UK).

2.1.2. Reagents used for miR-21 induction

Insulin and glucose were from Sigma-Aldrich (Poole, Dorset, UK). Recombinant human PDGF-BB, TGF- β , IL-1 α , and TNF- α , were all purchased from Life Technologies (Paisley, UK).

2.1.3. RNA extraction and RT-PCR

Aurum total RNA mini kit was from BioRAD (Hemel Hempstead, UK) and RNase away spray bottle from Fisher Scientific Ltd (Loughborough, Leicestershire, UK).

TaqMan microRNA assays U6 SnRNA (product# 001973), has-miR-21 (product# 000397) TaqMan micro primers, TaqMan® Gene expression Master Mix, TaqMan® Universal Master Mix II, no UNG, TaqMan® microRNA Reverse Transcription Kit and all PCR primers listed in Table 2.1 were purchased from Life Technologies (Paisley, UK). Reverse gene transcription system was obtained from Promega (Southampton, UK). 96 well- PCR plate and 0.2 ml PCR tubes were from STARLAB (Milton Keynes, UK). RT² Profiler human atherosclerosis PCR array (12 plates, Cat# PAHS-038ZA-12) and RT² SYBR Green/ROX PCR master mix (Cat# 330522) were from QIAGEN (Manchester, UK).

Table 2.1 Real time-PCR primers

| TaqMan primers | Cat number | Exon boundary | Amplicon length |
|-----------------------|-------------------|----------------------|------------------------|
| Col 1A1 | Hs00164004_m1 | 1 - 2 | 66 |
| MMP-1 | Hs00233958_m1 | 6 - 7 | 133 |
| MMP-2 | Hs00234422_m1 | 12 - 13 | 83 |
| MMP-3 | Hs00968305_m1 | 6 - 7 | 126 |
| RECK | Hs01019179_m1 | 20 - 21 | 69 |
| IL-1 α | Hs00174092_m1 | 6 - 7 | 69 |
| GAPDH | Hs99999905_m1 | 3 - 3 | 122 |

Table 2.2 Atherosclerosis array layout (96-well)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|----------|--------|-------|-------|-------|-------|--------|--------|---------|-------|--------|----------|
| A | ABCA1 | ACE | APOA1 | APOB | APOE | BAX | BCL2 | BCL2A1 | BCL2L1 | BID | BIRC3 | CCL2 |
| B | CCL5 | CCR1 | CCR2 | CD44 | CDH5 | CFLAR | COL3A1 | CSF1 | CSF2 | CTGF | EGR1 | ELN |
| C | ENG | FABP3 | FAS | FGA | FGF2 | FN1 | HBEGF | ICAM1 | IFNAR2 | IFNG | IL1A | IL1R1 |
| D | IL1R2 | IL2 | IL3 | IL4 | IL5 | ITGA2 | ITGA5 | ITGAX | ITGB2 | KDR | KLF2 | LAMA1 |
| E | LDLR | LIF | LPA | LPL | MMP1 | MMP3 | MSR1 | NFKB1 | NOS3 | NPY | NR1H3 | PDGFA |
| F | PDGFB | PDGFRB | PLIN2 | PPARA | PPARD | PPARG | PTGS1 | RXRA | SELE | SELL | SELPLG | SERPINB2 |
| G | SERPINE1 | SOD1 | SPP1 | TGFB1 | TGFB2 | THBS4 | TNC | TNF | TNFAIP3 | VCAM1 | VEGFA | VWF |
| H | ACTB | B2M | GAPDH | HPRT1 | RPLP0 | HGDC | RTC | RTC | RTC | PPC | PPC | PPC |

ABCA1: ATP-Binding Cassette A1; ACE: Angiotensin I Converting Enzyme; APOA1: Apolipoprotein A-1; APOB: Apolipoprotein B; APOE: Apolipoprotein E; BAX: BCL2-Associated X Protein; BCL2: B-Cell CLL/Lymphoma 2; BCL2A1: B-Cell CLL/Lymphoma 2 -Related Protein A1; BCL2L1: B-Cell CLL/Lymphoma 2 -Like 1; BID: BH3 Interacting Domain Death Agonist; BIRC3: Baculoviral IAP Repeat Containing 3; CCL2: Chemokine (C-C Motif) Ligand 2; CCL5: Chemokine (C-C Motif) Ligand 5; CCR1: Chemokine (C-C Motif) Receptor 1; CCR2: Chemokine (C-C Motif) Receptor 2; CD44: CD44 Molecule (Indian Blood Group); CDH5: Cadherin 5, Type 2 (Vascular Endothelium); CFLAR: CASP8 And FADD-Like Apoptosis Regulator; COL3A1: Collagen, Type III, α 1; CSF1: Colony Stimulating Factor 1 (Macrophage); CSF2: Colony Stimulating Factor 2 (Granulocyte-Macrophage); CTGF: Connective Tissue Growth Factor; EGR1: Early Growth Response 1; ELN: Elastin; ENG: Endoglin; FABP3: Fatty Acid Binding Protein 3, Muscle And Heart; FAS: Fas Cell Surface Death Receptor; FGA: Fibrinogen α Chain; FGF2: Fibroblast GF 2; FN1: Fibronectin 1; HBEGF: Heparin-Binding EGF-Like Growth Factor; ICAM1: Intercellular Adhesion Molecule 1; IFNAR2: Interferon (α , β And Ω) Receptor 2; IFNG: Interferon, γ ; IL1A: Interleukin 1, α ; IL1R1: Interleukin 1 Receptor, Type I; IL1R2: Interleukin 1 Receptor, Type II; IL2: Interleukin 2; IL3: Interleukin 3; IL4: Interleukin 4; IL5: Interleukin 5; ITGA2: Integrin, α 2; ITGA5: Integrin, α 5; ITGAX: Integrin, α X; ITGB2: Integrin, β 2; KDR: Kinase Insert Domain Receptor; KLF2: Kruppel-Like Factor 2; LAMA1: Laminin, α 1; LDLR: Low Density Lipoprotein Receptor; LIF: Leukemia Inhibitory Factor; LPA: Lipoprotein, Lp(A); LPL: Lipoprotein Lipase; MMP1: Matrix Metalloproteinase 1; MMP3: Matrix Metalloproteinase 3; MSR1: Macrophage Scavenger Receptor 1; NFKB1: Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1; NOS3: Nitric Oxide Synthase 3 (Endothelial Cell); NPY: Neuropeptide Y; NR1H3: Nuclear Receptor Subfamily 1, Group H, Member 3; PDGFA: Platelet-Derived Growth Factor α Polypeptide; PDGFB: PDFG β Polypeptide; PDGFRB: Platelet-Derived Growth Factor Receptor, β Polypeptide; PLIN2: Perilipin 2; PPARA: Peroxisome Proliferator-Activated Receptor α ; PPARD: Peroxisome Proliferator-Activated Receptor δ ; PPARG: Peroxisome Proliferator-Activated Receptor γ ; PTGS1: Prostaglandin-Endoperoxide Synthase 1; RXRA: Retinoid X Receptor, α ; SELE: Selectin E; SELL: Selectin L; SELPLG: Selectin P Ligand; SERPINB2: Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 2; SERPINE1: Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 1; SOD1: Superoxide Dismutase 1; SPP1: Secreted Phosphoprotein 1; TGFB1: Transforming Growth Factor, β 1; TGFB2: Transforming Growth Factor, β 2; THBS4: Thrombospondin 4; TNC: Tenascin C; TNF: Tumor Necrosis Factor; TNFAIP3: TNF α - Induced Protein 3; VCAM1: Vascular Cell Adhesion Molecule 1; VEGFA: Vascular Endothelial Growth Factor A; VWF: Von Willebrand Factor; ACTB: Actin, β ; B2M: β -2-Microglobulin; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HPRT1: Hypoxanthine Phosphoribosyltransferase 1; RPLP0: Ribosomal Protein, Large, P0; HGDC: Human genomic DNA contamination; RTC: Reverse transcription control; PPC: Positive PCR control.

2.1.4. Western blotting reagents

Pierce BCA protein assay was from Life Technologies (Paisley, UK), copper (II) sulphate solution, PMSF, sodium vanadate, SDS, TEMED, and APS were purchased from Sigma-Aldrich (Poole, Dorset, UK). ProtoFLOWgel Acrylamide was from Flowgen Bioscience (Nottingham, UK) and molecular weight marker precision plus protein from Bio-RAD Laboratories (Hemel Hempstead, UK). PVDF transfer Immobilon-P membrane from Merck Millipore (Nottingham, UK).

Primary monoclonal antibodies were all purchased from Cell Signaling Technology New England Biolabs (Hitchin, Hertfordshire, UK), except β -actin that was from Abcam (Cambridge, UK). ECL Horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit and anti-mouse IgG, and Amersham ECL Prime Western blotting detection reagent were all from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Developer and fixer solutions were from Kodak GBX (Rochester, NY, USA).

2.1.5. Modulation of miR-21 levels in SV-SMC

Lipofectamine® 2000 reagent was purchased from Invitrogen (Paisley, UK). Anti-miR miRNA inhibitor (has-miR-21-5p; product# AM17000), anti-miR negative control (product# AM17010), pre-miR miRNA precursor (has-miR-21-5p; product# AM17100) and premiR negative control (product# AM17110) were all obtained from Life Technologies (Paisley, UK).

2.1.6. Staining reagents

Glass coverslips (10 mm diameter) were obtained from Scientific Laboratory Supplies (Hessle, UK). Paraformaldehyde, Triton X-100, normal goat serum and mouse monoclonal anti- α -SMA clone 1A4 were from Sigma-Aldrich (Poole, Dorset UK). SM-MHC antibody was from Cambridge (UK) and Vectashield® mounting media with DAPI counterstain was from Vector Laboratories (California, USA). The fluorescent cyanine dye (Cy3), and tagged FITC secondary IgG antibodies were obtained from Jackson ImmunoResearch (Newmarket, Suffolk, UK). Rhodamine phalloidin was from Life Technologies (Paisley, UK).

2.2. Methods

2.2.1. Cell culture

Human SV specimens were obtained from T2DM and ND patients undergoing elective CABG surgery at the General Infirmary of Leeds. Patient consent was obtained before each surgery and local ethical committee guidelines were followed (LREC reference is CA01/040). SV was chopped into small fragments and cultured in 2 ml of 10% FCS in DMEM, containing normal concentration of D-glucose (5.5 mM), 1% antibiotic-antimicotic and glutamine (full growth media; FGM) in 25 cm² tissue culture flask with a constant supply of 5% CO₂ at 37 °C in a humidified incubator. Cells were then explanted and allowed to grow to confluence in FGM using an explant technique (Porter et al., 2002) as shown in Figure 2.1. Half media was changed twice a week and when confluence was reached, the cells were subcultured to passage number 1 into a 75 cm² tissue culture flask and after confluence, cells divided for the second passage of 3 75 cm² flasks. All following experiments were performed using cells at passages 3 to 5 and all repeat experiments were performed on cells derived from different individuals.

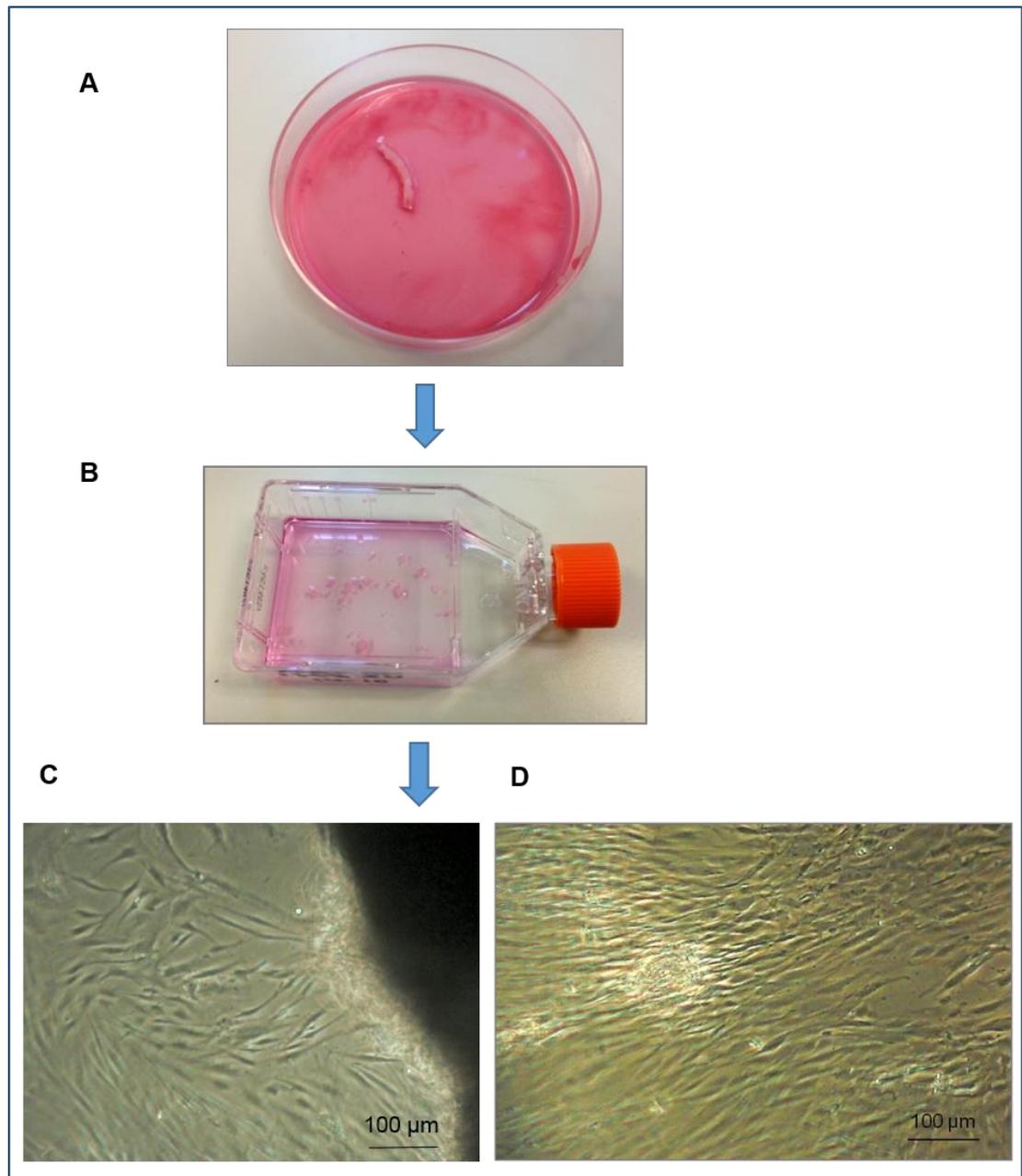


Figure 2.1: Diagram shows SMC explants from intact SV segment.

SV segment was obtained from a patient undergoing CABG surgery (A), chopped SV was cultured in 2 ml of FGM in 25 cm² tissue culture flask (B). Representative phase contrast microscopic image of SMC explanting from small fragments (C) until they reach confluence (D), original magnification (x100).

2.2.2. Modulation of miR-21 levels in SV-SMC

Under sterile conditions, SV-SMC of ND and T2DM patients were plated in six-well plates at a density of 70,000 cells in 2 ml of FGM and grown for 24 h before transfection.

For miR-21 overexpression, ND SMC were transfected with premiR-21 at a final concentration of 30 nM according to the protocol listed in Table 2.3. For miR-21 knockdown, cells were transfected with anti-miR-21 at a final concentration of 100 nM as described in Table 2.4. In each case, reagents in tube 1 were mixed and allowed to incubate for 5 min, this was then added to the mixture in tube 2 and incubated for 20 min at room temperature (RT). Pre-warmed (37 °C) 0.4% FCS media; which is the minimum growth media (MGM) was then added (2 ml) to transfection reagents in tube 2, this will make 4 ml total volume of each treatment and subsequently 2 ml of the mixture was loaded into the cells in each well after being washed once in PBS. The plate was then incubated for 6 h, old media was replaced by fresh MGM, and cells harvested after 72 h or at a particular time point.

Table 2.3 PremiR-21 transfection reagents

| Reagent | Tube 1 | | | Tube 2 | |
|----------------|-----------------|-----------------|---------|---------------|---------|
| | 30 nM PremiR-21 | 30 nM PremiR-ve | Optimem | Lipofectamine | Optimem |
| Un-transfected | - | - | 1 ml | - | 1 ml |
| Mock | - | - | 1 ml | 10 µl | 990 µl |
| PremiR-ve | - | 6 µl | 994 µl | 10 µl | 990 µl |
| PremiR-21 | 6 µl | - | 994 µl | 10 µl | 990 µl |

Table 2.4 AntimiR-21 transfection reagents

| | Tube 1 | | | Tube 2 | |
|------------|----------------------|---------------------------|-------------|---------------|-------------|
| Reagent | 100 nM AntimiR-21 | 100 nM AntimiR- -ve | Optimem | Lipofectamine | Optimem |
| AntimiR-ve | - | 20 μ l | 980 μ l | 10 μ l | 990 μ l |
| AntimiR-21 | 20 μ l | - | 980 μ l | 10 μ l | 990 μ l |

2.2.3. Characterisation of SMC

A. α -SMA and SM-MHC immunostaining

SMC were seeded on 10 mm glass coverslips in a 24 well plate and serum-starved for 3 days. Coverslips were then washed with PBS and cells were fixed with 200 μ l of 4% paraformaldehyde in PBS for 20 min on the rocking plate. After washing with PBS (x3), cells were permeabilised with 0.1% Triton X-100 in PBS for 30 min followed by washing twice with 5% BSA-PBS-Tween. Nonspecific binding was subsequently blocked by incubating the coverslips in 5% BSA-PBS-Tween-20 for 15 min at RT, then coverslips were washed and incubated overnight with primary antibody (1:200 for α -SMA and 1:100 for SM-MHC) at 4 $^{\circ}$ C in a humidified chamber.

After 24 h, coverslips were washed and incubated with the secondary fluorescent tagged FITC IgG with a dilution of 1: 200 (anti-mouse) and 1:400 (anti-rabbit) antibodies in PBS-Tween for 4 h at RT in the dark. After this time, the slips were washed with PBS-Tween (x3), PBS (x2) and distilled water (x1) and finally mounted using ProLong[®] Gold Antifade Mountant with the nuclear stain; DAPI and cells were visualised using LSM510 Upright Confocal microscope with x200 magnification power. Cy3 excitation wavelength of 550 nm and 570 nm emission and FITC excitation wavelength of 495 and emission at 519 nm.

B. F-actin staining with Rhodamine phalloidin

Cells were grown on glass coverslips, transfected with premiR-21 and antimiR-21 (as described in section 2.2.2) and incubated with MGM for 72 h. They were washed and fixed as previously mentioned (section 2.2.3A). The cells were then permeabilised and blocked for nonspecific binding for 5 min using 10% goat serum in 0.05% Triton

X-100 in PBS. Cells were incubated for 20 min in the dark with rhodamine phalloidin (1:40) in PBS that was utilised to label F-actin fibres. Coverslips were washed and mounted and F-actin fibres were visualised using a LSM510 Upright Confocal microscope.

2.2.4. Cell proliferation

SV-SMC proliferation was determined by *in vitro* cell counting using a haemocytometer. Cells were seeded at a density of 1×10^4 cells/well in 1 ml of FGM in 24-well tissue culture plate in quadruplicate wells and incubated for 24 h to allow cell attachment. The next day, cells were incubated in serum-free media (SFM) for 72 h. For cell counting, media was removed, cells were washed with 1 ml PBS, gently detached by 200 μ l trypsin then pelleted in 800 μ l FGM by centrifugation at $600 \times g$ for 6 min at 4 °C. 50 μ l of the precipitate was resuspended with 50 μ l of trypan blue dye and cells were counted using the haemocytometer under the microscope (x200 power). This was designated as day 0, and SFM in remaining cells was replaced by 1 ml of FGM containing appropriate stimuli to allow cell growth. Afterwards, the number of cells was counted for the days specified to construct the proliferation curve.

2.2.5. Cell morphology

Light microscopic images were captured for subconfluent cells (~60%) in a T75 culture flask under the light microscope at x100 magnification that was connected to the camera. For each patient, cell area was measured by tracing the areas of 50 cells using imageJ program (1.46r, USA; <http://rsb.info.nih.gov/ij/>). Fluorescence and immunostaining images were visualised using a LSM510 Upright Confocal microscope.

2.2.6. Boyden chamber assay

Migration and invasion of SV-SMC were assayed using a modified Boyden chamber technique (Figure 2.2) in a 24 well-specialised plate, in which the upper and lower compartments were separated by 6.4 mm diameter filter membrane with 8 μm pores.

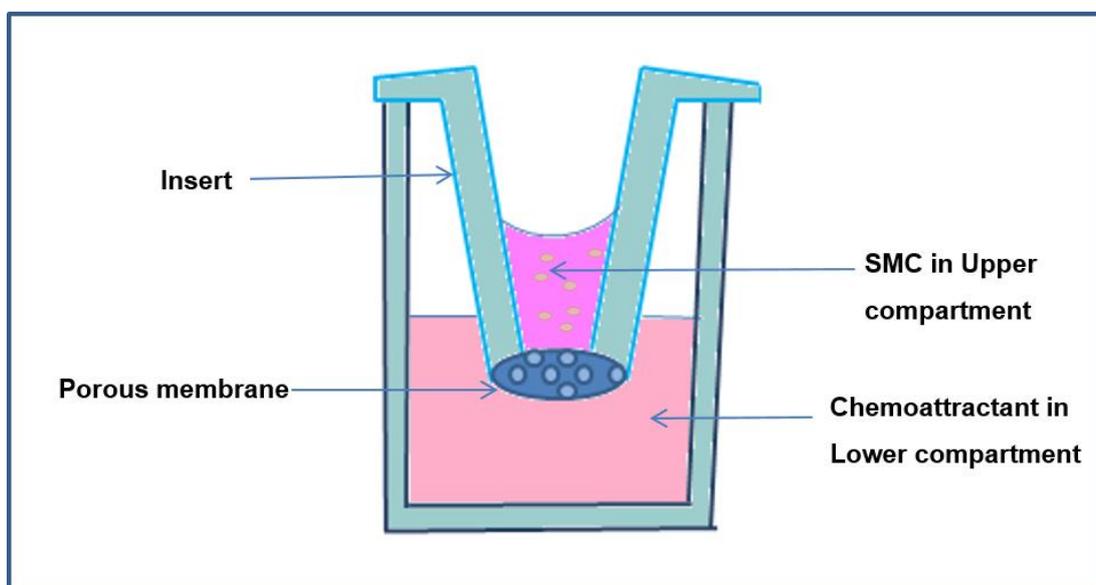


Figure 2.2: A modified Boyden chamber Diagram.

SMC were placed in the upper compartment of the insert and allowed to migrate through the porous membrane toward the media containing chemoattractant in the lower compartment.

A. Migration assay

ND SV-SMC were serum starved for 48 h prior to being trypsinised and resuspended in FGM. The resultant cell suspension was then centrifuged at 600 x g for 6 min at 4 °C and the pellet was re-centrifuged in SFM and finally suspended in MGM.

Chemoattractant PDGF-BB (10 ng/ml) was added to 0.75 ml of MGM in the lower chamber of the 24 well plate to stimulate cell migration towards the lower compartment. Cells migrated towards MGM alone as a control group was also carried out. 1×10^5 cells in each insert in 0.5 ml of cell suspension of MGM were gently added to the top chamber and migration was allowed to proceed for 6 h inside the incubator.

Cells were fixed in pre-cooled (-20 °C) 70% Ethanol for 30 min, then chambers were rinsed with water 3 times and non-migrating cells in the upper compartment were scraped off by cotton swabs and washed out.

Migrated cells on the underside of the chamber were stained with haematoxylin-purple to stain the nuclei then eosin-orange colour solutions to stain the proteins and cytoplasm of the cell, each for 30 seconds (s) and inserts were dipped in water between the 2 stains. The stained membrane was then briefly dried, peeled off the insert and mounted on a labelled glass microscope slide, using warm glycerol gelatin drops and pressed by coverslips. The number of migrated cells in 10 random fields was finally determined by counting the number of stained nuclei. Data were expressed as the average number of migrated cells (10 high-power fields/well) per view (at x200 magnification power) on duplicate membranes for each condition.

B. Invasion assay

The invasion assay was essentially the same as the migration assay, except it used inserts in which the 8 μm pores were pre-occluded with collagen I or III and was performed by incubation for 24 h to allow cells to digest the collagen before migrating towards the chemoattractant. Conditioned media (CM) in the upper compartment were collected to measure MMP-1 secreted from invaded cells, centrifuged to pellet out the debris, snap frozen and stored at $-80\text{ }^{\circ}\text{C}$. Invaded cells were counted as described in the previous paragraph.

2.2.7. Measurement of MMP-1 protein using ELISA

A. Human Pro-MMP-1

The MMP-1 Human ELISA (Enzyme-Linked Immunosorbent Assay) is an *in vitro* assay for the quantitative measurement of human pro-MMP1 form in the CM collected from invading cells through Boyden chambers.

The assay was conducted according to the manufacturer's protocol. Briefly, standards (concentration from 0.156 - 10 ng/ml) were prepared from calibrator diluent RD5-19 and reconstituted Pro-MMP-1 standard. 100 μl of the assay diluent RD1-52 was added into the appropriate wells of the ELISA plate precoated with a monoclonal antibody specific for human pro-MMP-1. 100 μl of the standard and sample aliquots of CM were loaded into the specific wells in duplicate, the plate was sealed and incubated for 2 h at RT on a horizontal shaker.

The wells were washed four times using diluted washing buffer (25x diluted), by filling each well using a squirt bottle with complete removal of all washing buffer by inverting the plate over a piece of tissue and striking on the lab bench. 200 μl of the Pro-MMP-1 conjugate was added to the wells, then the plate was incubated for 2

hours (h) at RT. Wells were washed again and 200 µl of substrate solution was added and the plate incubated for 20 minutes (min) in the dark.

Finally, 50 µl of stop solution was added to the substrate, making the colour change from blue to yellow. Finally, the optical density was determined within 30 min using a microplate reader at 450 nm wavelength. Then the standard curve was constructed and average readings for standard and samples were measured. If any sample has been diluted, the concentration reading was multiplied by the dilution factor.

B. Human active MMP-1 ELISA

This was a fluorimetric assay, in which the fluorescence intensity represented the MMP-1 activity. In brief, the MMP-1 standard was reconstituted with calibrator diluent RD5-25 and standard serial dilution was processed from highest concentration 25 ng/ml to lowest 0.39 ng/ml. 100 µl of assay diluent was added to each well of the monoclonal antibody pre-coated MMP-1 black microplate, followed by 150 µl of the standard or samples in duplicate. The plate was covered and incubated for 3 h at RT on a horizontal shaker. After washing away any unbound substances as mentioned in section 2.2.7A, an activation reagent p-Amino Phenyl Mercuric Acetate (APMA) was added to the standard wells and 200 µl reagent diluents 2 was added to samples.

The plate was then sealed and incubated for 2 h at 37 °C in a humidified incubator. Wells were washed and 200 µl of fluorogenic substrate linked to a quencher molecule was added to all wells in a low light environment and the plate was incubated for 18 h in a dark humidified incubator. Any available active enzyme would split the peptide bond between the fluorophore and the quencher molecule. This cleavage produced a fluorescent signal that was measured at a wavelength of 320 nm and an emission wavelength 405 nm. The relative fluorescence units were assessed by using Fluostar Omega reader BMG Labtech (Offenburg, Germany).

2.2.8. Reverse transcription real time –polymerase chain reaction (RT-PCR)

To analyse mRNA expression, three main steps were performed. Firstly RNA was extracted from pelleted cells (A), secondly the complementary DNA (cDNA) was synthesised from RNA (B), and finally amplification of cDNA by real time- PCR was performed using specific TaqMan primers/ probes (C). All steps were carried out in a DNA-free environment to avoid amplification of contaminating DNA.

A. Pellet preparation and RNA extraction

For cells in 6 and 12 well plates: wells were washed with 2 ml PBS then cells were trypsinised with 0.5 ml trypsin and cells collected in 6 ml (3 ml each time) of cold (4 °C) PBS in a 15 ml falcon tube. Cells were centrifuged at 600 g for 6 min, the supernatant was poured off and tubes were drained upside down before storage at -80 °C.

Total RNA was extracted using the BioRad Aurum Total RNA mini kit. According to the manufacturer's instructions, the pellet was treated with 350 µl lysis solution and 350 µl RNase-free 70% ethanol and the mixture was transferred to the corresponding labelled total RNA mini-column within a collection tube, then centrifuged at 13,000 g for 30 s. The flow-through was discarded, then the column was washed with 700 µl low stringency wash solution and centrifuged for 30 s. To remove genomic DNA contamination, 80 µl of diluted DNase (5 µl of DNase 1 : 75 µl DNA dilution solution) was added to each column and incubated at RT for 15 min. Afterwards, the column was centrifuged at 13,000 g for 30 s. The column was washed with 700 µl high stringency solution followed by 700 µl low stringency wash solution and centrifuged twice to dry the column. Finally, 80 µl of preheated (70 °C) elution solution was added to the column, incubated for 1 min and microfuged into a clean microcentrifuge tube at 13,000 g for 2 min. RNA samples were stored at -80 °C until required.

B. Real time PCR for gene expression

Step 1: Generating cDNA and reverse transcription

cDNA was prepared from RNA according to the Promega Reverse Transcription System manufacturer's protocol. The extracted RNA was thawed and 5 µl in 0.2 ml PCR tube was preheated using the pre-RT program on BIO-RAD T100 Thermal cycler, in which the RNA was heated up to 70 °C for 10 min then cooled back to 4 °C to remove any secondary structure produced by the RNA extraction process. For each RNA sample, reverse transcription was carried out by preparation of master mix from the following reagents listed in Table 2.5.

Table 2.5 Master mix reagents used for RNA reverse transcription

| Reagents | Volume (μl) | Function |
|-------------------------|---------------------------------------|--------------------------------------------------------------------------------|
| 25 mM MgCl ₂ | 4 | Helps primer annealing and acts as a cofactor for reverse transcriptase |
| RT 10X buffer | 2 | Regulates pH of the reaction at 8.5 |
| 10 mM dNTP | 2 | Building blocks for new cDNA strand |
| RNAsin | 0.5 | Inhibits ribonuclease, to minimise RNA degradation in the reaction |
| AMV RT | 0.6 | Reverse transcriptase enzyme, uses RNA template to synthesise new cDNA strand |
| Random primers | 1 | For RT enzyme to anneal to the RNA and provides 3'OH group as a starting point |
| H ₂ O | 7.9 | Makes the total volume up to 18 μ l |

To generate cDNA in a 20 μ l reaction, 2 μ l from each heated RNA sample was mixed with 18 μ l of the master mix in an individual 0.2 ml PCR tube and loaded in the thermal cycler on the reverse transcription program. In which total 33 min, divided between 10 min at 25 $^{\circ}$ C to permit the random primers to anneal to the RNA, 15 min at 42 $^{\circ}$ C to reverse transcribe the second strand, 5 min at 95 $^{\circ}$ C to terminate the reaction and denature the reverse transcriptase enzyme and finally hold at 4 $^{\circ}$ C.

Step 2: Gene expression analysis

Applied Biosystems TaqMan[®] Gene expression Assays, were used to amplify cDNA. TaqMan probes consist of a specific TaqMan[®] probe with a fluorogenic FAM reporter dye label on the 5' end and a nonfluorescent quencher at the 3' end and primers specific to the gene of interest. In a small tube, 10 μ l of TaqMan Universal MasterMix II containing UNG (to prevent DNA contamination) was mixed with 0.5 μ l specific TaqMan primer/probe (primers used are listed in table 2.1) and 7.5 μ l nuclease free water. 18 μ l of this mixture was loaded into each well in a 96-well plate plus 2 μ l of each cDNA was used to measure target and control genes. To ensure accurate measurements, each sample was measured in triplicate.

The plate was sealed and centrifuged for 10 s to pull down all ingredients, then RT-PCR was performed using 7500 Real-Time PCR System under the following conditions: heating the samples up to 50 $^{\circ}$ C for 2 min (for optimal AmpErase[®] UNG activity), then 95 $^{\circ}$ C for 10 min for activation of DNA polymerase and then 50 cycles of reaction at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for one min to enable primer/ probe annealing and DNA synthesis. When the complementary second strand was synthesised, the DNA polymerase cleaved the probe 5' to 3', liberating the fluorescent FAM from the 3' quencher. Results were analysed using 7500 System SDS software version 1.2.3.

Fluorescent signals were normalised to an internal reference and the threshold cycle number (C_t) calculated, which is the number of amplification cycles of the PCR reaction at which fluorescence reached a defined threshold in the linear region of the curve (see Figure 2.3). The difference between C_t values for the target gene and endogenous control (GAPDH) gene is referred as ΔC_t (i.e. $\Delta C_t = C_t$ (target) – C_t (control)). The difference between ΔC_t values for the target and endogenous control reflect the relative amount of target gene in comparison to the control and it is calculated as the following:

$$\Delta\Delta C_t = \Delta C_t \text{ (target gene)} - \Delta C_t \text{ (endogenous control)}$$

Relative quantification (RQ) represents relative quantification based on the relative expression of a target gene versus a reference gene and it was calculated by the formula $(RQ) = 2^{-\Delta\Delta C_t}$.

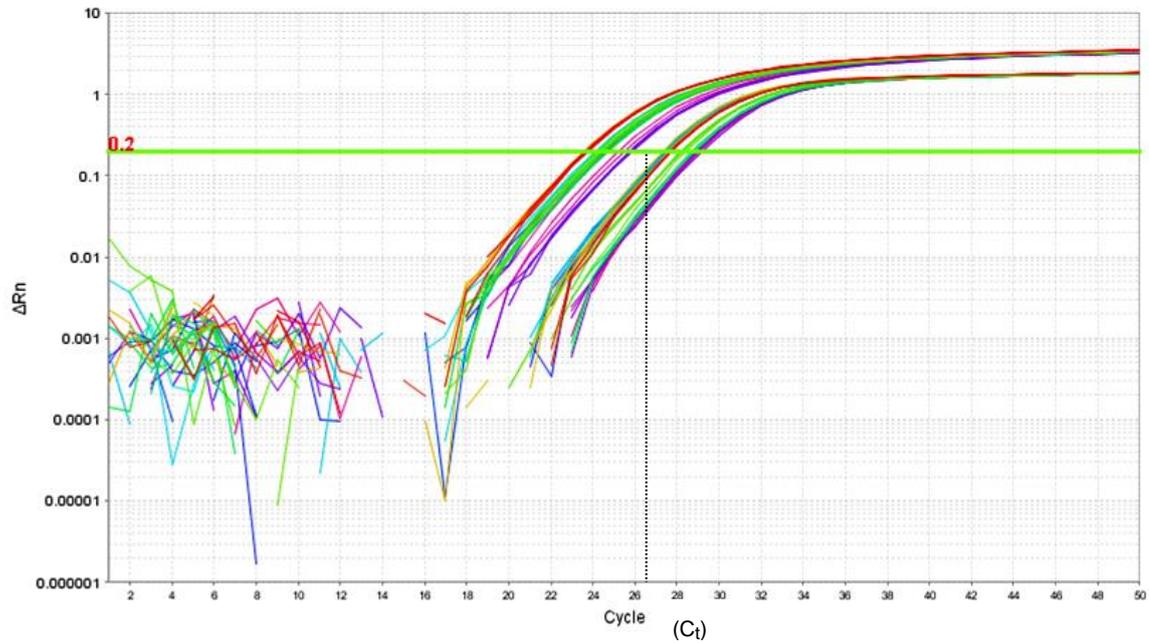


Figure 2.3: Amplification plots for RT-PCR.

Amplification plots showing the fluorescent signal from each sample plotted against cycle number (C_t). The threshold line was fixed at 0.2 in the exponential amplification region for both control and sample C_t .

2.2.9. Specific RT-PCR for microRNA-21 expression

Step 1: Reverse transcription for miR-21 expression

To detect miR-21 expression levels in SV-SMC relative to U6 (internal control), RNA was first reverse transcribed with TaqMan® MicroRNA Reverse Transcription Kit to produce cDNA. Master mix was prepared (each reaction) by mixing 0.08 µl dNTP (100 mM) (source of dNTPs to form the new cDNA strand), 0.54 µl multiscribe™ reverse transcriptase enzyme (50 U/ µl) (to synthesize the new strand) 0.8 µl 10 x RT buffer, 0.103 µl RNase inhibitor (20 U/ µl) (to prevent any RNase in the reaction) plus 3.815 µl nuclease free water. 5.34 µl of the prepared master mix was loaded into labelled 0.2 ml PCR tubes, 1.05 µl of each RNA sample was added into the PCR tube and 1.6 µl of TaqMan specific primer (miR-21 and U6; x 5).

Contents were mixed and tubes were then centrifuged for 10 s, kept on ice for 2 min and finally placed in a BIO-RAD T100 Thermal cycler and exposed to microRNAs reverse transcription program, which heats the RNA up to 16 °C for 30 min to help random primers to anneal to RNA, then to 42 °C for 30 min to allow reverse transcriptase enzyme to catalyse synthesis of the second strand and 85 °C for 5 min to stop the reaction and denature the reverse transcriptase enzyme. Finally, tubes were held at 4° C to be used for the next step.

Step 2: Measuring miR-21 expression

This was carried out by mixing (for 1 reaction) 10 µl of TaqMan® Universal Master Mix II (no UNG) plus 1 µl of the fluorescent TaqMan specific primer (miR-21 or U6; x 20) and 7 µl nuclease free water. Then 18 µl of these two mixtures was added to the specified well in the 96 well PCR plate and 2 µl of the cDNA was added subsequently then real-time PCR was performed as previously described in step 2 section 2.2.8.

$\% \text{ Relative to U6} = 2^{-\Delta\Delta C_t} \times 100$ was used to calculate expression level of miR-21 normalised against U6 and an average of 5 experiments with a standard error of the mean is commonly presented.

2.2.10. Atherosclerosis microarray analysis

The purpose of the array was to detect the potential cardiovascular genes that can be regulated by using RNA samples from premiR-21 transfected cells. An individual full RT² Profiler human atherosclerosis PCR microarray (96 well plate) was utilised for each RNA sample of the premiR-negative and premiR-21 transfected cells of 4 different patients. 2.5 µl of the preheated RNA sample was added to the master mix that was prepared as listed in Table 2.5. cDNA was then diluted 6 times with distilled H₂O into 121 µl diluted cDNA and 2 µl of it was used for GAPDH RT-PCR analysis test to check the quality of cDNA before being loaded onto the plate. RT² SYBR Green/ROX PCR master mix (1350 µl) was added to 102 µl of the diluted cDNA and 1248 µl of dH₂O (experimental cocktail). 25 µl of this cocktail was loaded to wells of the microarray plate in which each well contains different primers of genes involved in cardiovascular related diseases. 5 wells for housekeeping genes were also included in each plate and others that act as positive and negative controls and one for DNA contamination. The plate was then covered by the adhesive film, briefly centrifuged and placed in the 7500 RT-PCR System (See figure 2.4).

The SYBR Green PCR array protocol was followed which involved first 2 stages set at 95 °C at 10 min (for DNA denaturation) and at 15 s (for primers annealing and DNA extension) for 50 cycles then the third stage at 60 °C for 1 min for detection. For each patient, the difference between ΔC_t values for the target and the average of 5 endogenous controls reflect the relative amount of target gene in comparison to the control: $\Delta\Delta C_t = \Delta C_t (\text{Target gene}) - \Delta C_t (\text{average of housekeeping genes})$.

Ct values were exported to an excel file to create a table of Ct value for each gene in each pair. RQ represents relative quantification based on the relative expression of a target gene versus a reference gene and it was calculated manually by the formula $(RQ) = 2^{-\Delta\Delta C_t}$.

The cut-off value was measured for each plate by adding 12 (arbitrary number to take into account the undetectable Ct values above 35 cycles) to the average geometric Ct value of their housekeeping genes and this value was then replaced any test Ct value above the cut-off. Finally, fold change between $2^{-\Delta\Delta C_t}$ of the test (premiR-21) over control (premiR-negative) was calculated manually for each pair and genes that showed sizeable and consistent fold change in premiR-21 transfected cells were then verified by RT-PCR using TaqMan specific primers.

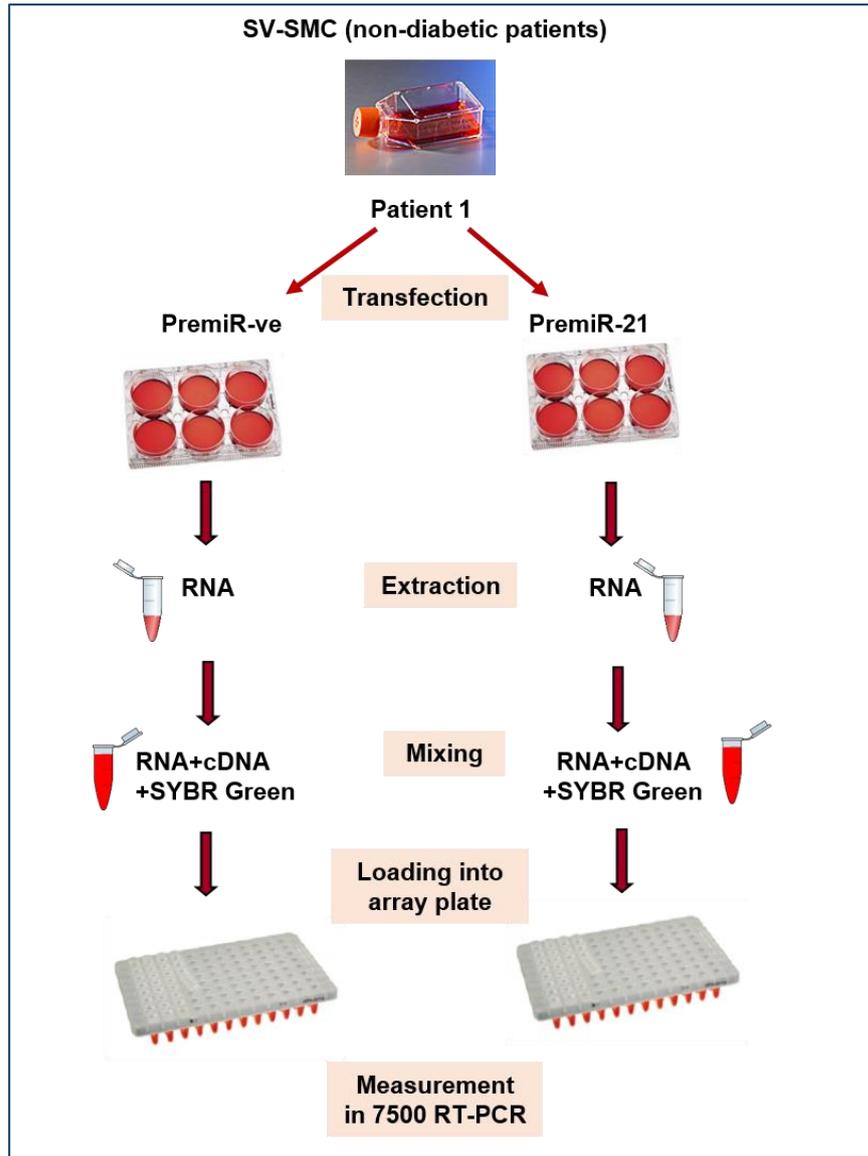


Figure 2.4: Protocol showing the PCR array procedure.

SV-SMC of ND patients were transfected with 30 nM of premiR-21 or premiR-negative for 6 h followed by 72 h incubation in MGM. Cells were then pelleted, RNA extracted and mixed with the master mix to form cDNA. This was then diluted with water and mixed with RT² SYBR Green/ROX PCR master mix. 25 μ l of this cocktail was loaded to wells of the microarray plate which was placed in the 7500 RT-PCR System to measure amplification of genes.

2.2.11. Western immunoblot analysis

Proteins isolated from SV-SMC were determined by western blot analysis, in which 70,000 cells/ well were cultured in a 6-well plate and harvested at a particular time point using sample lysis buffer, separated by gel electrophoresis, protein transferred onto PVDF membranes and finally probed with the appropriate antibody.

A. Cell lysate

Cells were washed once with 2 ml of cold PBS (4 °C) and lysed in 80 µl of SDS-PAGE sample lysis buffer (Table 2.6) together with 0.5% PMSF (protease inhibitor) and 1% sodium vanadate (phosphatase inhibitor). For example; for 2 wells, 0.25 ml of sample buffer was mixed with 1.25 µl of PMSF and 2.5 µl of sodium vanadate.

Table 2.6 SDS-PAGE sample lysis buffer preparation

| Reagent | Volume |
|-------------------------|--------|
| 500 mM Tris-HCl, pH 6.8 | 25 ml |
| Glycerol | 20 ml |
| dH ₂ O | 43 ml |
| 100 mM EDTA | 2 ml |
| 20% SDS | 2 ml |

After mixing with sample buffer using a cell scraper, cells were collected in 1.5 ml screw-cap eppendorf tubes and samples were then boiled for 5 min in a water bath. 8 μ l (1 tenth of the total volume) of cooled protein was removed into a separate tube for subsequent protein analysis. β -mercaptoethanol and bromophenol blue solution was mixed in a ratio of 10:1 and 8 μ l of this mixture were added to each sample tube. Samples were then mixed, boiled again for 4 min, cooled and frozen at -20 °C until analysis.

B. Protein assay

Protein concentrations of total cell lysates were measured using Pierce bicinchoninic acid (BCA) protein assay kit. Protein samples were diluted 1:8 with dH₂O by adding 56 μ l water to the 8 μ l of protein samples. A standard curve was then prepared in 96 well protein assay plate using 1 mg/ml BSA by aliquoting 25 μ l of standard samples of 0, 31.25, 62.5, 125, 250, 500 and 1000 μ g/ml and experimental diluted samples all in duplicate wells. 200 μ l of BCA protein assay (a ratio of 50:1 mixture of BCA and 4% copper II sulphate solution) was added to each well using a multichannel pipette and the plate incubated at 37 °C for 30 min. Absorbance was measured at 562 nm using (BIORAD iMark) microplate reader, a standard curve was generated and protein content of samples was estimated from the standard curve.

C. SDS-PAGE immunoblotting

After assembling gel equipment with 1 mm casting cassettes, separating and stacking gels were prepared from reagents listed in Table 2.7, and TEMED was added immediately before pouring the gels.

Separating buffer gel was poured into the double glass plate and top surface was covered with thin layer of water-saturated isobutanol. After polymerisation, isobutanol was poured off and any remaining dried with filter paper, stacking buffer gel was added into the top layer, the comb was placed directly over it and gel allowed to polymerise for 20 min.

Afterwards, 350 ml of running buffer was prepared from 70 ml 5x running buffer (144 g of glycine, 30 g of Tris-base and 10 g of SDS made up to 2 litres with dH₂O) and 280 dH₂O and poured to fill the bottom of assembled gel apparatus with the 2 plates of gel facing each other inside the tank. For immunoblotting, an equal amount of protein of cell lysates (20 - 30 μ g) per well was loaded onto the SDS-PAGE 10% polyacrylamide gels (unless otherwise stated) with the addition of sample buffer in the blank wells and pre-stained molecular weight markers. Electrophoresis was then run at 150 V for 55 min until the blue dye front reached the bottom of the gel.

Table 2.7 Polyacrylamide gel (10 and 7.5 %) ingredients

| Reagents | Separating gel | | Stacking gel |
|----------------------------|----------------|----------|--------------|
| | 10% gel | 7.5% gel | |
| dH ₂ O | 6 ml | 7.5 ml | 3.05 ml |
| ProtoFLOWgel Acrylamide | 5 ml | 3.5 ml | 0.65 ml |
| Separating gel buffer | 3.75 ml | 3.75 ml | -- |
| Stacking gel buffer | -- | -- | 1.25 ml |
| 10% SDS | 150 µl | 150 µl | 50 µl |
| 10% APS (100 mg/ml) | 75 µl | 75 µl | 25 µl |
| TEMED | 30 µl | 30 µl | 20 µl |

Separated proteins were transferred onto pre-wetted (methanol, dH₂O, then transfer buffer, 5 min each) labelled PVDF membranes that were stacked in the transfer cassette in the middle of 2 wet sponges and 6 filter papers for each gel, this was subjected to electrophoresis at 100 V for 2 h (on ice) in a tank filled with transfer buffer (188 mM glycine, 25 mM tris-base, and 20% methanol).

Following transfer, membranes were allowed to dry for at least 30 min to maximise protein binding, rewetted with methanol (10 s), dH₂O (5 min), then with TBS-Tween (5 min) and then blocked for 1 h at RT with 5% of non-fat dried milk diluted with 40 ml TBS-Tween (blocking solution) on a horizontal rocker. 10x TBS was prepared by adding 12.1 g (0.1 M) of tris-base plus 87.65 g (1.5 M) of NaCl to 1 litre dH₂O (pH 7.4). One litre of TBS-Tween was then prepared by diluting 1:10 of 10x TBS in dH₂O plus 20 µl of Tween-20.

The membrane was incubated with specific primary antibody in blocking solution overnight on a roller at 4 °C (see Table 2.8 for primary antibody dilution and species). Membranes were then washed with TBS-Tween for 4 min (x3) before being incubated

with HRP-conjugated secondary (anti-rabbit or anti-mouse) antibody at a dilution of 1:4000 in blocking solution rotating for 1 h at RT. After 3 washes, each membrane was incubated in 1.5 ml of ECL Prime western blotting detection reagent (mixing equal volumes of reagent A and B) for 2 min. The membrane was then exposed to x-ray film using developer and fixer Kodak solutions. Toward the end of the lab work, blot imaging of RECK protein were measured by SynGENE G:BOX System (Cambridge, UK).

Furthermore, the suitable concentration of the gel utilised for PTEN, PDCD4 and Sprty-1 that have molecular weight of 35 – 60 kDa was 10% acrylamide gel and β -Actin (42 kDa molecular weight) was used as a loading control. Analysis of RECK protein with a high molecular weight of 110 kDa required the use of 7.5% gel concentration. The loading control β -Actin was undetectable on the lower concentration gel thus, it was replaced with α -tubulin which has a 52 kDa molecular weight.

Finally, the membrane was allowed to dry after washing off ECL for 5 min (with water and methanol). Bands intensities were then quantified by densitometry using imageJ and normalised with image densities of β -actin or α -tubulin which were used as loading controls. The molecular mass of sample proteins was always estimated by comparison with precision plus protein molecular weight markers.

Table 2.8 Primary antibodies used in immunoblotting

| Primary antibody | Dilution | Secondary |
|--------------------------------|-----------------|------------------|
| Akt1 Cat# 2967 | 1:1000 | Mouse |
| α -Tubulin Cat# 3873 | 1:1000 | Mouse |
| β -actin Cat# ab8226 | 1:1000 | Mouse |
| PDCD4 Cat# 9535S | 1:1000 | Rabbit |
| PTEN Cat# 9188S | 1:2000 | Rabbit |
| RECK Cat# 3433S | 1:1000 | Rabbit |
| REK-MAP kinase Cat# 9102 | 1:1000 | Rabbit |
| Sprouty 1 Cat# 13013S | 1:200 | Rabbit |

2.2.12. Statistical analysis

All results presented are expressed as a mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 by one-way ANOVA, followed by Newman-Keuls multiple comparisons post hoc-test. An unpaired t-test was used to compare 2 different group of patients (ND and T2DM) and paired ratio t-test was performed on log transforms of all normalised data to test statistical difference as appropriate. P values of < 0.05 were considered statistically significant.

Chapters 3 - 6
RESULTS

Chapter 3

The role of miR-21 in changing morphological and functional characteristics in saphenous vein smooth muscle cells from diabetic and nondiabetic donors

3.1. Introduction

Diabetes is the most common metabolic disorder and is considered to be one of the most serious health burdens worldwide. Patients with T2DM account for more than 90% of adult diabetes cases and such individuals are at increased risk of cardiovascular complications and SV graft failure following CABG surgery (Berry et al., 2007, Guay et al., 2011). Despite the co-expression of α -SMA and SM-MHC in SMC from both diabetic and ND populations (Owens et al., 2004), distinct morphologies of human SV-SMC cultured from T2DM patients have been observed when compared to ND cells. Non diabetic cells exhibited a classical spindle shape while diabetic cells had a predominantly rhomboidal shape morphology (Madi et al., 2009, Riches et al., 2014). In addition, the appearance of disrupted F-actin cytoskeleton and truncated fibres in T2DM cells was observed in comparison to the classical helical F-actin filaments in ND cells (Riches et al., 2014).

Vascular SMC compose the majority of the wall of blood vessels mainly in the underlying media layer, and their principal function is to maintain prominent plasticity and adjust the calibre of the blood vessels in the body (Newby and Zaltsman, 2000). Under normal physiological conditions, vascular SMC proliferate at a very low rate and remain in a differentiated, contractile quiescent state to regulate blood pressure and flow. They exhibit remarkable plasticity and can switch between two distinct phenotypes upon injury, such as that which occurs moderately in the case of revascularisation procedures or excessively in diseases such as intimal hyperplasia and atherosclerosis (Rzucidlo, 2009). Thus, SMC can become dedifferentiated and transform into a proliferative and migratory phenotype (Alexander and Owens, 2012) and the mechanism by which this phenotypic switching from the quiescent contractile state to the synthetic and migratory phenotype, still needs to be further investigated. Whilst currently available pharmacological therapies are highly efficient in treating cardiovascular remodelling problems, the mortality rate following coronary intervention remains high. Therefore, a better understanding of the biological

mechanisms underlying the vascular SMC response to injury could disclose novel therapeutic targets.

MicroRNAs are a recently discovered class of endogenous, small, single-stranded non-coding sequences of RNA that control nearly 30% of all genes. They negatively regulate gene expression and protein synthesis by promoting either translational inhibition and/or degradation of their target mRNAs, acting either alone or in combination (Ji et al., 2007). One miR can control the expression of multiple target genes which have related to cellular function and can regulate genes according to different environmental conditions (Bartel, 2009). They play an important part in the normal physiological development of the pancreas, insulin signalling in target tissues, apoptosis, vascular SMC phenotypic changes, development and regulation (Seyhan, 2015). In addition to their physiological contributions, the pivotal role of miRs in the pathological processes of SMC dedifferentiation and proliferation is documented in various studies (Albinsson et al., 2010, Urbich et al., 2008).

The nature of miR expression is tissue-specific and highly affected by the severity of diseases (Lagos-Quintana et al., 2002). Recent studies have implicated miRs in both the pathogenesis of diabetes and its related cardiovascular complications (Shantikumar et al., 2012).

A High Throughput Sequencing approach (Vertis Biotechnologie AG) was carried out in our laboratory to analyse the miR transcriptome using RNA samples from SV-SMC from 6 ND (average age 65.7, range 47-82 years) and 6 T2DM (average age 63.4, range 50-85 years) donors. From this, the top five most differentially regulated miRs were obtained (listed in Table 3.1).

Despite the apparently highest fold increase in T2DM versus ND SMC (Table 3.1), being for miR-1260a/b there are no data regarding the role of either miR-1260a/b or miR-1280 in the vasculature or cardiovascular conditions, although miR-1280 was studied in bladder cancer and shown to inhibit cell invasion (Majid et al., 2012). Whilst miR-145 and miR-21 were also among the highest-ranked significant miRs in T2DM versus ND cells, miR-145 is known to be highly expressed in the vasculature and it has been thoroughly explored by several groups. In both *in vitro* studies using murine aortic SMC (Cordes et al., 2009) and in human SMC studies, miR-145 has been shown to drive cell differentiation and phenotypic changes, aspects that have been previously studied by others (Cheng et al., 2009a) and in our own research group (Riches et al., 2014).

Table 3.1 MicroRNAs showing the greatest fold increase in expression in SV-SMC of T2DM relative to ND donors

| MiRs | Fold increase in T2DM |
|-------------|------------------------------|
| miR-1260b | 320 |
| miR-1260a | 292 |
| miR-1280 | 56 |
| miR-145 | 45 |
| miR-21 | 41 |

Out sourced to Vertis Biotechnology (2012)

MiR-21 is known to be involved in various physiological conditions (Zhang, 2008) and is expressed in several tissues including the vasculature. Experimental animal studies showed that miR-21 is upregulated in different types of pathology such as fibrosis (Thum et al., 2008), diabetes complications (Guay et al., 2011), cancer (Jazbutyte and Thum, 2010), cardiovascular diseases including cardiac hypertrophy (Syed et al., 2015) and atherosclerosis (Cheng and Zhang, 2010). Furthermore, it has been shown that miR-21 is upregulated in rat balloon-injured arteries and in mouse ligation models, inducing a proliferative vascular SMC effect through targeting various genes (Ji et al., 2007, Stein et al., 2014). MiR-21 has also been found to be highly upregulated and play important roles in human atherosclerotic plaques (Raitoharju et al., 2011) and arteriosclerosis by modulating human artery SMC proliferation and migration (Ruan et al., 2011). However, the role of miR-21 in inducing human vascular remodelling particularly in diabetic SV grafting has received little attention.

To be able to detect changes in cells induced by miR-21 modulation, we utilised the transfection technique. MiR-21 was artificially overexpressed using premiR-21 and knocked down using antimiR-21 in SV-SMC. PremiRs are double-stranded RNAs with sequence analogous to the mature miR of interest, for example, miR-21. One strand is identical to and effectively mimics the endogenous mature miR-21. Thus, when premiRs enter the cell they are processed into mature miR, bind RISC then post-transcriptionally inhibit the target mRNA. In contrast, the antimiRs are single-stranded RNA-based inhibitors. They have a complimentary sequence to the miR of interest

such that they bind to and prevent complementary pairing of a mature miR with its target mRNA (Patel and Nouredine, 2012) (see Figures 1.9 A/B).

We confirmed the upregulation of miR-21 observed in SV-SMC from T2DM patients obtained using a high throughput screening approach (Table 3.1), by real-time PCR using Bio-Rad and a SYBR Green-based miR-21 assay on 5 different patients. On average, the relative expression levels of miR-21 were 60% higher in T2DM SMC than cells from ND donors (Figure 3.1, K. Riches, unpublished data 2012).

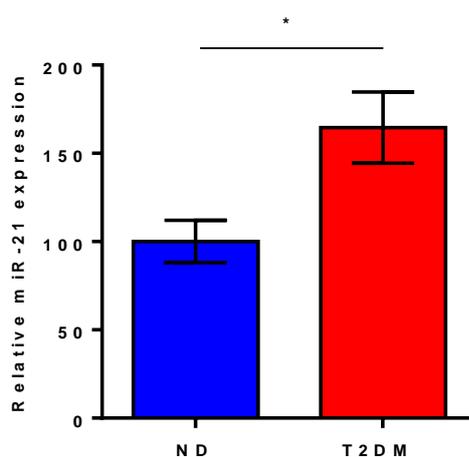


Figure 3.1: Expression of miR-21 in cultured SV-SMC from ND and T2DM donors.

Bar chart showing difference in miR-21 expression between SV-SMC from T2DM and ND donors, to confirm result obtained from high throughput sequencing data. Error bars denote SEM; * $P < 0.05$; unpaired t-test, two tailed. MiR-21 was normalised to U6 as an internal control ($n = 5$ / group). Data provided by Dr K Riches, 2012.

3.2. Aims

The aims of this chapter were:

- To determine functional differences between human SV-SMC cultured from T2DM and ND donors
- To investigate whether manipulation of miR-21 levels in human SV-SMC play a role in impaired cell function.

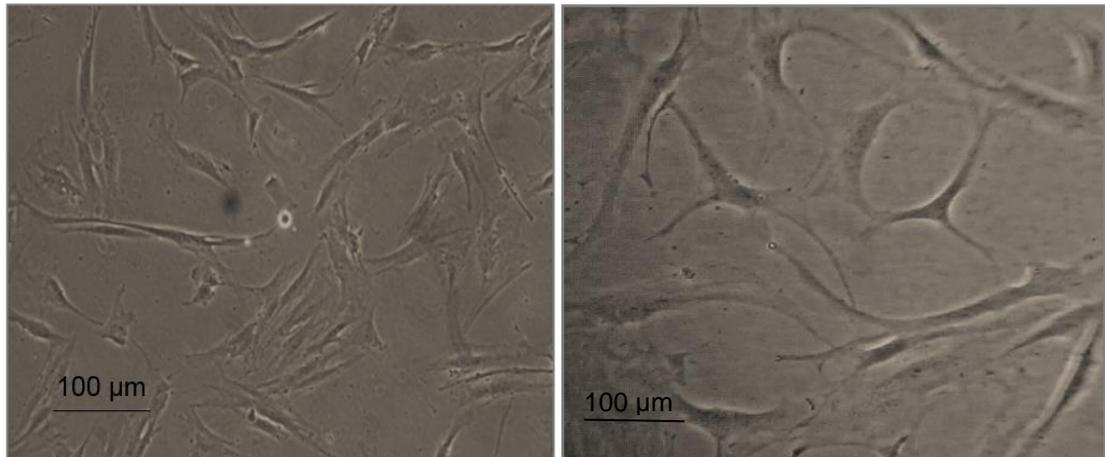
3.3. Comparison between T2DM and ND SV-SMC

3.3.1. Morphology

SV-SMC were harvested from T2DM and ND patients undergoing CABG surgery. Cells were cultured in a T75 flask and grown to sub-confluence using the method described in 2.2.1. Cell images were captured at approximately 50% confluence using phase contrast microscopy and all experiments were conducted on cells from equal passage numbers (3 - 5). Images were obtained for eight different patients in each group from 20 random fields of view. Distinct morphology was exhibited between SV-SMC cultured from T2DM and ND patients (Figure 3.2). SV-SMC from ND donors predominantly exhibited classical spindle-shaped elongated SMC morphology as presented in Figure 3.2A, whilst T2DM cells were mainly rhomboid in shape (Figure 3.2B).

To confirm the SMC specificity and characterisation, α -SMA and SM-MHC were visualised by immunostaining. T2DM and ND SV-SMC were plated at an equal density on glass coverslips in 24 well plates in FGM as described in section 2.2.3A. The co-expression of α -SMA and SM-MHC positively identified the cells as SMC in both ND and T2DM patients. Additionally, α -SMA staining clearly demonstrated spindle-shaped fibres in ND patients (Figure 3.3A). In contrast, the cells obtained from T2DM patients, clearly showed an increase in cell size with more pronounced α -SMA fibres (Figure 3.3B).

A- Non-diabetic



B- Diabetic

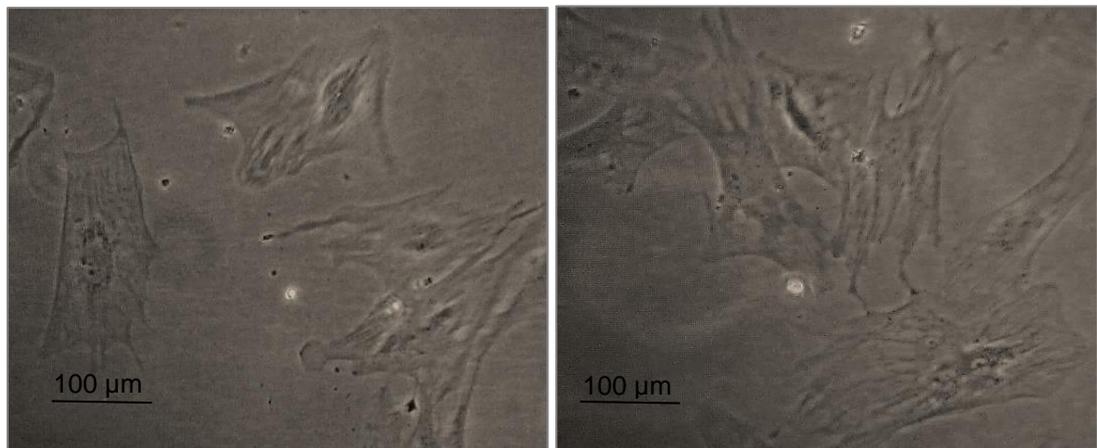
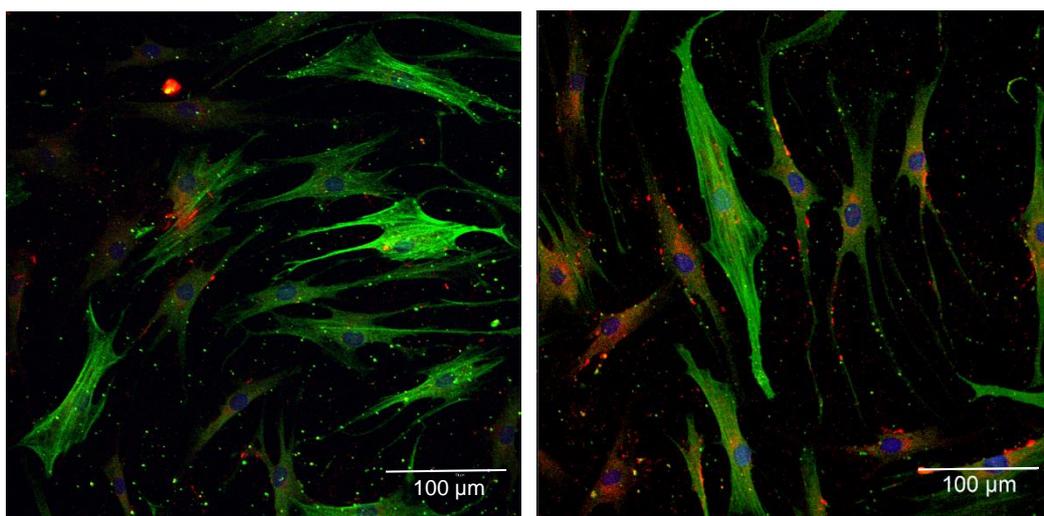


Figure 3.2: Representative phase contrast images of cultured human ND and T2DM SV-SMC.

SV-SMC of two representative ND (A) and two T2DM donors (B). Original magnification (x100) and scale bar =100 μm.

A- Non-diabetic



B- Diabetic

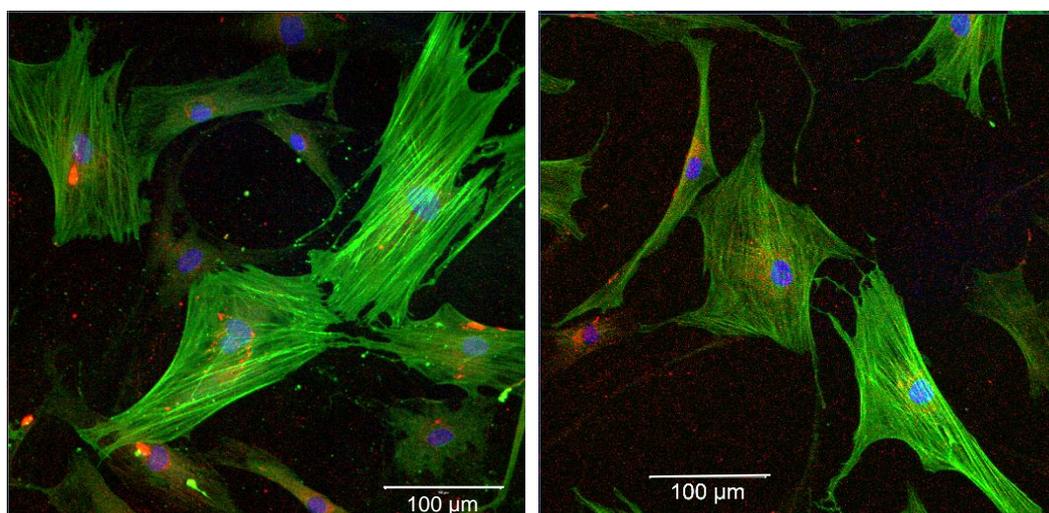


Figure 3.3: Representative fluorescent microscopy images of cultured ND and T2DM SV-SMC.

Representative fluorescence images after staining ND SV-SMC (A) and T2DM SV SMC (B) with DAPI (nucleus, blue), α -smooth muscle actin (α -SMA, green), and smooth muscle myosin heavy chain (SM-MHC, red) antibodies. Power magnification x200 and scale bar =100 μ m.

3.3.2. Proliferation and cell area

Given the role of SMC proliferation in effective adaptation during arterialisatation and vascular remodelling, proliferation rate was measured in SV-SMC obtained from T2DM and ND patients in a parallel manner. Cell proliferation was determined by cell counting as described in Section 2.2.4 over a 7-day time course.

To compare proliferation between T2DM and ND SV-SMC, equal passage numbers of SMC were used in a parallel for six T2DM and six ND patients. SV -SMC were plated at a density of 1×10^4 cells/well in quadruplicate in a 24-well plate in FGM and incubated overnight, then quiesced in SFM for 72 h. SMC cultured from T2DM had a slower proliferation rate than cells from their ND counterparts, this difference became significant from day 4 onwards ($P < 0.05$) (Figure 3.4A). Area under the proliferation curve (AUC) analysis showed a significant reduction (approximately 50%) in AUC of T2DM compared to ND SV-SMC (Figure 3.4B). This finding implies that diabetes can impair the rate of cell proliferation.

To investigate cell morphology, multiple images of parallel passage number (3 and 4) SV-SMC were captured under light microscopy. Cell area of 50 cells per patient were measured by drawing around the perimeter of each cell and measured using ImageJ. Average cell area was calculated and mean cell area and distribution curves were constructed. The distribution of size graph plotted for T2DM was wider and shifted to the right compared to the graph obtained from ND SV-SMC (Figure 3.4C). Additionally, there was a significant increase in the average cell area of cells from T2DM compared to ND individuals. Average cell area of SV-SMC in ND donors was $8518 \mu\text{m}^2$ whilst in T2DM it reached $11623 \mu\text{m}^2$ showing a 1.4 fold increase relative to ND (Figure 3.4D, $n = 8$, $P < 0.05$).

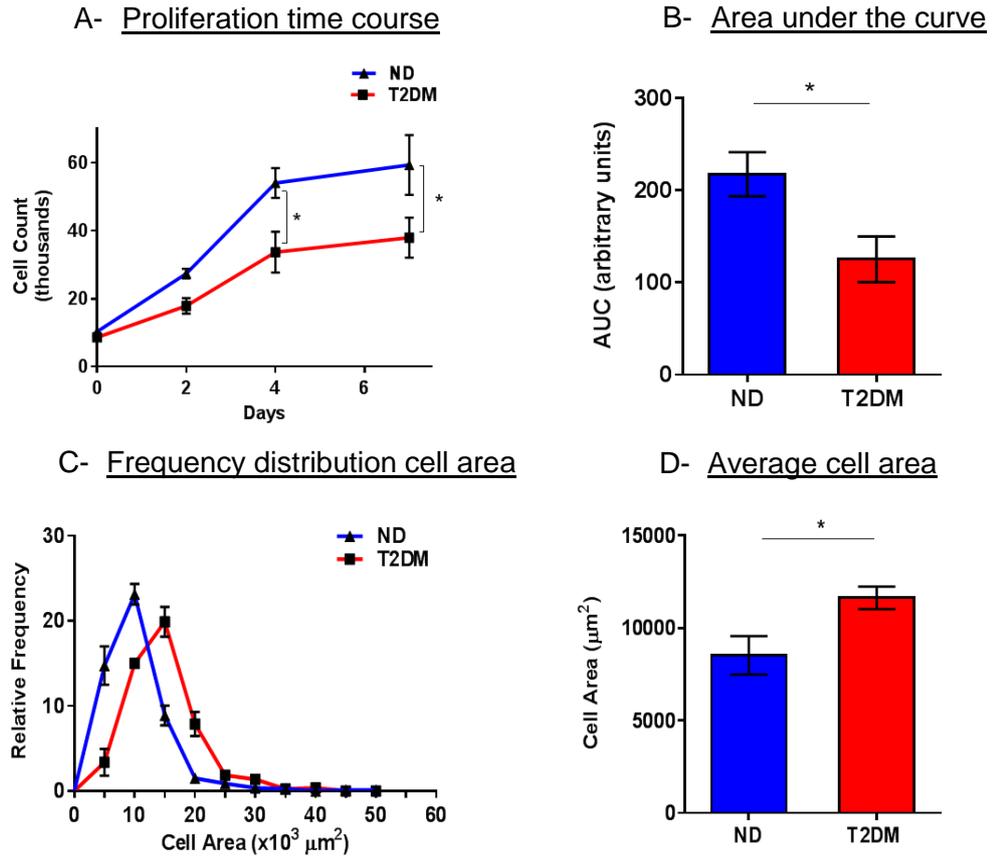


Figure 3.4: Differential proliferation and average cell area of SV-SMC from ND and T2DM patients.

A- Proliferation rate graph showing increase in cell number over 7 days. SV-SMC of T2DM patients proliferated more slowly to cells from ND donors (* $P < 0.05$, $n = 6$; mean \pm SEM; Two-way ANOVA and Newman-Keuls multiple comparisons test). B- Area under the curve (AUC) of counted cells from ND and T2DM donors (* $P < 0.05$, $n = 6$, unpaired t-test). C- Frequency distribution of cell areas in 5000 μm^2 intervals for ND and T2DM SV-SMC ($n = 8$). D- Average cell area comparison between cells from ND and T2DM donors (mean \pm SEM; * $P < 0.05$, unpaired t-test, two-tailed, $n = 8$).

3.4. Manipulation of miR-21 levels in ND SV-SMC

Having shown that miR-21 was elevated in SV-SMC of T2DM, we artificially overexpressed miR-21 in ND SV-SMC in an effort to determine whether miR-21 could induce phenotypic changes similar to those observed in cells from T2DM. Cells of six different ND patients were plated at a density of 70,000 cells/ well in a 6-well plate and transfected with 30 nM premiR-21 or premiR-negative (control) for 6 h and then incubated with MGM for 72 h according to the protocol in Section 2.2.2. This was carried out alongside untransfected and mock groups to check if the transfecting reagents themselves induced any effect on the cells. Cells were trypsinised and pelleted for subsequent RNA extraction and RT-PCR which revealed that premiR-21 transfected cells showed significant upregulation of miR-21 expression levels compared to the three control groups ($P < 0.001$, Figure 3.5).

3.5. Downregulation of miR-21 in ND SV-SMC

Having obtained a sizeable overexpression of miR-21 using 30 nM premiR-21, 30 nM antimiR-21 was selected at first to attempt to downregulate miR-21 expression in ND native SV-SMC. Following 72 h cells were pelleted and RNA was extracted to measure miR-21 expression using RT-PCR. The knock down of miR-21 was not sufficient under these conditions, thus to optimise the antimiR-21 concentration and time of sample collection, the concentration was increased to 60-500 nM for up to 7 days. However, none of these trials showed significant or strong miR-21 knockdown (data not shown).

Owing to the low level of endogenous miR-21 in native ND SV-SMC, we decided to overexpress miR-21 to provide a bigger “window” of miR-21 expression against which we could test the effectiveness of knockdown with antimiR-21. Thus, SV-SMC from four different donors were seeded in a 6-well plate and antimiR-21 (100 nM) was combined together with premiR-21 in the same well. Overexpression of miR-21 by using 30 nM premiR-21 in SV-SMC significantly upregulated miR-21 expression (approximately 3100-fold increase) relative to premiR-negative 72 h after transfection (Figure 3.6). Furthermore, the addition of antimiR-21 to premiR-21 induced a significant 95% reduction in miR-21 expression in SV-SMC compared with cells transfected with premiR-21 alone thus indicating the strong efficacy of 100 nM antimiR-21 concentration in knocking down overexpressed miR-21 (Figure 3.6).

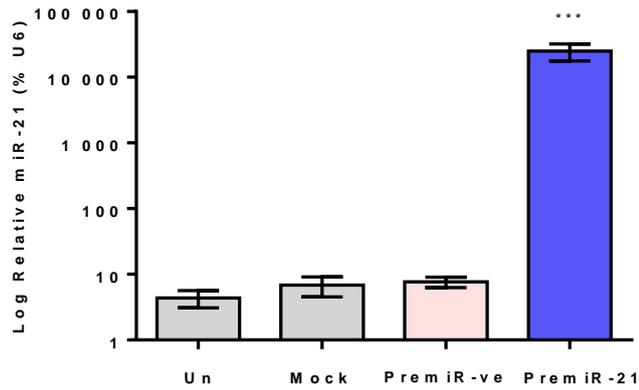


Figure 3.5: Overexpression of miR-21 in SV-SMC from ND donors.

ND SV-SMC were transfected with either 30 nM premiR-21, 30 nM premiR-negative (PremiR-ve), mock (lipofectamine), or untransfected (un; Optimem). Bars showing RT-PCR for miR-21 expression after 72 h (** $P < 0.001$, One way ANOVA and Newman-Keuls multiple comparisons test, $n = 6$). Expression of miR-21 was normalised to U6 and error bars denote SEM. Note the log scale on the y-axis.

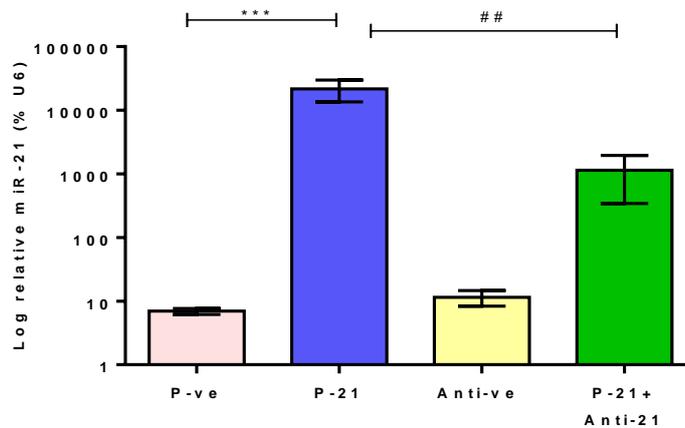


Figure 3.6: Effect of antimiR-21 on ND SV-SMC overexpressing miR-21.

ND SV-SMC were transfected with either 30 nM premiR-negative (P-ve), 30 nM premiR-21 (P-21), 100 nM antimiR-ve (Anti-ve) or 30 nM premiR-21 + 100 nM antimiR-21. RT-PCR for miR-21 expression after 72 h (** $P < 0.001$, $###P < 0.01$, One way ANOVA and Newman-Keuls multiple comparisons test, $n = 4$). Expression of miR-21 was normalised to U6 and error bars denote SEM. Note the log scale on the y-axis.

3.6. Knockdown of miR-21 in T2DM SV-SMC

Having shown the increased expression of miR-21 in T2DM relative to ND SV-SMC, knockdown of miR-21 was performed in T2DM SV-SMC to try to alter the phenotypic characteristics to those of ND cells. Because the 500 nM concentration of anti-miR-21 did not show an extra knockdown of miR-21 compared with either 60 nM or 100 nM anti-miR-21, cells from five different T2DM patients were transfected with anti-miR-21 or anti-miR-negative at a final concentration of 100 nM. Additionally, to determine the optimal time to achieve significant knockdown of miR-21 expression, a time course experiment was performed. Cells were harvested daily from T2DM transfected SV-SMC up to day 7, RNA extracted and RT-PCR for miR-21 expression was performed. An approximate 40-50% reduction in miR-21 expression was achieved by anti-miR-21 relative to anti-miR-negative, with the maximum drop in miR-21 expression obtained at day 5 (Figure 3.7A, $P < 0.05$).

To compare the total amount of miR-21 expression between the two groups, AUC for these samples was measured for the 7 days and a significant 47% total reduction in the AUC of anti-miR-21 transfected cells was obtained relative to anti-miR-negative transfected cells, indicating that around 50% reduction of miR-21 expression was achieved in the anti-miR-21 transfected cells relative to basal controls (Figure 3.7B, $P < 0.01$).

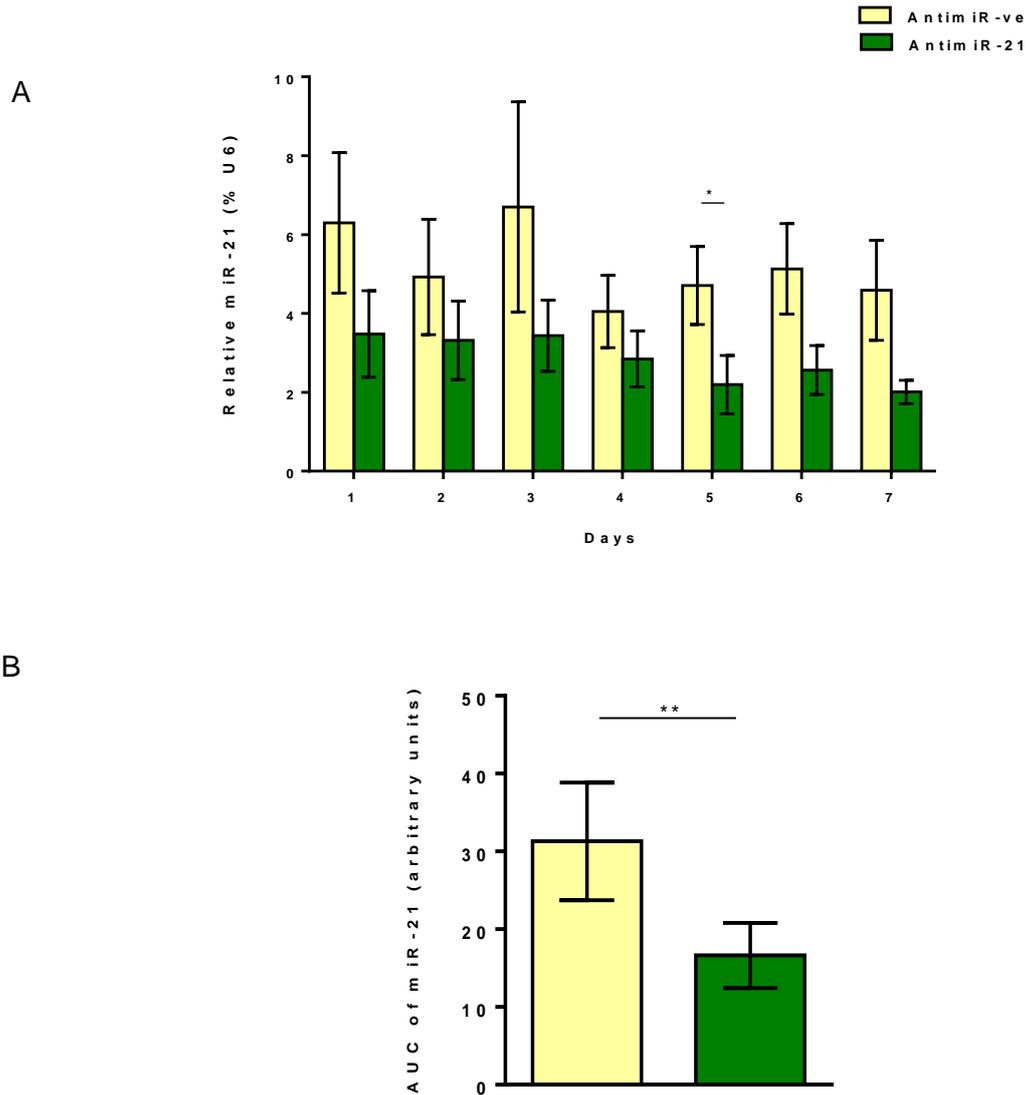


Figure 3.7: Time course comparison and AUC of knockdown of miR-21 in SV-SMC from T2DM donors.

A- T2DM SV-SMC were transfected with 100 nM antimiR-21 or antimiR-negative (antimiR-ve). Bars showing RT-PCR measurement of miR-21 expression in cells at 24 h intervals for 7 days (* $P < 0.05$, 2 way ANOVA and Newman-Keuls multiple comparisons test). Expression of miR-21 was normalised to U6. B- AUC of miR-21 knockdown in the antimiR-21-transfected cells relative to antimiR-negative transfected T2DM cells for a total 7 days (** $P < 0.01$, paired t-test), $n = 5$ and error bars denote SEM.

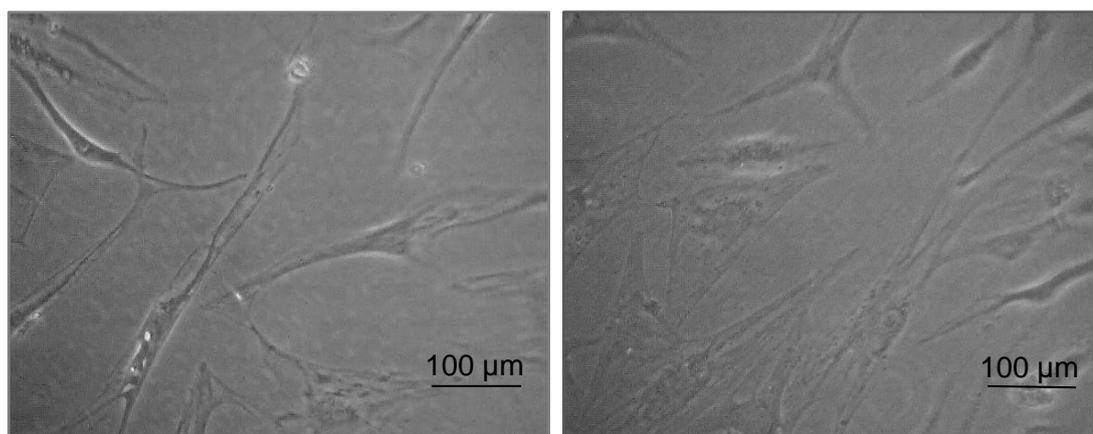
3.7. Effect of overexpression miR-21 levels in SV-SMC on cell morphology

Since the level of miR-21 is reportedly upregulated in different cardiovascular diseases and T2DM is one of the major risk factors, we investigated whether miR-21 overexpression could drive the phenotypic changes similar to those observed in T2DM SMC. PremiR-21 and premiR-negative transfected SV-SMC were seeded in 6-well plates in FGM and 72 h after transfection (Section 2.2.2) images of the cells were captured and the imageJ program was used to calculate average cell area (Section 2.2.5). Representative images presented in Figure 3.8A/B revealed the relatively enlarged cell area in the premiR-21 transfected ND cells as compared to premiR-negative transfected SV-SMC. The cells overexpressing miR-21 showed a 1.7-fold increase in cell area compared to negative control, indicating the important role of miR-21 in inducing phenotypic changes in SV-SMC (Figure 3.8C, $P < 0.001$, $n = 7$).

Studies have shown that F-actin bundling into fibres is related to SMC differentiation, and phenotypic modulation of vascular SMC is accompanied by changes in actin cytoskeleton alignment (Han et al., 2009). Thus, F-actin fibres were visualised to test their organisation and as a sign of differentiation status in the premiR-21 and antimiR-21 transfected SV-SMC. To visualise F-actin fibres, premiR-21 and antimiR-21 transfected cells were seeded on coverslips and stained with rhodamine-phalloidin as in Section 2.2.3B. Representative images were obtained by fluorescent microscopy and revealed premiR-21 transfected cells (Figure 3.9B) did exhibit a slight enlargement in F-actin fibres area compared to premiR-negative transfected cells (Figure 3.9A), with the maintenance of the characteristic well-aligned F-actin fibres of ND SV-SMC. This suggests that miR-21 overexpression did not disorganise the alignment of F-actin in SV-SMC.

A- PremiR-negative

B- PremiR-21



C

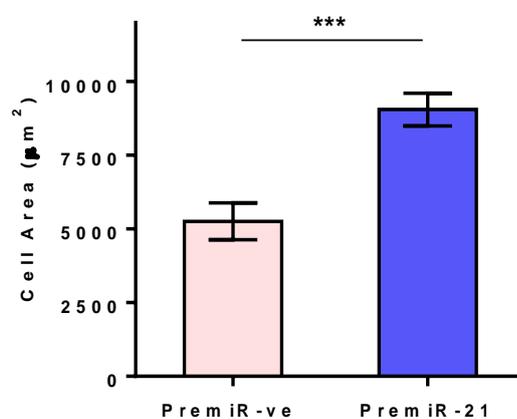
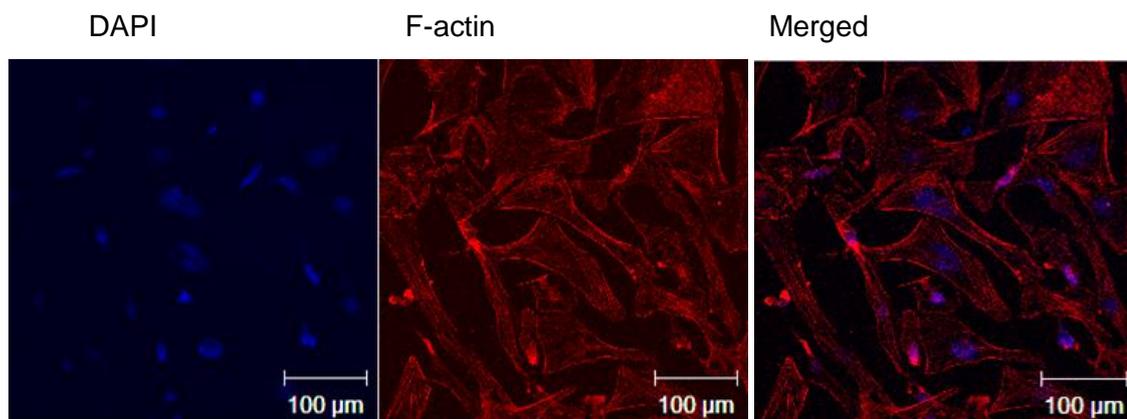


Figure 3.8: Representative phase contrast images and average cell area of the effect of miR-21 overexpression on ND SV-SMC.

Representative phase contrast images were captured for ND SV-SMC 72 h following miR-21 overexpression using 30 nM premiR-negative (A) or premiR-21 (B). Scale bar =100 μm. C- Average cell area of SV-SMC overexpressing miR-21 relative to premiR-negative transfected cells (**P < 0.001, paired t-test, two-tailed, n= 7, 50 cells for each). Error bars denote SEM.

A-PremiR-negative



B-PremiR-21

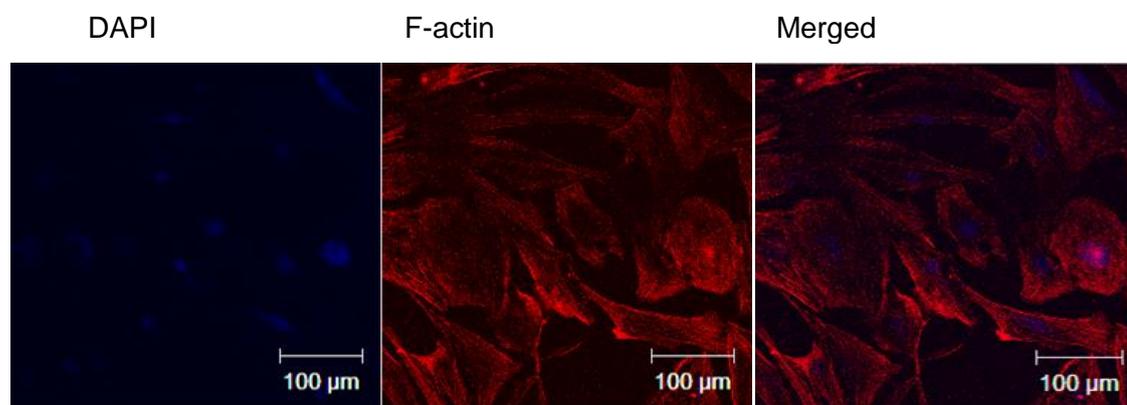


Figure 3.9: Representative fluorescent microscopy images showing the effect of miR-21 overexpression on the F-actin cytoskeleton in ND SV-SMC.

Representative fluorescent microscopy images of F-actin cytoskeletons captured for ND SV-SMC 72 h following transfection with 30 nM premiR-negative (A) or premiR-21 (B) and stained with rhodamine phalloidin. Cells were visualised using LSM510 Upright Confocal microscope (x200) and scale bar =100 µm.

3.8. Effect of knockdown of miR-21 in T2DM SV-SMC on cell morphology

Whilst high levels of miR-21 are documented in cardiovascular diseases (Jazbutyte and Thum, 2010), we performed knockdown of miR-21 expression in SV-SMC from five different T2DM donors to determine whether the cells could be rescued to the ND phenotype. SMC were cultured in six-well plates and transfected with 100 nM antimiR-21 or antimiR-negative control. After 72 h, microscopic images were taken and ImageJ was utilised to measure cell area over 7 days. A representative image at day 3 of the antimiR-21 transfected T2DM cells showed a general reduction in cell area in comparison to the control cells (Figure 3.10A/B). This indicates the important role of miR-21 in mediating phenotypic changes in SV-SMC, particularly increasing cell area.

There was a trend for a reduction in average cell area in the antimiR-21 transfected SV-SMC compared to antimiR-negative controls at all time points. The average cell area was reduced by 14 – 34 % across the whole time course (Figure 3.10C).

To visualise F-actin fibres images were obtained using fluorescent microscopy taken 72 h after transfection. A representative image of antimiR-21 transfected cells showing indistinguishable changes in F-actin fibres size or shape from control is shown in Figure 3.11A/B, suggesting an insignificant role of miR-21 knockdown in mediating changes in F-actin fibre cytoskeleton in SV-SMC.

A- AntimiR-negative

B- AntimiR-21

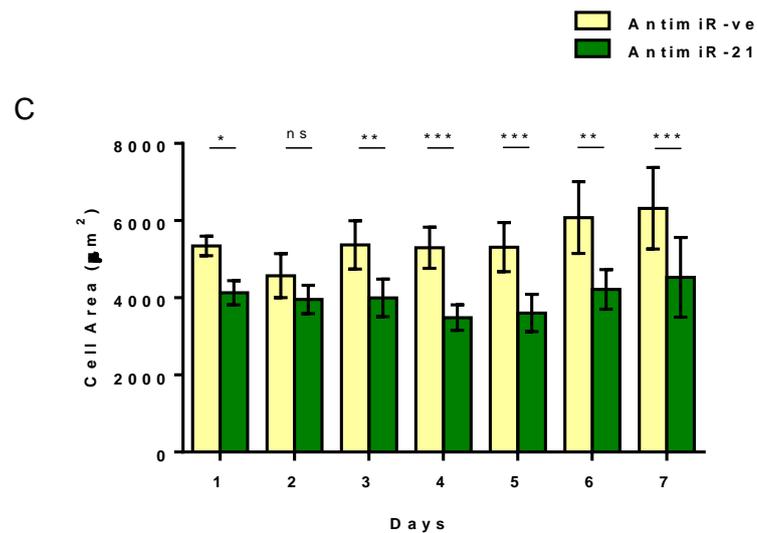
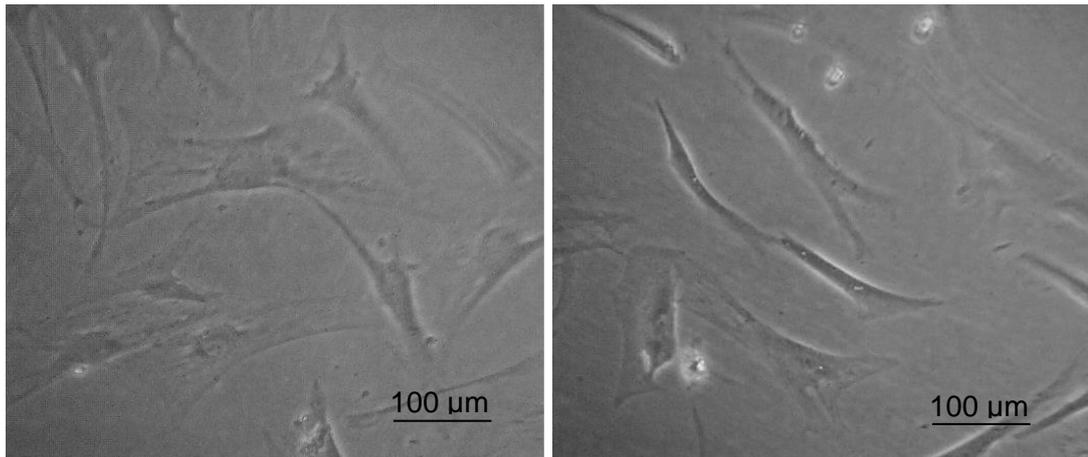


Figure 3.10: Representative phase contrast images and effect of miR-21 knockdown on cell area in T2DM SV-SMC.

Representative phase contrast images captured at 72 h for T2DM SV-SMC following transfection with either 100 nM anti-miR-negative control (A) or anti-miR-21 (B). Scale bar =100 μm. C- Effect of anti-miR-21 transfection on average cell area relative to control over a 7 day period (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; 2 way ANOVA and Newman-Keuls multiple comparisons test, $n = 5$). Error bars denote SEM.

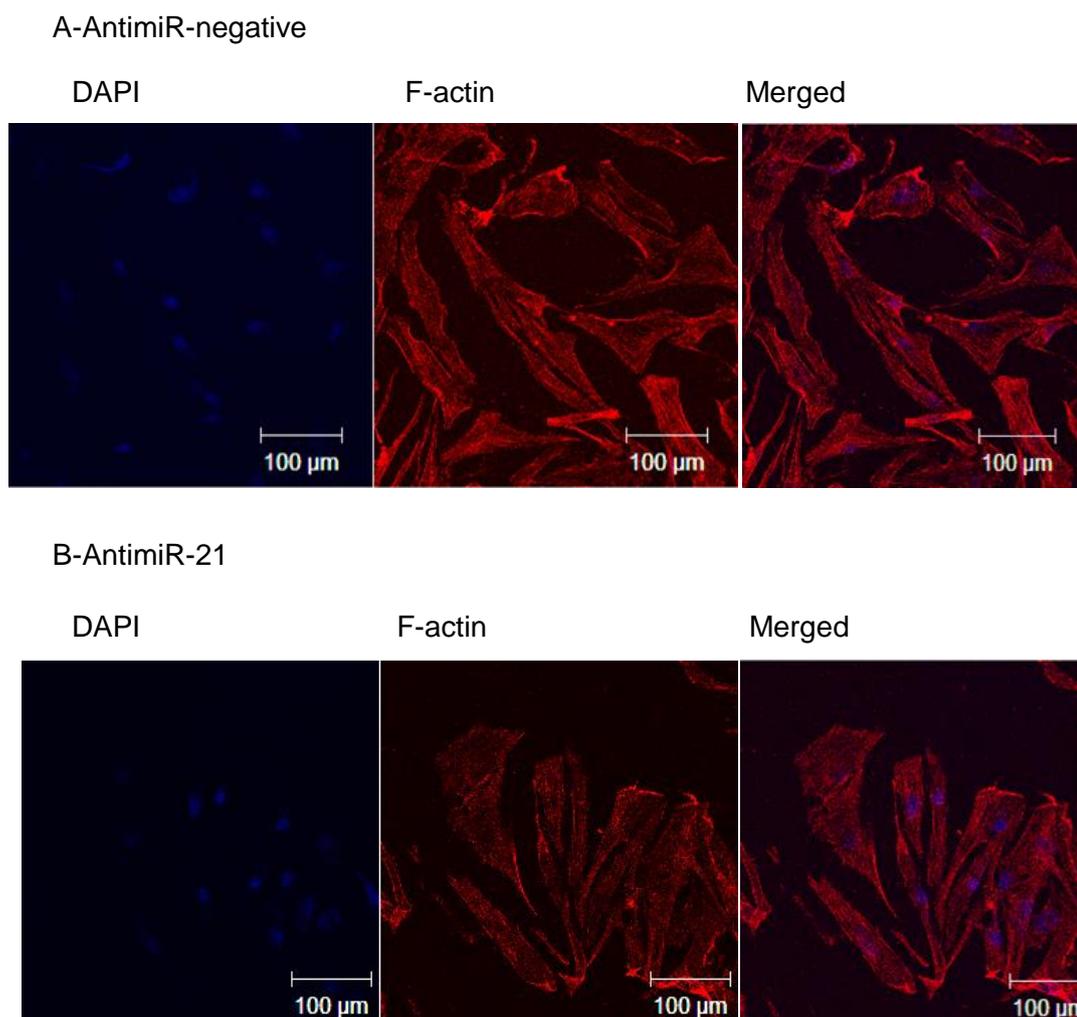


Figure 3.11: Representative fluorescence microscopic images of the effect of knocking down miR-21 on F-actin cytoskeleton in T2DM SV-SMC.

Fluorescent microscopy images of F-actin cytoskeleton for T2DM SV-SMC 72 h following transfection with 100 nM antimiR-negative (A) or antimiR-21 (B) stained with rhodamine phalloidin. F-actin fibres were visualised using LSM510 Upright Confocal microscope at a magnification x200 and scale bar =100 µm.

3.9. Differential gene expression measurement between ND and T2DM SV-SMC

Because of the established role of activation of MMPs such as MMP-2 and MMP-3 and decomposition of collagen in vascular remodelling and intimal lesion formation (de Kleijn et al., 2001), the difference in type I collagen $\alpha 1$ (COL1A1) and matrix regulatory gene expression between T2DM and ND SV-SMC was tested in eight different patients. Cells from both groups were collected and RNA samples were extracted for gene analysis using RT-PCR. There was no significant difference between SV-SMC obtained from T2DM and ND patients (Figure 3.12) with respect to COL1A1 (A), MMP2 (B) and MMP3 (C) mRNA expression. This indicates that the expression of these genes, in our SV-SMC samples utilised, is not regulated by the diabetic milieu or any regulation *in vivo* is not maintained in culture.

3.10. Correlation between miR-21 and collagen 1A1, MMP2 and MMP3 in SV-SMC obtained from ND and T2DM patients

Having obtained no significant difference in COL1A1, MMP2 and MMP3 gene expression between ND and T2DM SV-SMC we proceeded to analyse the correlation between miR-21 expression level and expression of these genes in SV-SMC of both diabetic and ND patients. Correlation figures were compiled showing a high correlation between miR-21 expression and COL1A1 mRNA ($R^2 = 0.81$, Figure 3.13A) in ND cells but this correlation was absent in cells obtained from T2DM patients ($R^2 = 0.33$, Figure 3.13D). In normal healthy cells there is a strong correlation between miR-21 and COL1A1 mRNA. However, this relationship appears to have been lost in T2DM as evident by the lack of correlation. Expression of the other two genes, MMP2 (Figure 3.13 B and E) and MMP3 (Figure 3.13 C and F) showed no correlation with miR-21 expression in either ND or T2DM SV-SMC ($n = 8$ / each group).

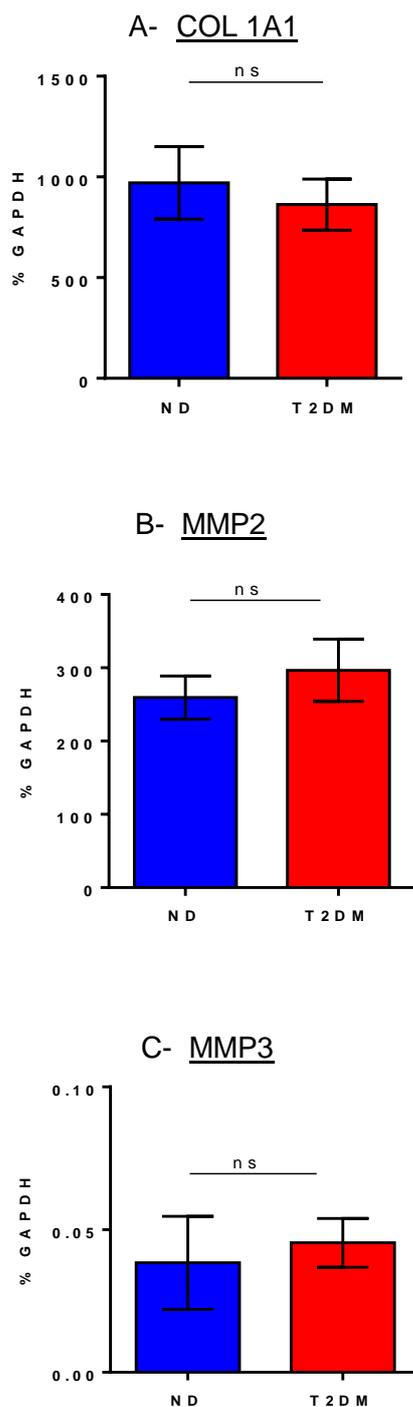


Figure 3.12: Comparison of collagen 1A1, MMP2 and MMP3 mRNA levels between ND and T2DM SV-SMC.

Bar chart showing a comparison between ND and T2DM SV-SMC for collagen 1A1 (A), MMP2 (B) and MMP3 (C) mRNA expression using RT-PCR. (ns= not significant, unpaired t-test, n= 8). Gene expression was normalised to the endogenous control GAPDH and error bars denote SEM.

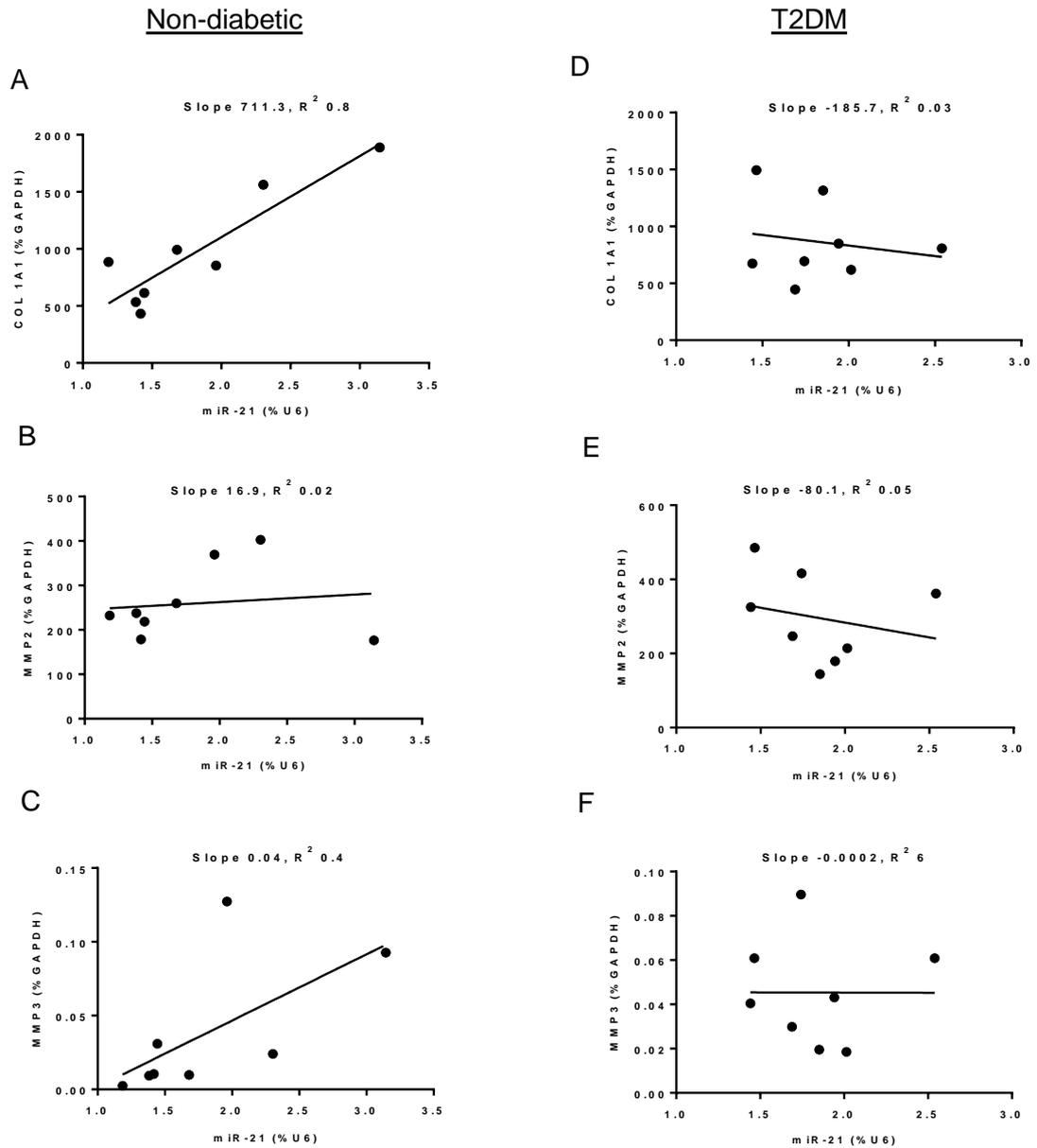


Figure 3.13: Correlation between miR-21 expression and Collagen 1A1, MMP-2 and MMP-3 in ND and T2DM SV-SMC.

Correlation between miR-21 expression in ND SV-SMC and COL1A1 (A), MMP2 (B) and MMP3 (C) and T2DM SV-SMC and COL1A1 (D), MMP-2 (E) and MMP-3 mRNA (F) (each group n= 8).

3.11. Effect of overexpression/ knockdown of miR-21 on collagen 1A1 expression in ND SV-SMC

To examine the effect of miR-21 manipulation in ND SV-SMC on COL1A1 expression (which accounts for 85% of the ECM content (Kong et al., 2013), RT-PCR was performed on RNA samples from miR-21 overexpressing or underexpressing cells to measure COL1A1 mRNA levels. Overexpression (Figure 3.14A, n= 7) or knockdown of miR-21 levels (Figure 3.14B, n= 4) did not induce changes in COL1A1 expression relative to premiR-negative and antimiR-21 transfected control cells.

3.12. Comparison of miR-21 expression levels between ND and T2DM patients in different cardiovascular cell types

Several studies have documented the increased miR-21 expression in cardiovascular diseases and one particular study also showed the upregulation of miR-21 expression in mice after SV grafting (McDonald et al., 2013). As comparable results were obtained for COL 1A1, MMP2 and MMP3 expression in SV-SMC obtained from ND and T2DM donors, we decided to measure the miR-21 expression level in a larger population (n= 11/ each group) to check the level of miR-21 expression between the two groups. The samples used in our previous pilot data (Figure 3.1) were tested again using the TaqMan-based assay by ABI 7500 Real-Time PCR System. As shown in Figure 3.15A there was a significant increase in miR-21 in the diabetic SV-SMC compared to ND counterparts. In contrast to the results obtained from the small sample size, when using a larger population of eleven samples the real-time PCR analysis of the RNA samples revealed comparable levels of miR-21 expression between the two groups (

Figure 3.15B). Lastly, even when the samples of the pilot data were added, the expression of miR-21 between T2DM and ND SV-SMC was similar.

Other cardiovascular tissues were utilised to compare miR-21 expression between T2DM and ND donors; namely cardiac fibroblasts and endothelial cells. The real-time PCR analysis of miR-21 expression showed a trend toward an increase in miR-21 expression in the RNA samples from T2DM cardiac fibroblasts (

Figure 3.15C) and no difference in miR-21 expression in the endothelial cells (Figure 3.15D) compared to the RNA samples obtained from ND patients (n= 6).

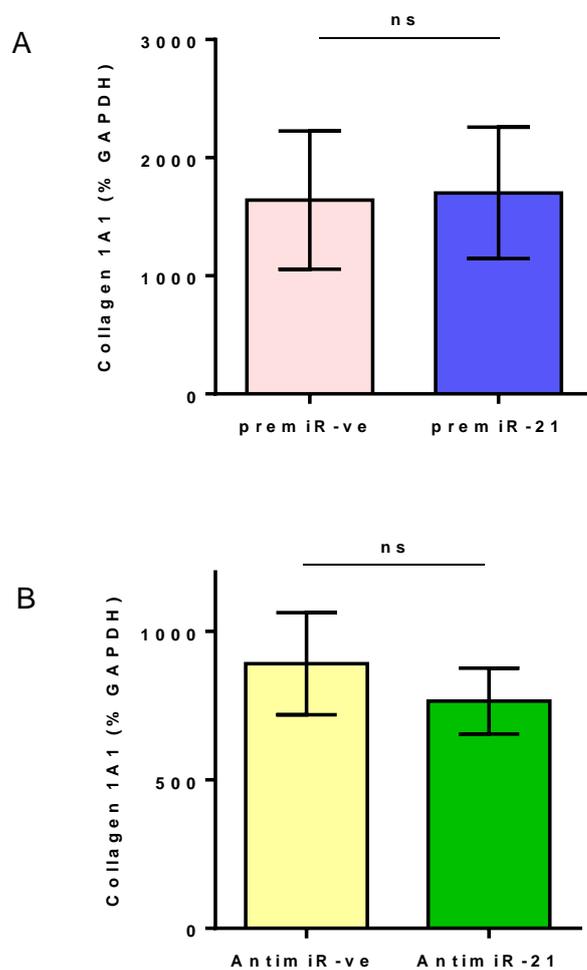


Figure 3.14: Measurement of collagen 1A1 mRNA in premiR-21 and anti-miR-21 transfected ND SV-SMC.

RT-PCR measurement of COL1A1 mRNA expression for SV-SMC 72 h following transfection with either 30 nM premiR-21 or premiR-negative (A, n= 7) and 100 nM anti-miR-21 or anti-miR-negative (B, n= 4). Gene expression was normalised to GAPDH and error bars denote SEM (ns= not significant, paired t-test).

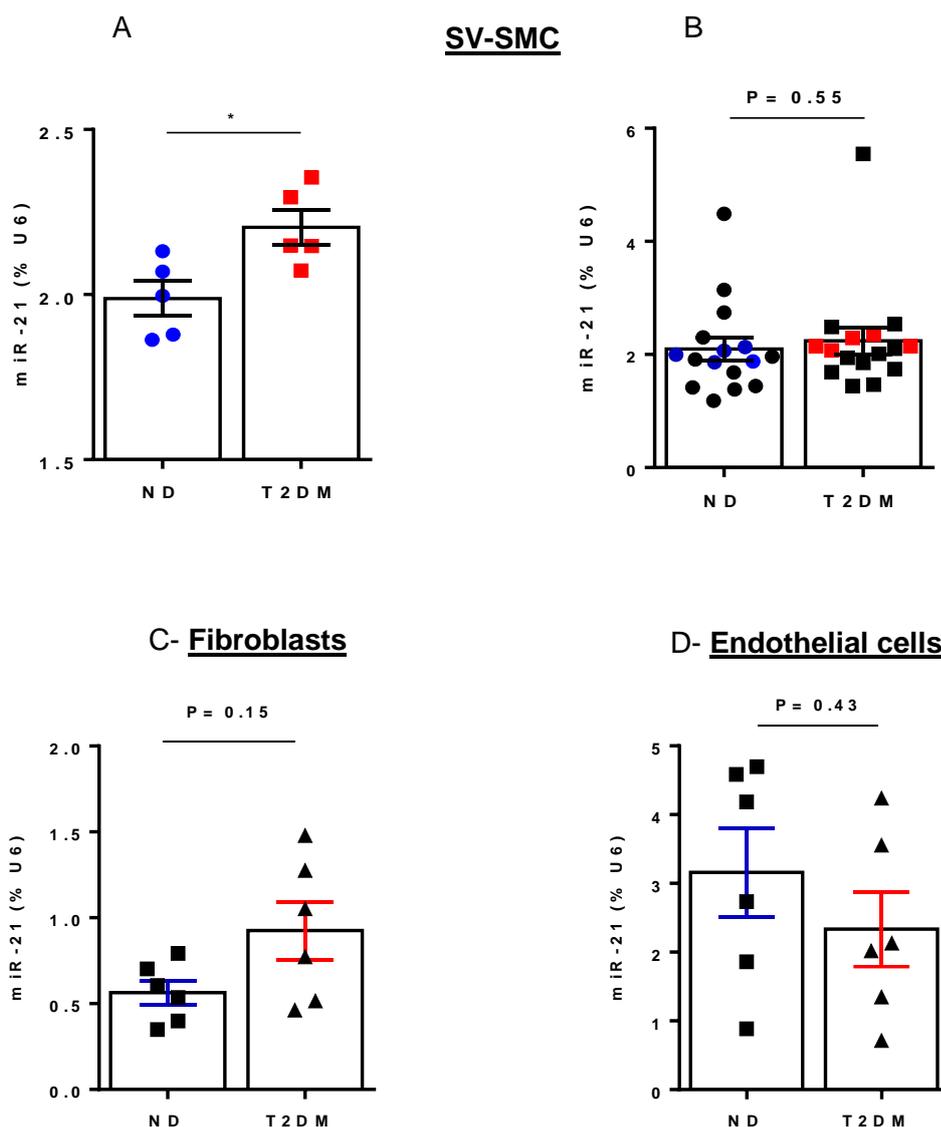


Figure 3.15: Comparison measurement of miR-21 in ND and T2DM SV-SMC, cardiac fibroblasts and endothelial cells.

RT-PCR for miR-21 expression levels in SV-SMC of T2DM compared to ND donors (A, * $P < 0.05$, $n = 5$). B- MiR-21 expression after adding the 5 samples in (A) into 11 different ND or T2DM SV-SMC samples (each $n = 16$). MiR-21 expression level in cardiac fibroblasts (C, each $n = 6$) and endothelial cells (D, each $n = 6$) obtained from T2DM relative to ND patients (unpaired t-test). Expression of miR-21 was normalised to U6 and error bars denote SEM.

3.13. Discussion

The results in this chapter have highlighted the distinct appearance of SV-SMC cultured from T2DM relative to ND donors. The diabetic cells were predominantly distinguishable by their rhomboidal shape as compared to the classical spindle shape of cells from ND counterparts and this is consistent with the previously published work from our department (Madi et al., 2009). Furthermore, cells from T2DM donors had a significantly lower proliferative rate, which was also shown by Riches et al., 2014, and increased spread cell area compared to ND counterparts. Confirmation that all cultured cells were SMC was demonstrated by immunostaining that showed co-expression of α -SMA and SM-MHC in both ND and T2DM SV-SMC. Overexpressing miR-21 in ND SV-SMC led to a significant increase in average cell area, whereas knocking down miR-21 in T2DM cells significantly reduced cell area versus negative controls.

A number of miRs have been implicated in vascular remodelling and cardiovascular diseases, such as miR-21, miR-210 and miR-34a which were implicated in human atherosclerosis (Raitoharju et al., 2011) and miR-208 and miR-499 which were involved in rat and human ischemia (Corsten et al., 2010, Fukushima et al., 2011). The outcome of the high throughput screen showed an obvious upregulation of miR-1260 in cells from T2DM patients. However, a thorough search of the literature failed to identify a potential role of miR-1260 in cardiovascular diseases. Despite miR-1260a and miR-1260b showing an approximate 300-fold increase in T2DM SV-SMC relative to ND in our pilot data (Table 3.1), there are just three published studies that focus exclusively on upregulation of miR-1260b in lung cancer cells mediating metastasis (Xu et al., 2015b), prostate cancer (Hirata et al., 2014a) and renal cancer (Hirata et al., 2014b). Another recent study proposed a possible diagnostic role for miR-1260a in asthmatic patients (Wang et al., 2015c). However, no studies have explored or confirmed the role of this miR in vascular remodelling.

Both miR-145 and miR-21 also showed high expression levels in T2DM relative to ND SV-SMC and recent evidence has supported the role of these miRs in inducing phenotypic changes of vascular SMC. Overexpression of miR-145 in (non-diabetic) SMC has proven to be involved in regulating SMC phenotypes such as mediating a reduction in the proliferation rate of rat aortic and pulmonary artery SMC (Cordes et al., 2009). Additionally, it has been confirmed by our group that T2DM SV-SMC showed elevated levels of miR-145 expression. Knocking down miR-145 in T2DM SV-SMC reverted cells into a ND phenotype and its overexpression in ND cells drove the diabetic phenotypes (Riches et al., 2014).

Emerging evidence has shown that miR-21 expression is one of the highly deregulated miRNAs in the cardiovascular system in diseases such as proliferative vascular disease, cardiac fibrosis, cardiac hypertrophy and ischaemic heart disease (Cheng and Zhang, 2010, Small and Olson, 2011), providing the cause-effect relevance between the expression level and regulation of miRNAs and cardiovascular diseases. Various studies have shown that miR-21 is significantly upregulated in most cardiovascular cell types and can mediate cardiovascular pathological problems. For example, miR-21 was found to be upregulated in vascular SMC mediating neointimal lesion formation (Ji et al., 2007), endothelial progenitor cells of atherosclerotic patients (Zuo et al., 2015), cardiomyocytes mediating cardiac hypertrophy in mice (Cheng et al., 2007) and cardiac fibroblasts inducing fibrosis (Roy et al., 2009). MiR-21 was also found to be increased 4.6-fold in atherosclerotic plaques in comparison to control human arteries (Raitoharju et al., 2011). Additionally, in a key study performed by McDonald et al. (2013), miR-21 was found to be significantly upregulated and mediated neointimal formation after vein grafting in mice. Therefore, miR-21 was selected in this project for investigating whether regulating its expression levels could alter SMC phenotypes in both ND and T2DM cells and contribute to SMC dysfunction that is relevant to vascular remodelling.

In addition to the divergent morphological features exhibited in T2DM SV-SMC, a disrupted F-actin cytoskeleton can also accompany the phenotypic changes mediated by overexpression of miR-145 (Riches et al., 2014). Overexpression of miR-21 in SV-SMC from ND donors significantly increased average cell areas by 56% (Figure 3.8). This rhomboidal shape is characteristic of the dedifferentiated SMC phenotype that was found to be dominant in atherosclerosis formation and has also been known to augment neointima thickness (Hao et al., 2003). However, this was not accompanied by F-actin fragmentation or disruption.

On the contrary, knocking down miR-21 in SV-SMC from T2DM donors reduced the average cell area to be more like a ND cell with a differentiated phenotype (Figure 3.10). These changes in cell area mediated by miR-21 manipulation underline the important role of miR-21 expression in cell morphology. As SMC compose the majority of cells in the blood vessel wall, the accumulation of these cells in the intima is greatly linked to blood vessel occlusion and restenosis following vascular injury. T2DM is considered as a major risk factor for cardiovascular diseases and contributes to poor prognosis, developing in-stent restenosis (Berry et al., 2007), reduction in long-term survival outcome and unfavourable consequences after SV revascularisation in CABG surgery (Kubal et al., 2005). This increase in SMC area induced by miR-21

may be important in T2DM or it could play an important role in driving phenotypic changes similar to these seen in T2DM or dedifferentiated SV-SMC phenotypes. Therefore, miR-21 could be the driving cause behind phenotypic changes such as the rhomboidal SMC shape associated with T2DM vascular SMC.

Having observed the significant upregulation of miR-21 using 30 nM premiR-21 (Figure 3.5), a series of optimisation experiments were carried out to achieve robust miR-21 knockdown in native SV-SMC. Because miR-21 has been suggested to be expressed at high levels in pathological conditions we decided to enhance the miR-21 (i.e. overexpress) and follow this with a reduction. The knockdown could not be detected unless we artificially overexpress miR-21 in which we obtained a 95% reduction (Figure 3.6).

Studies have shown a high correlation between diabetes and matrix alterations that is involved in poor wound healing (Brem and Tomic-Canic, 2007), vascular stiffness (Henry et al., 2003) and dysfunctional remodelling (Chow et al., 2013). Diabetes is a major risk factor that augments expression of ECM proteins in different organs (Law et al., 2012). The increased matrix protein cross-linking that is seen in T2DM enhances scaffold stiffness in both radial and circumferential directions in diabetic rats compared to ND (Chow et al., 2013). Thus, changes in ECM-related genes in vascular SMC can play a crucial role in cellular phenotypes including the process of SMC proliferation and migration which can lead to atherosclerosis or restenosis following CABG surgery. Collagen provides the main structural integrity to tissue and represents the major ECM component of the atherosclerotic plaque (Kong et al., 2013). Studies have shown that disturbances in collagen metabolism in diabetic patients contribute to poor wound healing (Knas et al., 2015). T2DM patients exhibited an increased deposition of matrix collagen in arteries and this was contributed to the downregulation of MMPs (Chung et al., 2007). Both MMP2 (gelatinase A) and MMP3 (stromelysin 1) play a major role in ECM degradation and collagen deposition. It has been shown that hyperglycaemia enhanced MMP2 mRNA expression and activity in rat aortic SMC (Hao and Yu, 2003). A study using human umbilical vein EC showed that hyperglycaemia induced upregulation of MMP2 and downregulation of MMP3 (Death et al., 2003). Moreover, as MMP3 has a high ability to degrade collagen (Chung et al., 2007), these two genes were selected to test their levels in SV-SMC of diabetic patients and whether their differential level contributed to collagen variation in diabetes relative to ND. Collagen, MMP2 and MMP3 are known to be involved in vascular restenosis and neointimal formation, surprisingly, our data revealed that the levels of collagen 1A1, MMP2 and MMP3 mRNA expression in SV-SMC were

comparable between the two populations (Figure 3.12). It could be that these genes levels are normalised into their basal levels in culture and their levels become similar to the ND sample because they are deprived of the diabetic milieu.

However, of particular interest, we did note a positive correlation between miR-21 and COL1A1 expression level in ND SV-SMC that was not observed in cells obtained from T2DM donors, suggesting that this correlation is lost. A study by Li et al. (2011) demonstrated that the upregulation of miR-21 was positively linked with collagen 1 accumulation in lung fibrogenesis. Studies have shown that miRs can regulate the expression of multiple collagens (Ji et al., 2010), therefore, we manipulated miR-21 expression levels in ND SMC to determine whether this could modulate COL1A1 expression. However, neither overexpressing nor knocking down miR-21 in ND-SMC affected COL1A1 expression (Figure 3.14). This confirms that COL1A1 is not directly and solely regulated by miR-21, but by other genes. The metabolic abnormalities associated with T2DM, including hyperglycaemia, hyperinsulinaemia, hyperlipidaemia and increased protein glycation perhaps modified SMC response to collagen and disturbed the positive association between miR-21 and collagen 1A1 levels. As a result of the metabolic disturbance in T2DM, expression of growth factors is also dysregulated (Aronson et al., 1996). This could also contribute to phenotypic changes in SMC leading to the vanished response observed in miR-21 correlation to collagen expression. Following on from this, the exact missing link in diabetes needs further investigation. Additionally, it is still unclear what the significance of this relationship is and therefore, further studies are required to determine its importance.

T2DM is a chronic metabolic disease and growing evidence has suggested that dysregulated miR expression is implicated in the pathogenesis of T2DM. MiRs have been shown to be upregulated in adipose tissue, liver and pancreatic beta cells of T2DM patients (McCullough et al., 2010). Furthermore, aberrant expression of miRs is implicated in several vascular pathologies including atherosclerosis, CAD, vascular inflammation, and other diabetic vascular complications such as the microvascular diseases (Arunachalam et al., 2015, Athyros et al., 2015). The early metabolic milieu may induce modulation in gene expression and aberrancies of vascular SMC function, which negatively affect SMC plasticity and contribute to poor outcome after CABG in T2DM patients (Hakala et al., 2005). Our initial experiment shown in Figure 3.1 revealed a 60% increase in miR-21 expression in SV-SMC of T2DM relative to ND when a small population sample was used (n= 5 per group). The present study has shown that this difference in miR-21 expression between the two populations was indistinguishable when the sample size was increased to 16. Additionally, a power

calculation of sample means revealed that to obtain 80% power of detecting a 50% difference between these 2 groups we needed a total of 11 per group. Therefore, the initial experiment on 5 samples was underpowered. A total of 16 samples in this assay were powered enough to conclude that there was no significant relationship between miR-21 expression in SV-SMC and diabetic status.

There is a high incidence of mortality and morbidity from cardiovascular complications following CABG surgery in diabetic patients (Berry et al., 2007). As miRs have a different expression profile in different tissues (Small and Olson, 2011) and after finding no difference in miR-21 expression in SV-SMC between T2DM and ND patients, the expression of miR-21 in other cardiovascular cell types was tested; namely cardiac fibroblasts and endothelial cells. These cell types, in addition to SMC, have an important role in inducing vascular restenosis after injury and cardiac fibrosis. The result showed comparable levels of miR-21 expression in both fibroblasts and endothelial cells between the two populations.

This chapter has highlighted an important role for miR-21 in SMC function as miR-21 mediated differentiation changes in SV-SMC by inducing a significant increase in cell area that is partially characteristic to the dedifferentiated phenotype. However, we did not observe any differences in miR-21 expression between T2DM and ND SV-SMC. Therefore, all of the experiments conducted in the subsequent chapters were performed only on SV-SMC from non-diabetic patients.

Chapter 4

Factors modulating miR-21 expression and identification of cardiovascular target genes in human ND SV-SMC

4.1. Introduction

Neointimal lesion formation in proliferative vascular disease shares similar molecular mechanisms with cancers, as both are characterised by excessive cell proliferation and migration (Owens et al., 2004). Given the abundance of research demonstrating the role of miRs in the development of cancer, it has been hypothesised that miRs could also play an important role in neointimal lesion formation after revascularisation; however, relatively few studies have been conducted to reveal the precise role of miRs in vascular remodelling. The level of miR expression is highly affected by the severity of diseases and their expression is tissue-specific (Cheng and Zhang, 2010). Indeed, one miR may be highly expressed in one tissue type, but have low or no expression in other tissues (Small and Olson, 2011). This will assign the potential role of a specific miR according to cell type or pathophysiological situation. Neointimal progression and vascular diseases may therefore potentially be resolved by normalising miR expression and preventing phenotypic modulation in a manner that may be detrimental.

The high induction of miR-21 in the vast majority of cancers (Selcuklu et al., 2009b), fibrosis (Thum et al., 2008) and cardiac disorders (Cheng and Zhang, 2010) has attracted the attention of researchers in an effort to assign potential mechanistic functions of miR-21 in the context of cardiac remodelling, atherosclerosis, aneurysms and restenosis injury (McDonald et al., 2013). It has been documented that miR-21 is significantly upregulated in different types of human tissues and diseases, acting through numerous targets and signalling mechanisms (Krichevsky and Gabriely, 2009). MiR-21 was one of the significantly upregulated miRs in human atherosclerotic plaque compared with healthy individuals and multiple target genes were downregulated (Raitoharju et al., 2011). However, further investigations to identify the precise role of miR-21 in T2DM and vascular diseases, and to ascertain the underlying mechanisms driving its upregulation, are challenging to uncover. In an experimental study, using a mouse insulin-secreting cell line (MIL6) treated with proinflammatory

cytokines; miR-21 was among the most highly expressed miRs and its subsequent blockade protected β -cells from death caused by cytokines (Roggli et al., 2010, Bravo-Egana et al., 2012). Therefore, cytokine-induced miR over production and the consequent recruitment of inflammatory mediators together with the diabetic milieu could be predicted to contribute to diabetic cardiovascular complications.

Following vascular injury and bypass grafting, the initial response to injury lies at the level of endothelial dysfunction, inflammation and platelet adhesion (Zernecke and Weber, 2010). This stimulates secretion of various growth factors such as PDGF and TGF- β , cytokine secretion (e.g., interleukins and TNF- α) and degradation and deposition of the ECM (Chung et al., 2007). When this exceeds the level required for healing and adaptation to the new environment of arterial haemodynamics, it can lead to uncontrolled SMC proliferation and migration towards the intima. This may lead to intimal hyperplasia, restenosis and subsequently, graft failure (Lafont and Faxon, 1998, Chen et al., 2012).

TGF- β has been shown to control miR-21 biosynthesis at the post-transcriptional level in human pulmonary artery SMC (Davis-Dusenbery and Hata, 2010); and treating SV-SMC with TGF- β led to a 2.5-fold increase in miR-143/145 after 48 h (Riches et al., 2014). PDGF-BB is among the growth factors known to be upregulated in response to vascular injury and following angioplasty (Sun et al., 2012b). It is also involved in tumour diseases and is a crucial stimulator of growth and motility in many cell types including EC, fibroblasts and SMC (Board and Jayson, 2005, Alvarez et al., 2006). PDGF-BB is known to induce dysregulation of miR expression in different cell types; inducing down-regulation of miR-145 and upregulation of miR-221 and -222 in vascular SMC (Cheng et al., 2009a), increasing miR-21 expression in mouse glioma cells (Polajeva et al., 2012) and increasing miR-21 expression in human hepatic cell lines (Wei et al., 2013). PDGF activates two well-characterised signalling pathways, mitogen-activated protein kinase extracellular regulated kinase 1/2 (MAPK/ERK1/2) and phosphatidylinositol 3-kinase/protein kinase B pathway (PI3K/Akt) (Xu et al., 2015a).

The expression of miRs in SMC is controlled by a number of different factors. As inflammatory cytokines and other diabetic mediators are elevated in the circulation of diabetic patients we sought to identify if any of these factors mediated the proposed increase in miR-21 expression observed in T2DM SV-SMC. In the present study, we selected various stimuli known to be altered in T2DM, such as IL-1 α , TNF- α , glucose, insulin, TGF- β , PDGF-BB and combinations thereof, and measured the effect of these on ND SV-SMC miR-21 expression.

The aims of this chapter were:

- To explore the ability of diabetic stimuli and other inflammatory mediators to modulate miR-21 expression in ND SV-SMC
- To investigate the downstream target genes affected by miR-21 upregulation in SV-SMC.

4.2. Effect of interleukin-1 α exposure on miR-21 expression in ND SV-SMC

IL-1 α is a proinflammatory cytokine that is usually elevated alongside other diabetic mediators, in the blood of T2DM patients and in various CVDs (Ruscitti et al., 2015). Initial time course experiments were conducted on ND SV-SMC that were cultured in 6 well plates (120,000 cells/ well) in FGM for 24 h. To determine the time point at which miR-21 expression was most significantly modulated by acute IL-1 α exposure, cells were quiesced in SFM prior to the addition of 10 ng/ml IL-1 α . Cells were then trypsinised, harvested and pelleted at intervals between 2 and 24 h and RNA extracted to measure miR-21 expression using RT-PCR (as described in Section 2.2.9). Interleukin-1-treated cells showed a decline in miR-21 expression at all time points compared to untreated control collected at time zero (Figure 4.1A). There was a significant reduction in miR-21 expression between 4 and 8 h ($P < 0.05$) and the maximum drop observed relative to control was at 6 h ($P < 0.01$, $n = 4$). This demonstrated the negative response mediated by IL-1 α on the expression of miR-21.

In another set of experiments, to compare the effect of short and long-term exposure of SV-SMC to IL-1 α , SV-SMC from 3 different donors were exposed to IL-1 α for either: a short time (4 h) prior to replenishment with SFM or continuous exposure to IL-1 α . Cells were harvested at 24, 48 and 72 h alongside untreated cells which had been maintained in parallel in MGM containing vehicle (0.1% BSA) and used as a control at each time point. Short-term exposure to IL-1 α caused a significant downregulation in miR-21 at 48 h ($P < 0.01$, Figure 4.1B), whilst continuous exposure to IL-1 α induced a reduction in miR-21 expression at an earlier time (24 h), but no comparable changes at later time points (Figure 4.1C). This suggested that the effect of IL-1 α on miR-21 is transient and lasts up to 48 h.

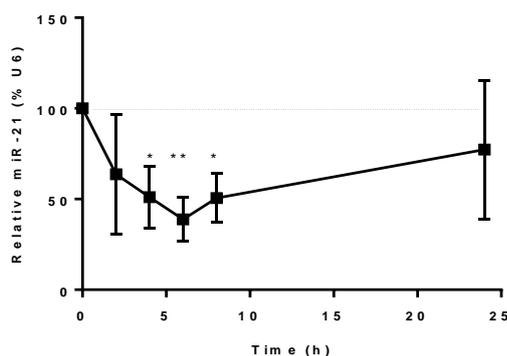
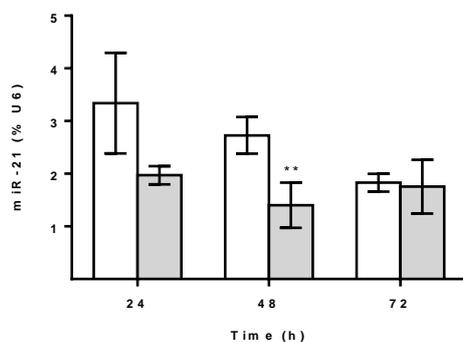
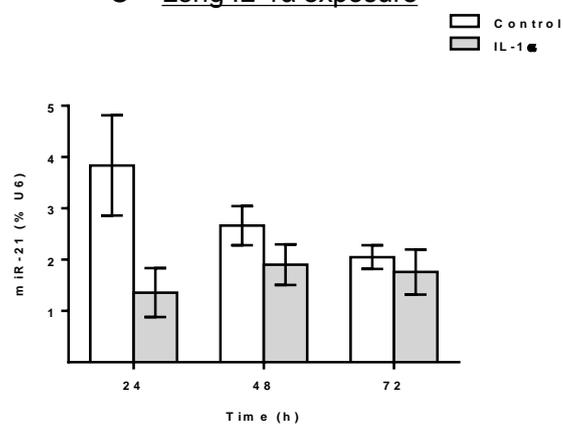
A- Acute IL-1 α exposureB- Short IL-1 α exposureC- Long IL-1 α exposure

Figure 4.1: MiR-21 expression in ND SV-SMC exposed to IL-1 α treatment.

RT-PCR for miR-21 expression in SV-SMC exposed to 10 ng/ml of IL-1 α . A. Effect of IL-1 α on miR-21 expression over a time course up to 24 h (* P < 0.05, ** P < 0.01, n = 4). B. Effect of short (4 h) IL- α treatment on expression of miR-21 at time points 24-72 h (** P < 0.01, n = 3). C. Effect of continuous treatment with IL-1 α from 24-72 h on miR-21 expression (n = 3). MiR-21 transcript levels were normalised to the endogenous control, U6 and error bars denote SEM; Newman-Keuls multiple comparisons test after One way ANOVA.

4.3. Effect of diabetic stimuli on miR-21 expression in ND SV-SMC

Most of the complications of T2DM are related to the cardiovascular system. Diabetes is considered a major risk factor for cardiovascular diseases and contributes to unfavourable consequences for CABG (Rong et al., 2016). Thus, we next investigated whether miR-21 expression could be modulated by “classical” diabetic mediators. SMC from five different ND donors were seeded in 6 well plates after being cultured in physiological low levels of glucose media (5.5 mM) for 14 days. Cells were then exposed to one of 5 different treatments over time points from 6 – 48 h in MGM containing (1) high glucose (25 mM), (2) 100 nM insulin, (3) IL-1 α and TNF- α (both 10 ng/ml), (4) 10 ng/ml of TGF- β or (5) a combination of high glucose with insulin, IL-1 α and TNF- α . Paired cells were cultured in parallel in low-glucose MGM as a control. At each time point, cells were pelleted and RNA extracted to measure miR-21 expression by RT-PCR. None of the diabetic stimuli (Figure 4.2A/B) and/or inflammatory mediators (Figure 4.3A/B) or the combination (Figure 4.3C) at the concentrations tested were capable of inducing significant changes in miR-21 expression levels as compared to their relevant controls.

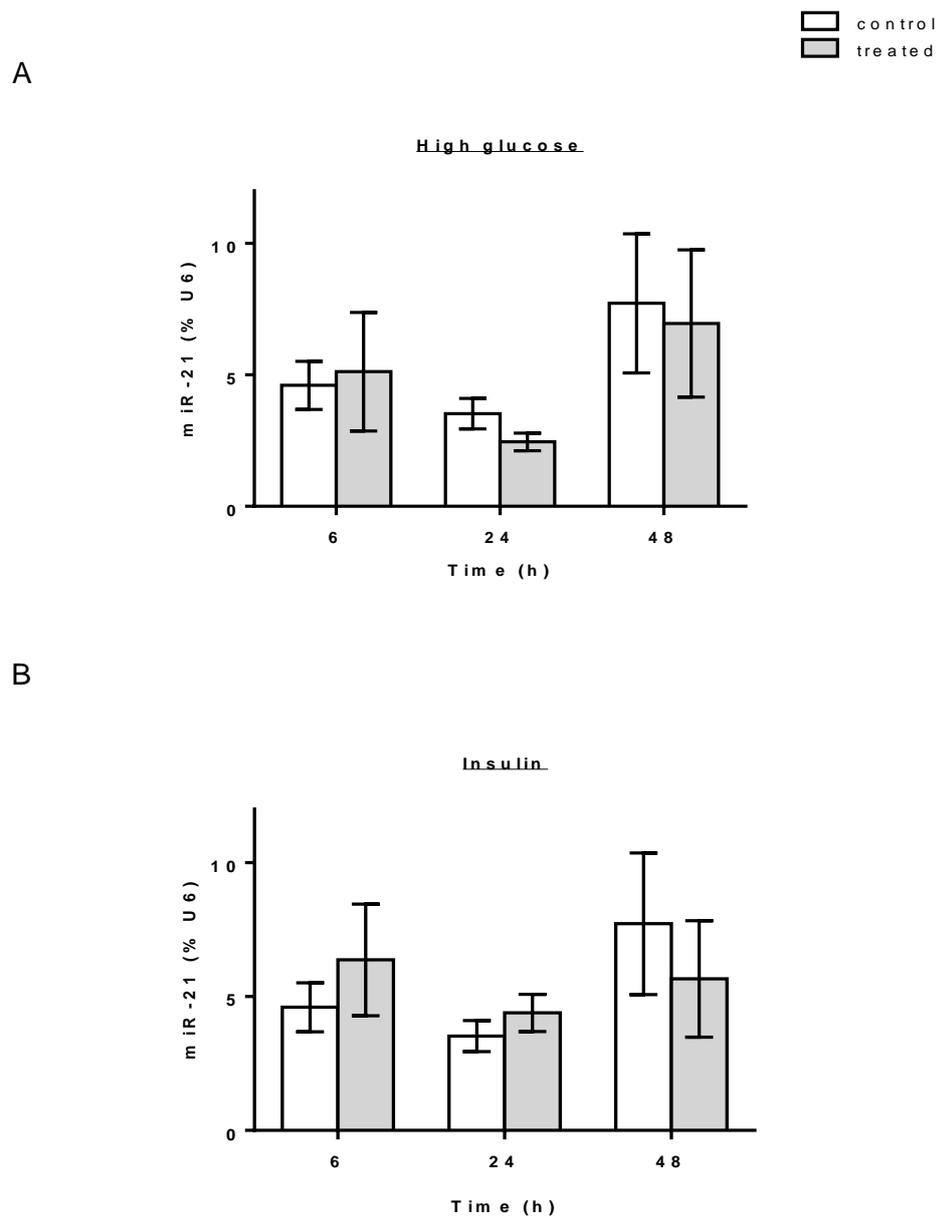


Figure 4.2: Effect of diabetic stimuli on miR-21 expression level in ND SV-SMC. RT-PCR of miR-21 expression in SV-SMC treated with 25 mM high glucose (A) or 100 nM insulin (B) for 6, 24, or 48 h. (n= 5, One way ANOVA and Newman-Keuls multiple comparisons test). MiR-21 transcript levels were normalised to the endogenous control U6 and error bars denote SEM.

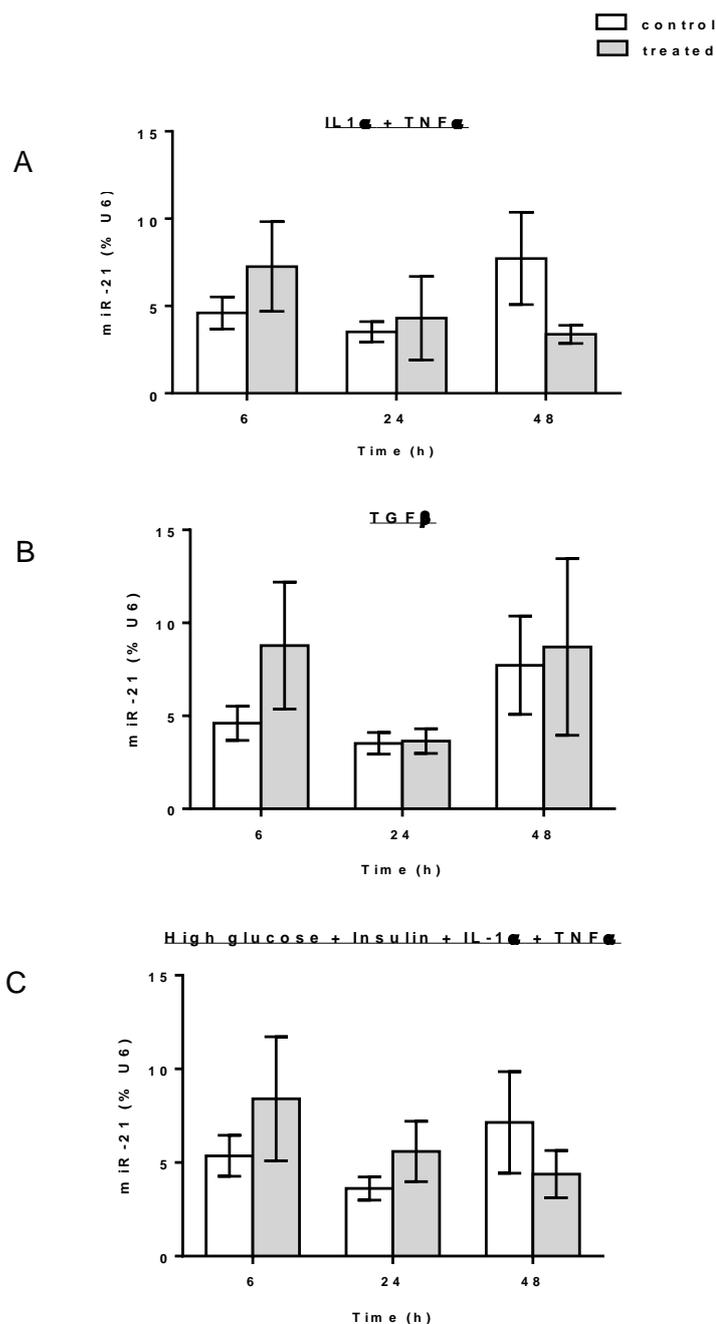


Figure 4.3: Effect of inflammatory mediators and diabetic stimuli on miR-21 expression level in ND SV-SMC.

RT-PCR of miR-21 expression in SV-SMC treated with 10 ng/ml of IL-1 α and TNF- α (A), 10 ng/ml of TGF- β (B) or combination of high glucose (25 mM), insulin (100 nM), IL-1 α and TNF- α (C) for 6, 24 or 48 h. (n= 5, One way ANOVA and Newman-Keuls multiple comparisons test). MiR-21 transcript levels were normalised to the endogenous control U6 and error bars denote SEM.

4.4. Effect of PDGF-BB exposure on miR-21 expression in ND SV-SMC

Other mediators known to be involved and upregulated in diabetes and vascular remodelling include the chemoattractant and mitogen PDGF-BB (Levrán and Hwang, 1990). To test its effect on regulation of miR-21 expression level, PDGF-BB was utilised in another set of experiments, in which SV-SMC were seeded in 6 well plate in FGM and incubated for 72 h. Cells were serum starved for 24 h and then stimulated with 1 or 10 ng/ml PDGF over a time course up to 72 h. Paired cells exposed to the vehicle (0.1 M acetic acid and 1% BSA in PBS) were cultured in parallel as controls. At each time point, cells were pelleted and RNA extracted for measurement of miR-21 expression by RT-PCR. Exposure of SV-SMC for 24 h to both low and high concentrations of PDGF led to approximately 60% and 30% increase in miR-21 expression, respectively (Figure 4.4A). By prolonging the exposure time to 48 and 72 h, both concentrations of PDGF-induced an 80% increase in miR-21 expression that reached statistical significance as compared to their respective controls (Figure 4.4B/C, $P < 0.05$, $P < 0.01$, $n = 5$).

4.5. Effect of PDGF-BB signalling inhibition on miR-21 expression in SV-SMC

Having demonstrated that miR-21 expression was elevated by PDGF-BB, we proceeded to investigate whether the PDGF signalling pathway was involved in this upregulation. Phosphorylation of Akt and ERK1/2 by PDGF was determined by using phospho-specific and expression antibodies. In order to confirm the effectiveness and specificity of PDGF signalling pathway inhibitors, SV-SMC were seeded in FGM and serum starved for 24 h. Cells were then incubated for 1 h with/without 10 μ M LY294002 (Akt inhibitor) or with/without 30 μ M PD98059 (ERK1/2 inhibitor) in MGM prior to 20 minutes stimulation with 1 ng/ml PDGF. Control groups were cultured alongside having been exposed to the relevant vehicle controls: DMSO with LY or PD and (0.1 M acetic acid with 1% BSA in PBS) with PDGF-BB. Cell lysates were prepared and protein was separated using SDS-PAGE 10% polyacrylamide gel and transferred into PVDF membrane. Membrane was then probed with the particular antibody as described in Section 2.2.11 and phospho- Akt and phospho- ERK1/2 protein levels were normalised to Akt and ERK1/2 expression, respectively. Western blots showed that PDGF-induced phosphorylation of Akt and ERK1/2 pathways

(Figure 4.5A/B) and that both Akt (Figure 4.5A) and ERK1/2 (Figure 4.5B) inhibitors selectively inhibited PDGF-induced phosphorylation of these two pathways.

Having shown that 48 h treatment with 1 ng/ml PDGF was sufficient to upregulate miR-21 expression, these conditions were utilised to test the effect of PDGF-BB in the presence of signalling pathway inhibitors. Cells were incubated with PD98059 and LY294002 for 1 h prior to the addition of PDGF for 48 h. Control cells were incubated with the inhibitors alone in parallel. Cells were pelleted and RNA extracted to measure miR-21 expression by RT-PCR. PDGF significantly upregulated miR-21 expression compared with control ($P < 0.05$) and the expression level of miR-21 was significantly reduced ($P < 0.05$) when either Akt or ERK1/2 signalling pathway was blocked as compared to cells treated with PDGF alone (Figure 4.5C), confirming that both pathways are important for PDGF-induced miR-21 upregulation.

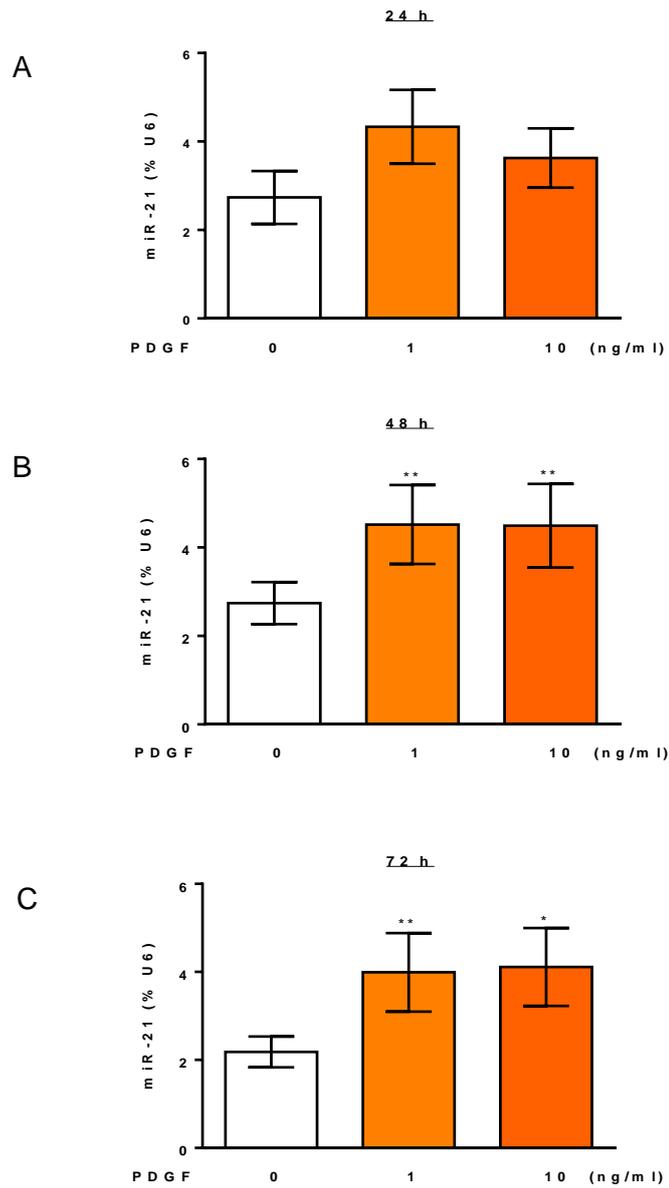


Figure 4.4: Effect of PDGF-BB on miR-21 expression level in ND SV-SMC.

RT-PCR of miR-21 expression in SV-SMC treated with either 1 or 10 ng/ml PDGF-BB and compared to vehicle-treated controls (0.1 M acetic acid and 1% BSA in PBS). Cells were incubated with PDGF for 24 h (A), 48 h (B) or 72 h (C). (n= 5, *P< 0.05, **P< 0.01, One way ANOVA and Newman-Keuls multiple comparisons test). MiR-21 transcript levels were normalised to the endogenous control U6 and error bars denote SEM.

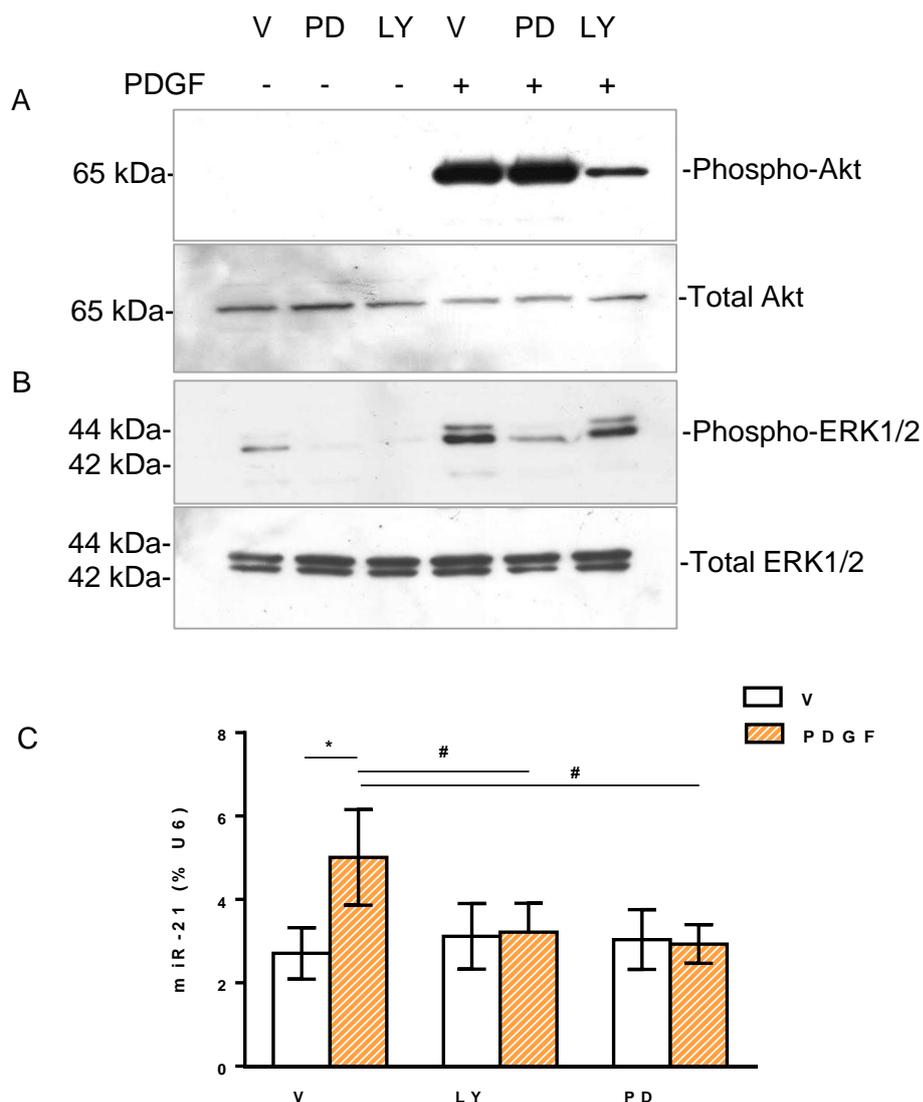


Figure 4.5: Representative blot of the effect of selective inhibition of Akt and ERK1/2 on PDGF-BB-induced phosphorylation of Akt and ERK1/2 and the effect of their inhibition on miR-21 expression in SV-SMC.

Representative blot of Akt (A) and ERK1/2 (B) phosphorylation by PDGF-BB in SV-SMC. Signalling pathways of 1 ng/ml PDGF were blocked by 10 μ M of LY294002 (Akt inhibitor) or 30 μ M PD98059 (ERK1/2 inhibitor), compared with vehicle (V, 0.1 M acetic acid and 1% BSA in PBS, or DMSO). (C) Effect of PDGF-BB signalling inhibition on miR-21 expression in SV-SMC. (n= 5, *P< 0.05 vs vehicle control, #P< 0.05 vs PDGF-BB, One Way ANOVA and Newman-Keuls multiple comparisons test). MiR-21 transcript levels were normalised to the endogenous control U6 and error bars denote SEM.

4.6. Human atherosclerosis microarray

Having identified PDGF-BB as a positive stimulator of miR-21 and owing to the pertinent role of PDGF-BB as a mediator known to be involved in atherosclerosis and vascular remodelling, we sought to determine the potential target genes mediating cardiovascular disorders that were affected by miR-21 upregulation by using a human atherosclerosis specific microarray. RNA samples of premiR-21 and premiR-negative (control) transfected SV-SMC of 4 different ND donors were analysed with a human atherosclerosis RT-PCR array. The array enables the investigation of 84 different genes involved in atherosclerosis and cardiovascular diseases and it was performed according to the manufacturer's instructions as described in method Section 2.2.10 and the genes included are listed in Table 2.2. The pattern of gene expression distribution affected by miR-21 overexpression is presented in a volcano scatter plot that displays the statistical significance versus fold change (Figure 4.6). Fold-change values greater than one indicate genes which are upregulated whilst fold-change values less than one indicate genes which are downregulated. Out of the 84 genes, 10 genes had a fold change $\geq \pm 1.5$ and these are listed in Table 4.1.

The genes that showed the highest fold changes were MMP1 and IL1A. MMP1, MMP3, IL1R2, PPARG and ITGB2 displayed ≥ 1.5 fold increase in the premiR-21 transfected cells relative to control SV-SMC whilst IL1A, KDR, BIRC3, VWF and IL5 showed > 1.5 fold decrease in the premiR-21 transfected cells relative to control cells. Fold change and P values for the 10 genes analysed by ratio paired t-test are presented in Table 4.1.

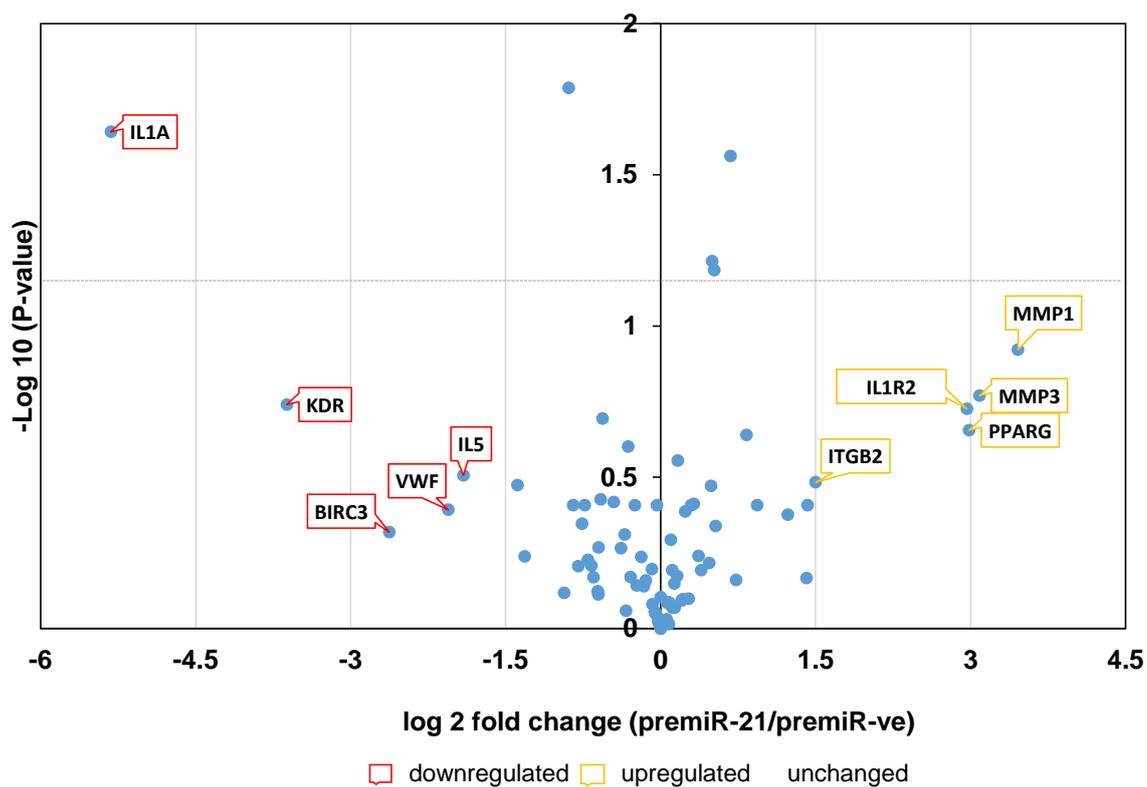


Figure 4.6: Volcano plot of atherosclerosis array data of premiR-21 versus premiR-negative transfected ND SV-SMC.

The difference in expression level on log₂ scale indicated in the x-axis and the y-axis indicates statistical significance in -log₁₀ scale. Values located above the dotted horizontal line indicating P value of < 0.05 (ratio paired t-test). Only genes that had ≥ +/-1.5 fold changes are labelled. Genes that are upregulated (yellow,) and downregulated (red) in RNA samples from SV-SMC transfected to overexpress miR-21 (using 30 nM premiR-21) 72 h after transfection compared to premiR-negative transfected SV-SMC, using RT² Profiler human atherosclerosis PCR microarray (n= 4 in each group).

Table 4.1 Human atherosclerosis array genes log 2 fold change \geq +/- 1.5 (up or down regulated) and P-values as a result of miR-21 overexpression relative to premiR-negative in SV-SMC.

| | Gene | Log 2 fold change (treated/control) | P-value Ratio paired t-test |
|-----------|-------------|------------------------------------------------|----------------------------------------|
| 1 | MMP1 | 3.46 | 0.12 |
| 2 | MMP3 | 3.08 | 0.17 |
| 3 | PPARG | 2.99 | 0.22 |
| 4 | IL1R2 | 2.96 | 0.19 |
| 5 | ITGB2 | 1.5 | 0.33 |
| 6 | IL5 | -1.91 | 0.34 |
| 7 | VWF | -2.06 | 0.41 |
| 8 | BIRC3 | -2.62 | 0.49 |
| 9 | KDR | -3.62 | 0.18 |
| 10 | IL1A | -5.32 | 0.02 |

The 10 genes that showed a fold change $\geq \pm 1.5$ in the premiR-21 relative to premiR-negative transfected SV-SMC were grouped according to function (Figure 4.7). RT-PCR analysis of the individual functional groups was also performed to examine the individual gene relative quantification and P values within each group. Four genes (IL1A, IL5, IL1R2 and ITGB2) were classified in immunity and inflammation category, 3 genes were involved in cell growth and proliferation (BIRC3, KDR and PPARG), 2 genes (MMP1 and MMP3) mediated ECM degradation and one gene (VWF) was involved in platelet adhesion and haemostasis (Figure 4.8). However, the result was variable and only the IL1A gene had a statistically significant reduction in premiR-21 transfected SV-SMC ($P < 0.05$).

Given the degree of variability and the limitations of the array of only having one primer set per well for each gene and the non-specific property of SYBR Green, the results were validated using specific TaqMan probes and RT-PCR using RNA samples from six different patients, including the four samples that were analysed on the array. RT-PCR of IL1A mRNA expression revealed a slight but non-significant reduction in the premiR-21 relative to the premiR-negative transfected control SV-SMC (Figure 4.9A). On the contrary, there was a significant increase in MMP1 expression in the premiR-21 versus control cells ($P < 0.05$, Figure 4.9B).

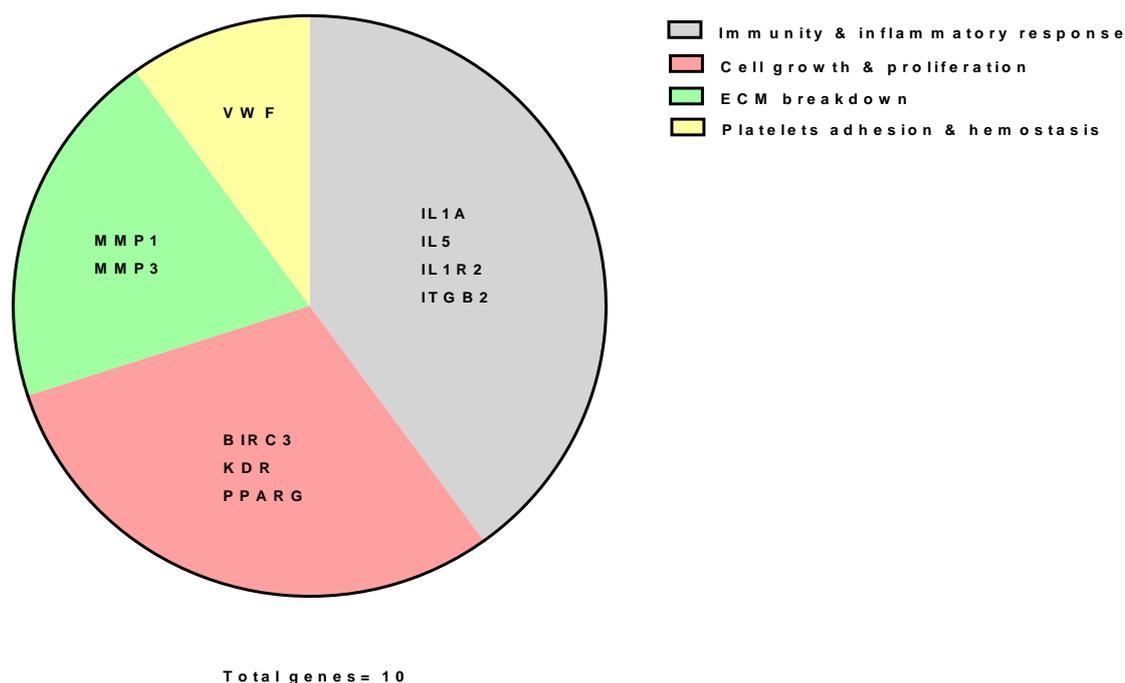


Figure 4.7: Pie chart of the genes affected by premiR-21 transfection using human atherosclerosis array organised according to gene function.

A pie chart showing the distribution of genes of SV-SMC transfected with 30 nM premiR-21 or premiR-negative using the human atherosclerosis array (n= 4). When the results were classified according to functional groups 40% of the genes were involved in immunity and inflammation, 30% modulate cell growth and proliferation, 20% mediate ECM degradation and 10% are involved in platelet adhesion and haemostasis.

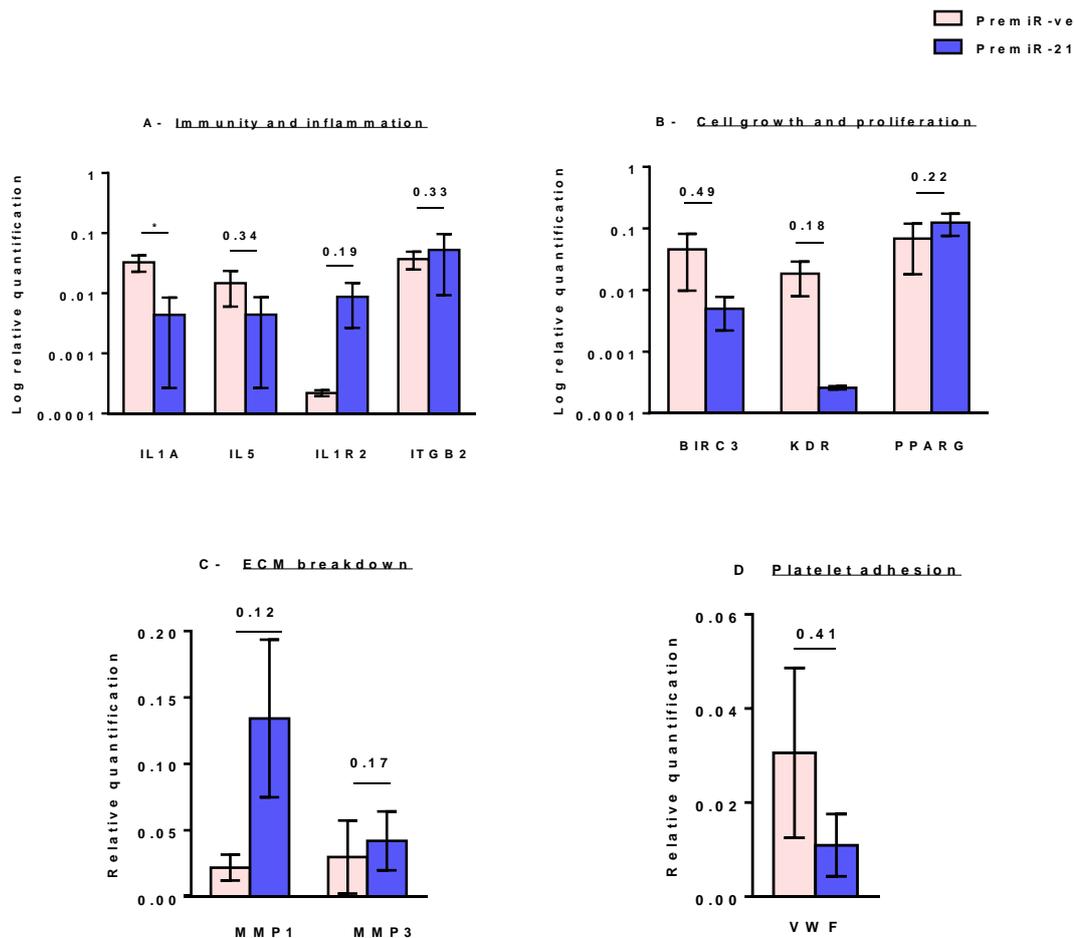


Figure 4.8: Effect of miR-21 overexpression on genes modulating immunity and inflammation, cell growth and proliferation, ECM breakdown and platelet adhesion using human atherosclerosis array.

Relative quantification in the premiR-21 relative to premiR-negative (control) transfected SV-SMC, using a human atherosclerosis array. Ten genes showed a fold change $\geq \pm 1.5$. The 10 genes are distributed in either immunity and inflammation, cell growth and proliferation, ECM breakdown or platelet adhesion ($n=4$, $*P < 0.05$, ratio paired t-test, error bars denote SEM). Note the log scale on the y-axis of figures A and B.

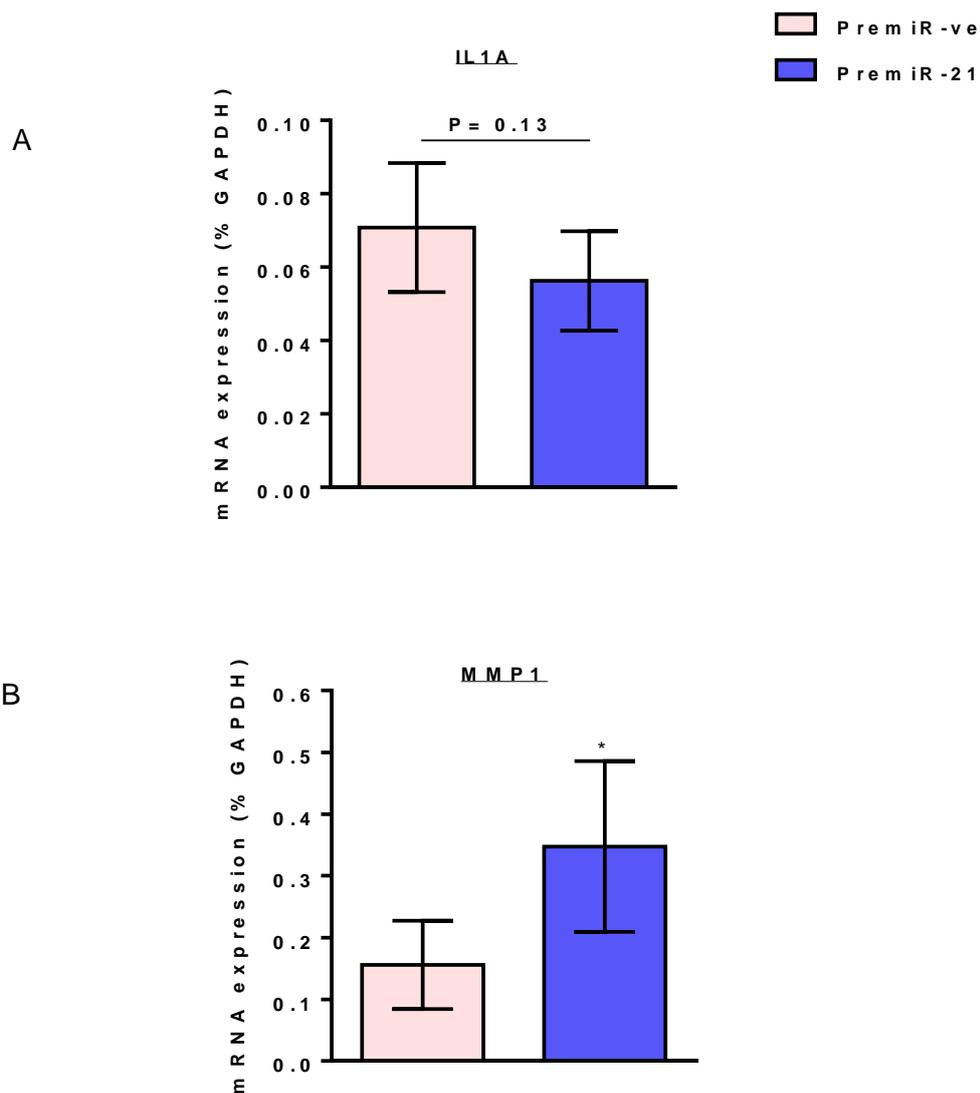


Figure 4.9: Effect of miR-21 overexpression on IL1 α and MMP1 mRNA expression in SV-SMC.

RT-PCR of IL-1A (A) and MMP1 (B) mRNA expression in SV-SMC transfected with 30 nM premiR-21 or premiR-negative (control). Both n= 6, *P< 0.05, ratio paired t test. Transcript levels were normalised to the endogenous control GAPDH and error bars denote SEM.

A number of the other genes that are linked to cardiovascular disorders and showed fold change $> \pm 2$ on the array were also followed up using RT-PCR. PPARG showed a 3-fold increase on the array in the premiR-21 relative to premiR-negative transfected (control) cells, however, its RT-PCR data showed no significant difference between the two groups (Figure 4.10A). Array data for KDR also revealed a 3.6 fold reduction in the premiR-21 transfected cells relative to control, and its RT-PCR follow-up showed a variable response (Figure 4.10B). Besides MMP1, other matrix modulating enzymes were also tested. MMP3 array data revealed a 3.1-fold increase in the premiR-21 transfected cells relative to control, however by using RT-PCR, there was a moderate 30% reduction in MMP3 expression in premiR-21 transfected cells (Figure 4.10C). Additionally, MMP2 (which was not included in the array) expression in the RT-PCR showed a slight increase in the premiR-21 transfected cells relative to control (Figure 4.10D), however, due to the high degree of variation between samples neither of these RT-PCR results reached statistical significance.

Therefore, having thoroughly studied by RT-PCR the genes highlighted to be modulated by miR-21 overexpression and finding a high degree of variability and no significant differences the previously discussed genes were eliminated from further assessment. Lastly, owing to the significant upregulation of MMP1 by miR-21 overexpression and its pertinent role in ECM degradation and vascular remodelling, MMP1 was selected for follow-up examination to study its mechanistic role in relation to miR-21 manipulation.

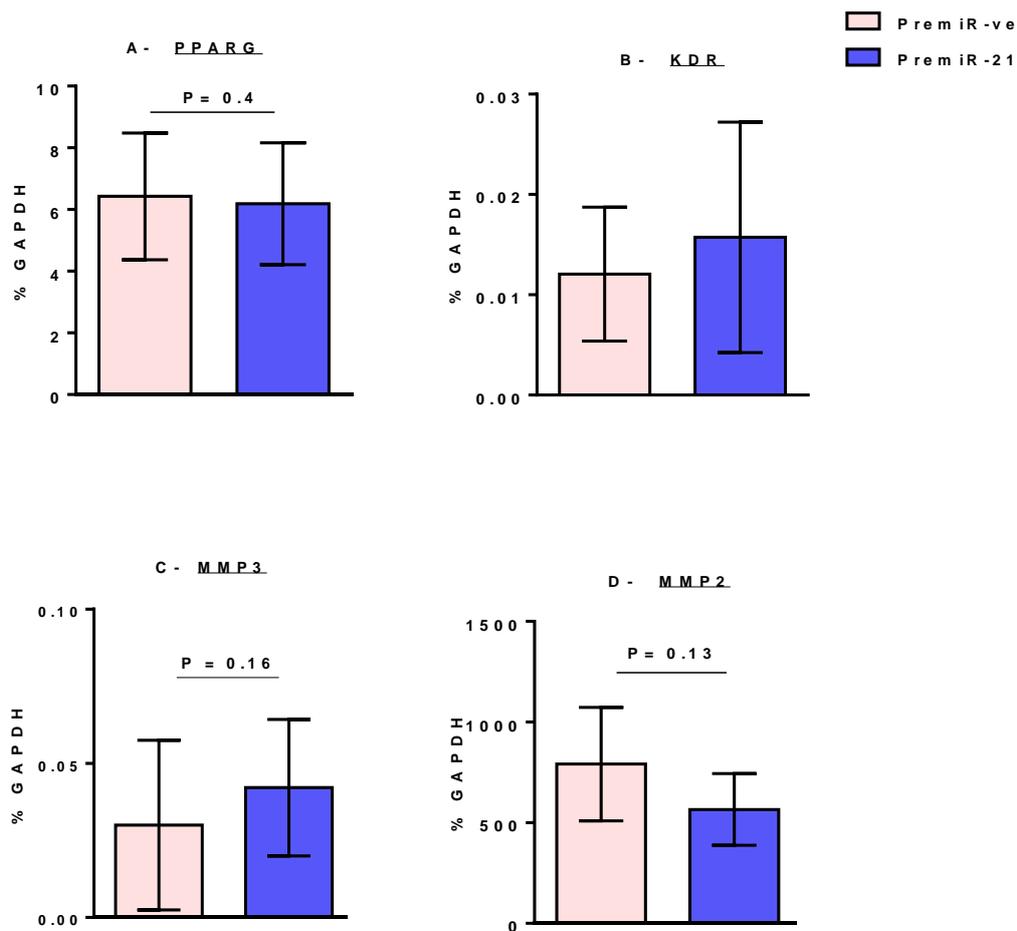


Figure 4.10: Effect of miR-21 overexpression on PPARG, KDR, MMP3 and MMP2 expression in SV-SMC.

RT-PCR of PPARG (A, n= 6), KDR (B, n= 2), MMP3 (C, n= 4) and MMP2 (D, n= 6) mRNA expression in SV-SMC transfected with 30 nM premiR-21 or premiR-negative control. Ratio paired t-test, transcript levels were normalised to the endogenous control GAPDH and error bars denote SEM.

4.7. Effect of PDGF-BB on IL1 α and MMP1

Having shown that PDGF-BB was able to increase miR-21 expression, and that miR-21 induced upregulation of MMP1 and downregulation of IL1 α expression as illustrated in Figure 4.11; we proceeded to explore a link between PDGF, miR-21 upregulation and expression of MMP1 and IL1 α . Thus to examine the effect of PDGF on the two genes affected by premiR-21 transfection; the expression of IL1 α and MMP1 was measured in PDGF-treated RNA samples of SV-SMC by RT-PCR. A high concentration of PDGF (10 ng/ml) was able to progressively impair IL1 α expression by 40%, 55% and 61% at 24, 48 and 72 h post treatment; respectively compared with control group in a manner similar to that produced by premiR-21 transfected cells (Figure 4.12).

Additionally, treating SV-SMC with a high concentration of PDGF-induced a 2.4-fold significant upregulation of MMP1 expression after 24 h treatment relative to control (Figure 4.13A). Although there was a high degree of variability, this effect of PDGF was maintained for 48 h with a modest transient 1.4-fold increase in MMP1 expression (Figure 4.13B) and declined to control levels by 72 h treatment (Figure 4.13C). Finally, the low concentration of PDGF (1 ng/ml) induced no notable difference in IL1 α (Figure 4.12) or MMP1 expression levels (Figure 4.13).

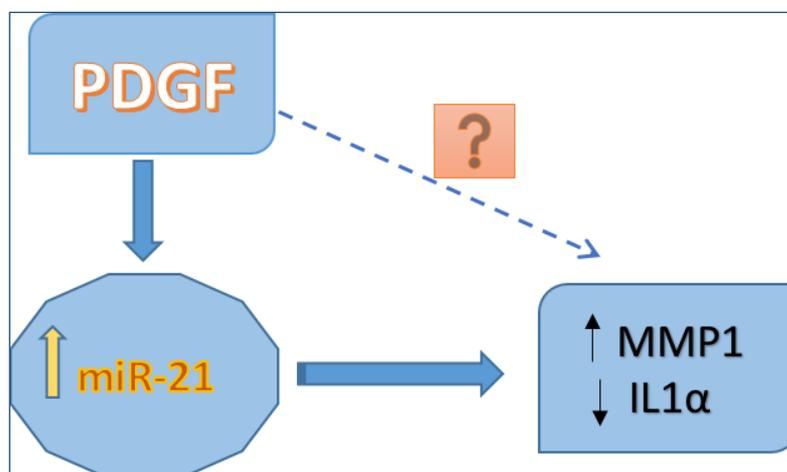


Figure 4.11: Schematic diagram showing the effect of PDGF-BB on miR-21 and its relationship with MMP-1 and IL-1 α .

Diagram illustrating PDGF-BB-induced upregulation of miR-21 expression in SV-SMC. The upregulation of miR-21 expression increased MMP-1 and decreased IL-1 α mRNA expression that could be linked to PDGF. Solid lines represent experimental results and dashed line indicates an unknown pathway.

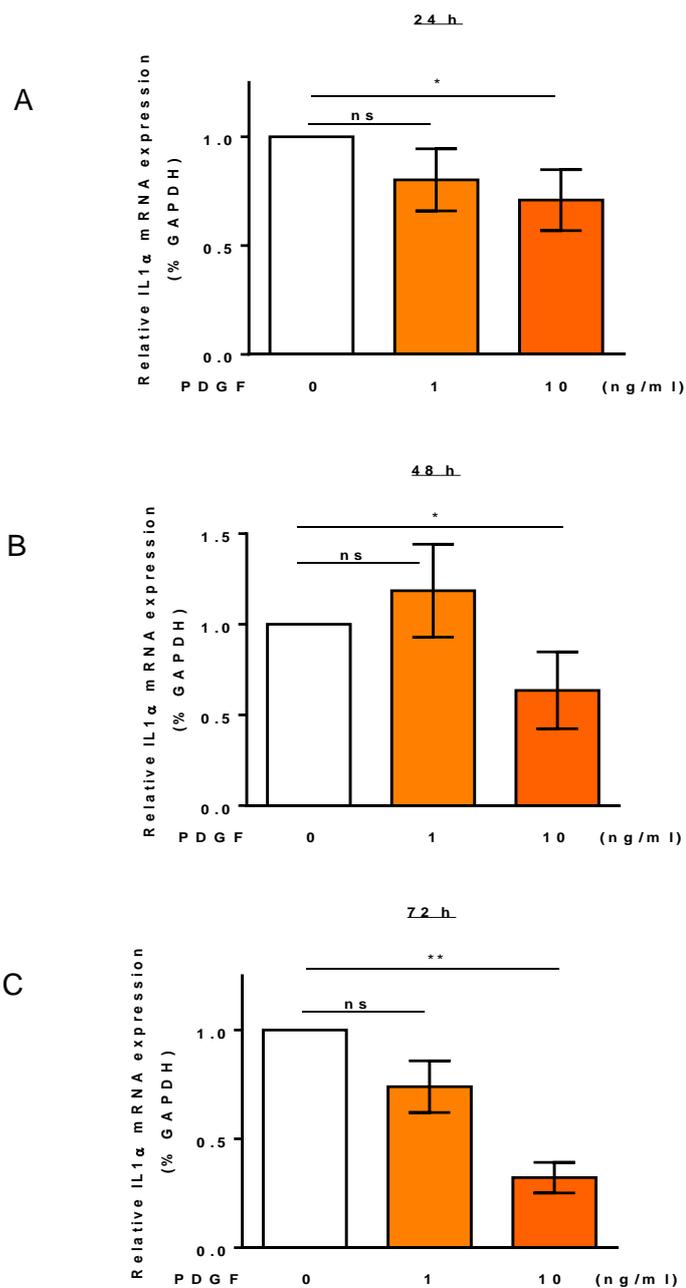


Figure 4.12: Effect of PDGF-BB on IL-1 α gene expression in SV-SMC.

RT-PCR of IL1 α expression in SV-SMC treated with low (1 ng/ml) or high (10 ng/ml) concentration of PDGF, for 24 (A), 48 (B) and 72 h (C), relative to vehicle control (acetic acid and 1% BSA in PBS). (n= 5, *P< 0.05, **P< 0.01, ns: not significant P> 0.05, One way ANOVA and Newman-Keuls multiple comparisons test). Transcript levels were normalised to the endogenous control GAPDH and error bars denote SEM.

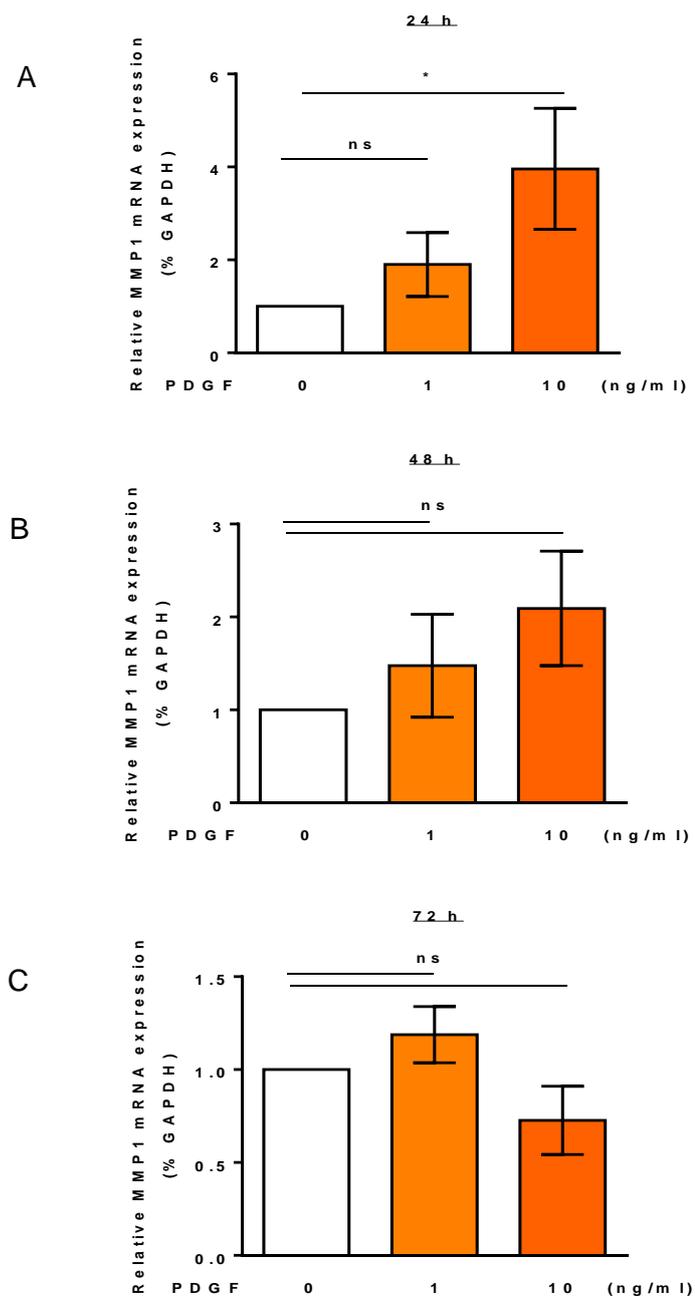


Figure 4.13: Effect of PDGF-BB on MMP1 gene expression in SV-SMC.

RT-PCR of MMP1 expression in SV-SMC treated with low (1 ng/ml) or high (10 ng/ml) concentration of PDGF, for 24 (A), 48 (B) or 72 h (C), relative to vehicle control (acetic acid and 1% BSA in PBS). (n= 5, *P< 0.05, ns: not significant P > 0.05, One way ANOVA and Newman-Keuls multiple comparisons test). Transcript levels were normalised to the endogenous control GAPDH and error bars denote SEM.

4.8. Discussion

In this chapter, the factors modulating miR-21 expression and downstream target genes of miR-21 were investigated. The principal finding was that miR-21 was significantly upregulated (2-fold) by PDGF-BB in SV-SMC and inhibition of either of the main signalling pathways of PDGF, Akt and ERK phosphorylation, completely prevented miR-21 induction. On the other hand, exposure of native SV-SMC to IL-1 α significantly reduced miR-21 expression by up to 50%. In addition to this, we have identified several genes that are regulated by miR-21 overexpression in SV-SMC. Analysis of genes involved in atherosclerosis revealed that miR-21 overexpression significantly downregulated IL1 α and surprisingly upregulated MMP1 expression. Lastly, comparable changes were mirrored with miR-21 overexpression in SV-SMC treated with PDGF-BB.

Whilst many cytokines and growth factors are secreted during vascular remodelling and vascular SMC proliferation and migration, PDGF-BB is one of the key players that is both mitogenic and chemotactic. PDGF-BB is widely expressed in several tissues, including SMC and belongs to a subfamily of PDGF-AA, PDGF-AB, PDGF-CC and PDGF-DD, which produce their effects through binding and activation of structurally related tyrosine kinase receptors, PDGFR α and PDGFR β (Heldin and Westermark, 1999). It plays an important role in physiological repair and is therefore implicated in SMC phenotype regulation and proliferative vascular diseases such as atherosclerosis and restenosis (Zemskov et al., 2012). Among the diabetic molecules and mediators tested in this study, only PDGF-BB markedly and significantly upregulated miR-21 in SV-SMC (Figure 4.4), suggesting the positive connection between PDGF and miR-21 and the possible important role of miR-21 in SMC behaviour in the pathogenesis of a variety of vascular diseases (for example atherosclerosis, cardiac hypertrophy, aneurysm and heart failure). Additionally, inhibition of PDGF-induced Akt and ERK1/2 phosphorylation in SV-SMC returned miR-21 expression to a level comparable with non-stimulated cells (Figure 4.5). This observation indicates that both pathways are required for PDGF to induce miR-21 upregulation and inhibition of either Akt or ERK1/2 can prevent PDGF-induced miR-21 expression. This positive link between PDGF and miR-21 is in agreement with one published study by Horita et al. (2011). They reported a 1.5-fold increase in miR-21 expression in rat aortic SMC treated with 20 ng/ml PDGF for 72 h, which concurs with the result obtained in human clinically relevant SV-SMC, where we observed a 2-fold increase in miR-21 expression induced by 1 and 10 ng/ml PDGF at 48 and 72 h.

Having demonstrated that miR-21 overexpression reduced IL-1 α and increased MMP-1, we then investigated whether PDGF could also modulate these two factors. With regards to the effect of PDGF on MMP1 upregulation, this was also consistent with another study that demonstrated the effect of PDGF alone and in combination with TGF- β inducing MMP1 upregulation through the ERK1/2 pathway (Ito et al., 2009). Although they did not relate their finding to miRs, as an extension of our own findings and the study by Ito et al. (2009), it would be interesting to identify whether the upregulation of MMP1 is associated with miR-21 upregulation as ERK1/2 was one of the important signalling pathways through which PDGF upregulated miR-21 expression (Figure 4.14A).

The use of the atherosclerosis array to provide relative quantification of differences in gene expression between the treated (premiR-21 transfected) and control (premiR-negative) RNA samples of SV-SMC was performed to provide a broad indication of the genes regulated by miR-21. However, often the results from the array could not be recapitulated when samples were reanalysed using specific TaqMan primers for RT-PCR. This can be explained by a number of limitations with the array. For example, genes on the array were normalised to the Ct values of the geometric mean of five housekeeping genes, which may show more variation than the standard housekeeping gene, GAPDH which was used to quantify expression using the TaqMan primers. Additionally, the fluorescence detection using SYBR Green has lower specificity and reproducibility compared to the specific TaqMan probe/primer (Petrov et al., 2014). Compared to TaqMan primers, SYBR Green dye is considerably more likely to yield false positive results if it binds to any nonspecific double-stranded DNA within the sample. Furthermore, the array was provided in 96 well plate format, such that only one replicate per gene was performed for each RNA sample, whereas all of the TaqMan specific RT-PCR experiments were performed in triplicate.

The ten genes most affected by miR-21 overexpression in SV-SMC were classified into four main categories namely (Figure 4.8); immunity and inflammation, cell growth and proliferation, ECM degradation and platelet adhesion and haemostasis. This may suggest the important role of miR-21 in affecting genes involved in cardiovascular diseases. The category with the greatest number of genes affected by miR-21 overexpression was immunity and inflammatory response, accounting for four of the ten genes. Samples overexpressing miR-21 showed ≥ 1.5 fold increase in IL1R2 and ITGB2 genes and < -1.5 fold decrease in IL1A and IL5 expression, this perhaps suggests the crucial role of miR-21 in immunity. However, the confirmatory RT-PCR of IL1A was not significant, suggesting that this was a false positive result. The trend

of reduction in IL1A mRNA expression induced by miR-21 upregulation was consistent in both the array and RT-PCR data. Perhaps it was a subtle change owing to the use of small sample size, and thus increasing sample size would confirm the effect of miR-21 overexpression on IL1A.

In the current study, it was intriguing to observe an unexpected upregulation of MMP1 induced by miR-21 overexpression both in the array and the confirmatory RT-PCR. This was a surprising result because miRs always act as negative regulators of gene expression at the post-transcriptional levels (Krol et al., 2010, Guo et al., 2010). There is no evidence in the literature to suggest that miR-21 has a direct positive regulatory effect on gene expression, therefore, it is possible that the effect of miR-21 on MMP1 is not direct, but perhaps through a mediator or possibly by de-repression of a gene that normally acts as a negative regulator of MMP1.

In support of an indirect relationship between miR-21 and MMPs, a previous study using a human glioma cell line showed that miR-21 significantly reduced mRNA and protein levels of two inhibitors of MMPs; namely RECK and TIMP3 (Gabriely et al., 2008). RECK is a membrane-anchored MMP inhibitor (Oh et al., 2001) and TIMP3 is a tissue inhibitor of MMPs (Qi et al., 2003); their inactivation increases cell invasion. Collectively, this suggests that miR-21 can directly downregulate TIMPs and RECK leading to upregulation of MMP-1 (Figure 4.14B). Therefore, further exploration of the downstream signalling pathway linking miR-21 and TIMPs inhibition would be useful to help explain the correlation between miR-21 and MMP1. TIMPs are tissue specific, endogenous inhibitors of MMPs, all are capable of inhibiting and modulating the activity of most known MMPs (Arpino et al., 2015); however, the activity of MMP inhibition varies with each TIMP (Baker et al., 2002). Therefore, future studies could use ELISA to measure TIMPs in the conditioned media collected from premiR-21 and anti-miR-21 transfected SV-SMC to indicate whether miR-21 modulates the secretion of TIMPs.

MiR-21 induction by cytokines and several diabetic mediators has been described in multiple studies in different types of tissues. MiR-21 has been documented to be upregulated by IL-6 and TGF- β in cervical tissues and cardiac fibroblasts (Bumrunghai et al., 2015, Lamoke et al., 2015). In this chapter, SV-SMC were subjected to different stimuli relevant to atherosclerosis and vascular remodelling. IL-1 α is one of the important cytokines that has proinflammatory effects. It is secreted into the extracellular milieu by activated macrophages following vascular injury in response to bypass surgery, atherosclerosis and neointimal hyperplasia (El Sayed et al., 2016). The result of this study showed that the exposure of SV-SMC to IL1 α

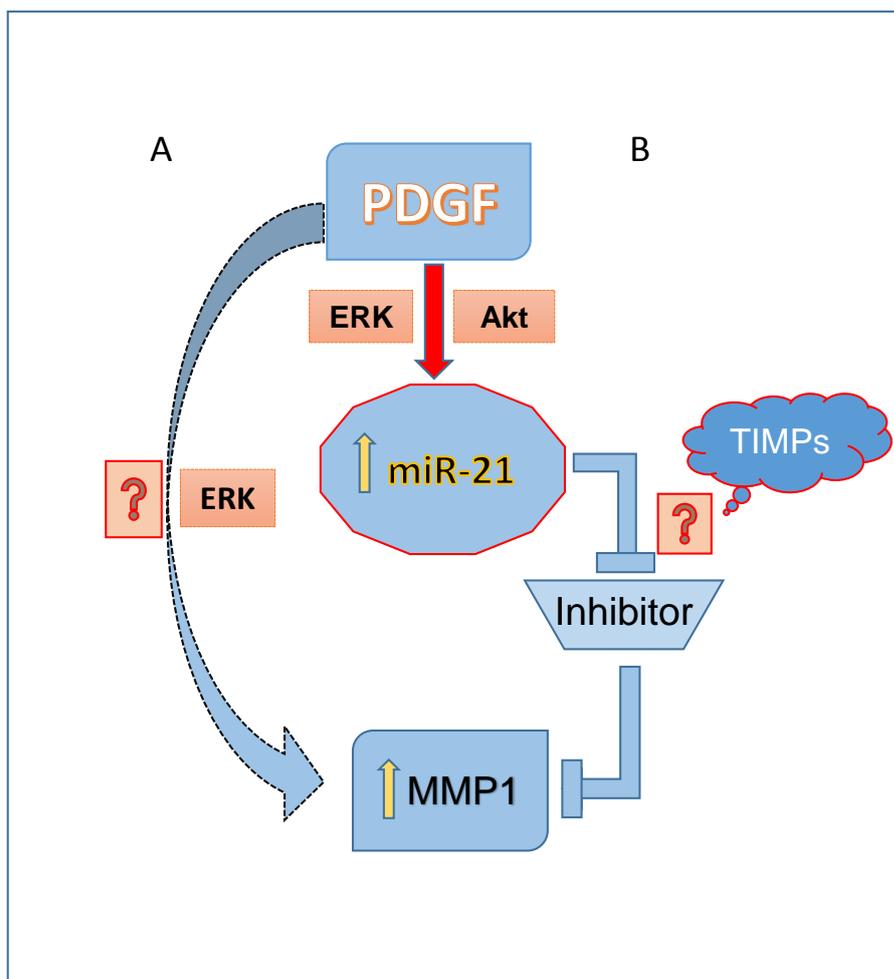


Figure 4.14: Diagram demonstrating the proposed relationship between PDGF, miR-21 and MMP1 expression.

PDGF increases miR-21 expression in SV-SMC and the upregulated miR-21 induced MMP1 overexpression and this could be via suppression of inhibitors of MMP1. The upregulation of MMP1 could be linked to PDGF through ERK1/2 signalling pathway together with miR-21 overexpression. The red arrow represents our experimental results and blue arrows are unknown pathways.

significantly repressed miR-21 expression, however, the mechanism involved in this effect and the consequence of miR-21 downregulation are still unclear. Thus, further investigation of the molecular mechanism by which IL1 α downregulated miR-21 and the importance of this relationship are required. This can be tested by knockdown of IL-1A in ND SV-SMC and measuring whether miR-21 expression could be affected in the presence and absence of IL-1 α .

We also studied the effect of other inflammatory mediators including TNF- α and TGF- β on miR-21 expression, nevertheless, none had any modulatory effect on miR-21 expression. These data were in contrast to other studies which showed that miR-21 was among miRs that were upregulated by 10 ng/ml of IL-1 β , TNF- α and INF- γ in mouse pancreatic β -cells (Roggli et al., 2010) and by TGF- β in human pulmonary SMC (Davis et al., 2008). Possible reasons for the discrepancy between our data and published research could be due to the tissue-specific expression of miRs and the differences in the response between animal and human cells for each miR (Ji et al., 2007).

The epidemic of diabetes is a severe and growing health problem around the world. In the UK, it is reported that 3.5 million people are diagnosed with T2DM and approximately half a million have the disease but are undiagnosed (https://www.diabetes.org.uk/About_us/What-we-say/Statistics/2015-as-published-2016/). Diabetic patients experienced long-term metabolic disturbances of hyperglycaemia, hyperinsulinaemia and increased inflammatory mediators even before being diagnosed. Additionally, studies have revealed that the fluctuated glucose levels can induce a significant increase in oxidative damage to the vascular endothelial layer and thus deteriorate the protective NO secretion (Chen et al., 2013a). Those patients are predisposed to cardiovascular complications due to their fluctuating metabolic abnormality as a result of untreated high glucose and high levels of insulin secretion as a compensatory mechanism of the pancreas.

Therefore, we sought to determine if exposure to diabetic factors could influence the expression of miR-21 in ND SV-SMC. MiR-21 has been shown to be upregulated *in vitro* by high glucose (25 mM glucose for 24 h) in glomerular mesangial and proximal tubular epithelial cells of diabetic mice (Dey et al., 2011). However, treating SV-SMC with 25 mM glucose for 24-72 h in the present study did not alter miR-21 expression levels. Although insulin can effectively treat hyperglycaemia, it can itself increase SMC proliferation and migration (Begum et al., 1998), and SV organ culture incubated with cell culture media supplemented with 100 nM insulin were shown to increase neointimal formation (Mughal et al., 2010). However, in our study treating

SV-SMC with diabetic stimuli (high concentration of either glucose or insulin or in combination with inflammatory mediators) produced no appreciable activation of miR-21 expression. This is most likely because miR-21 expression was not affected by the diabetic milieu and this is in agreement with the results obtained in the previous chapter that miR-21 expression levels was comparable in SV-SMC obtained from T2DM and ND counterparts (Figure 3.16B). Additionally, culturing SMC in the presence of hyperglycaemia, hyperinsulinemia or inflammatory cytokines for a short period of time (24 -72 h) fails to replicate the hallmarks of diabetic cells, which likely retain a metabolic memory of exposure to a diabetic milieu over an extended time period (e.g. years).

In summary, PDGF-BB significantly upregulated miR-21 expression in SV-SMC through activation of Akt and ERK pathways. Target genes of miR-21 were analysed using an atherosclerosis array and MMP-1 was surprisingly upregulated by miR-21 overexpression. Functionally, MMP1 is highly correlated with cell migration and invasion as it facilitates degradation of collagen and ECM and is also involved in atherosclerosis, intimal hyperplasia and vascular remodelling (Lee et al., 2011). Therefore, MMP1 is explored in detail in the following chapter to examine whether the upregulation of MMP-1 mRNA mediated by miR-21 overexpression was further inducing MMP-1 activity.

Chapter 5

Functional effect of miR-21 overexpression on SV-SMC migration

5.1. Introduction

Whilst miRs play a central role in controlling various physiological processes, there is accumulating evidence suggesting that dysregulation of miRs, including miR-21, is associated with the pathophysiology of many processes including cardiovascular disorders (Wang et al., 2011). MiR-21 is documented to be involved in the development of intimal hyperplasia following experimental balloon injury (Cheng et al., 2007) and in human arterial atherosclerosis formation (Raitoharju et al., 2011). It has been reported that miR-21 plays a crucial role in pulmonary artery SMC migration mediated by hypoxia (Sarkar et al., 2011). Additionally, in the field of cancer research, miR-21 overexpression in hepatocellular carcinoma has been shown to increase cell migration and invasion (Zhou et al., 2013).

Having previously confirmed the significant upregulation of MMP-1 mRNA induced by miR-21 overexpression using the human atherosclerosis array and by validating using TaqMan-specific RT-PCR primers we decided to study the effect of miR-21 on functional modulation of SMC through MMP-1. MMPs are a superfamily of zinc-dependent endopeptidases that play an essential role in ECM turnover and have been shown to be expressed in human atherosclerotic plaque (Shah et al., 1995). The ECM degradation process is triggered by the enzymatic activity of active collagenases (MMPs- 1, 8, 13 and 18) that digest collagens (Kar et al., 2010), followed by gelatinases (particularly MMP-2 and MMP-9) that digest collagen fragments, gelatins and basement membrane components such as collagen IV, laminin and elastin (Shi et al., 2009). Thus, the proteolytic effect of MMPs plays an essential role in tissue and vascular remodelling and cell migration during physiological processes and when this becomes dysregulated the resultant increase in cell migration, invasion and proliferation can lead to a variety of pathological conditions (Galis and Khatri, 2002). In particular, MMP-1 predominantly degrades the triple helical collagen thereby increasing its susceptibility to further degradation by the other collagenases and proteases. MMP-1 is capable of digesting collagens type I, II, and III, but has the highest affinity for type III (Kar et al., 2010).

Since intimal hyperplasia plays a key role in the complex process of vascular remodelling (Weintraub, 2007), and the uncontrolled proliferation and migration of SMC towards the intima mediates the development of vascular diseases such as atherosclerosis and restenosis following vein graft surgery (Doran et al., 2008), the focus of this chapter was therefore on the role of miR-21 in modulation of SV-SMC migration that might be regulated by MMP-1.

5.2. Effect of PDGF-BB on SV-SMC migration

To examine the effect of PDGF-BB on SV-SMC migration, Boyden chamber inserts were used (as described in Section 2.2.6A), in which the chemoattractant PDGF-BB (10 ng/ml) was contained in 750 μ l of MGM and loaded into the lower chamber in a 24-well plate. MGM alone acted as a negative control. ND SV-SMC (1×10^5 cells in 0.5 ml of MGM) were seeded in the Boyden chamber inserts, after being serum starved for 48 h. After 6 h, cells which had migrated towards the lower chamber were counted in duplicate wells per treatment. The representative image in Figure 5.1A shows a higher number of cells migrated towards PDGF versus control. PDGF induced a significant 2-fold increase in cell migration towards the lower chamber in comparison to control cells (Figure 5.1B, $P < 0.01$, $n = 4$).

5.3. Effect of occluding Boyden chamber membranes using increasing concentration of collagens on SV-SMC invasion

Owing to the indispensable role of ECM degradation for the remodelling of vascular structure, and to the effect of miR-21 on MMP-1 overexpression obtained in the previous chapter, a series of experiments were performed to optimise the coating of Boyden chambers with collagen. The MMP-1 substrates collagen I and III were tested at a range of concentrations (5-100 μ g) to select a suitable concentration. Membranes were coated with collagen type I or III solutions by adding 100 μ l of a serial dilution of the stock solution of each collagen. The gel was allowed to stabilise for 2-3 h at RT, then incubated at 37 $^{\circ}$ C to allow pore occlusion. After 24 h, the chamber was washed briefly with 0.5 ml of PBS and the remaining solvent and excess collagen were removed.

SV-SMC were resuspended in MGM and equivalent numbers of cells were seeded on the top chambers coated with the five different concentrations of collagen I or III in

comparison to wells coated with 0.01 M HCl (vehicle control). Cells were allowed to migrate for 24 h towards MGM supplemented with PDGF-BB (10 ng/ml) in the lower chamber and the cells which invaded were stained and counted as described in Section 2.2.6. As might be expected, the degree of cell invasion was inversely proportional to the concentration of collagen I (Figure 5.2A) and collagen III (Figure 5.2B). The concentration response curves of percent cells invaded versus collagen concentration were plotted and from this, the 50% inhibitory concentration (IC_{50}) for collagen I (Figure 5.3A) and collagen III (Figure 5.3B) were calculated to be approximately 35 μ g. This yielded a window of opportunity to observe the effect on cell migration and invasion (either increase or decrease) mediated by miR-21 manipulation.

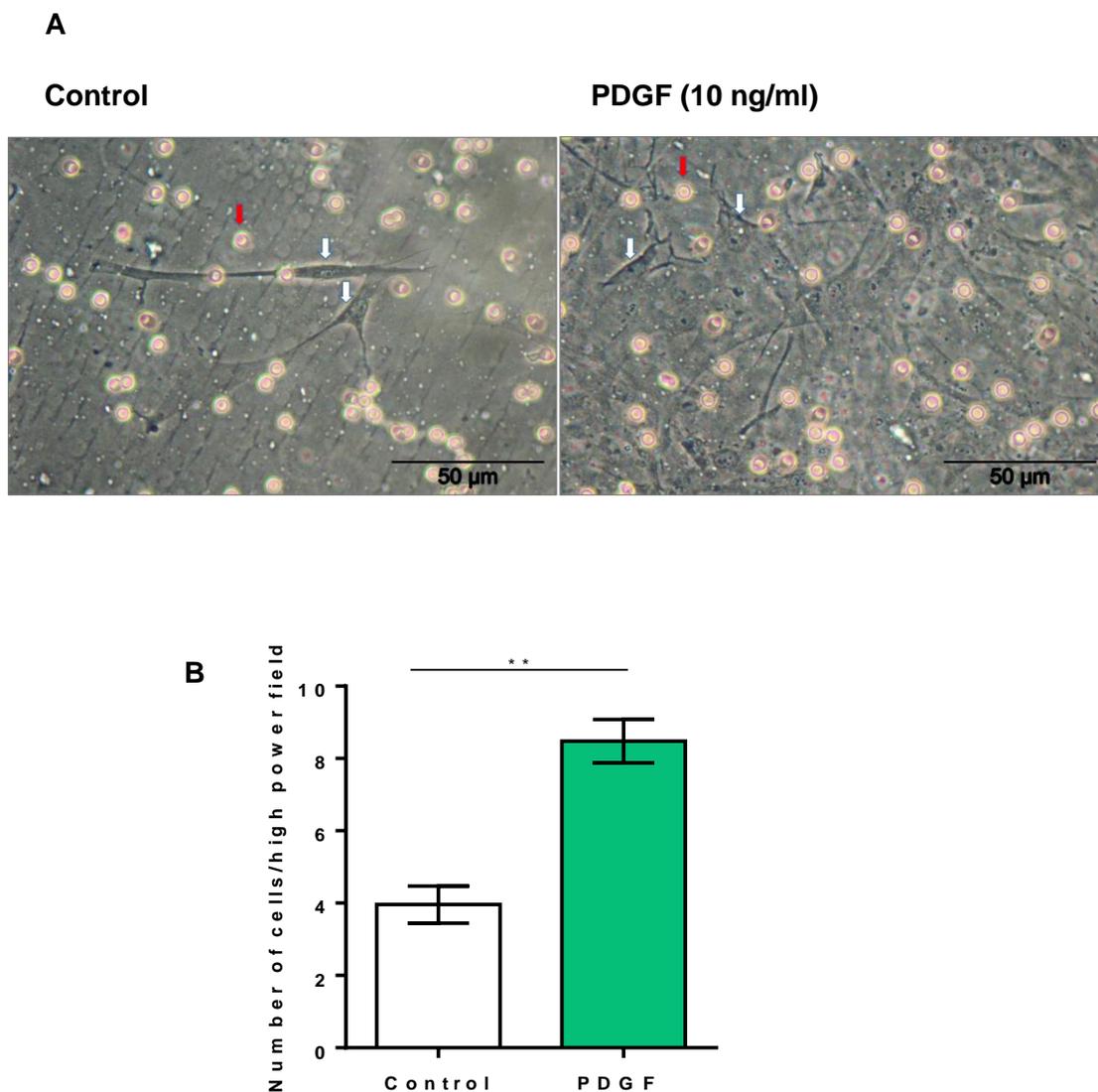


Figure 5.1: Effect of PDGF-BB on SV-SMC migration through Boyden chambers.

A- Representative images of the underside of the migration membrane showing the nuclei of cells which have migrated towards PDGF versus control (MGM). The white arrows indicate the nucleus and the red arrows indicate the pores of the membrane. Scale bar = 50 μm at x200 magnification. B- Average number of migrated SV-SMC using Boyden chamber inserts for 6 h towards the lower chamber of MGM alone or MGM containing 10 ng/ml of PDGF (n= 4, **P< 0.01, paired t-test).

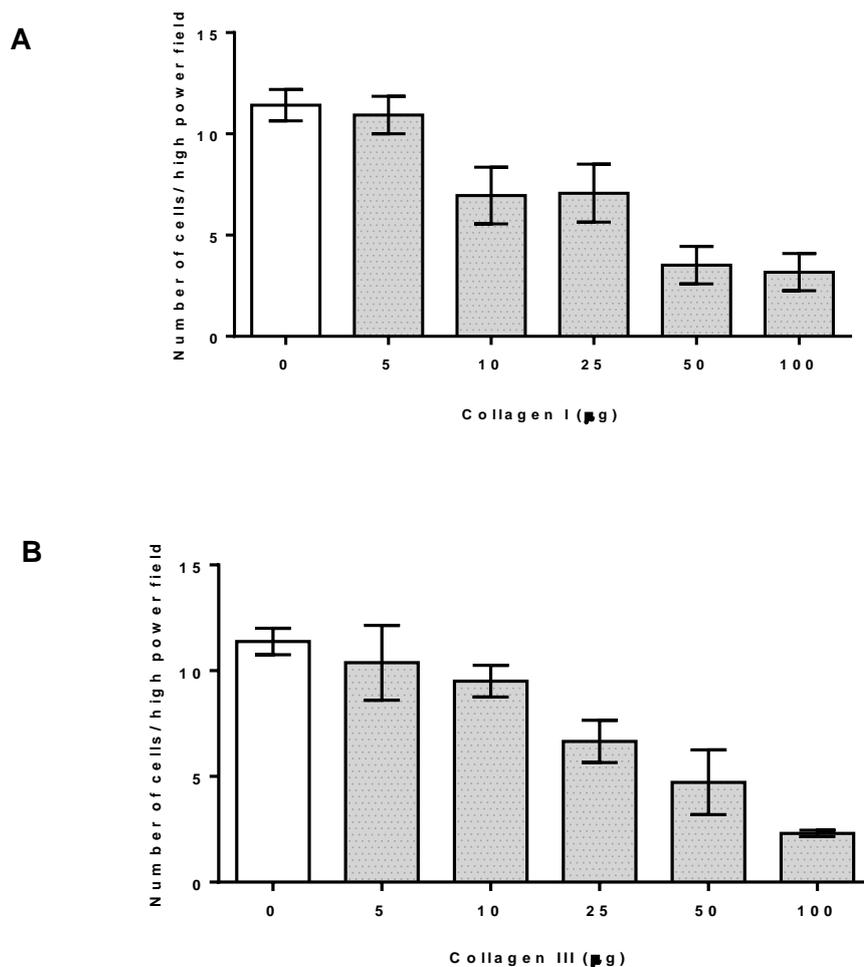


Figure 5.2: Concentration-dependent effect of coating Boyden chambers with collagens I and III on ND SV-SMC invasion.

Number of SV-SMC which migrated within 24 h through Boyden chamber inserts coated with collagen type I (A) or III (B) towards the lower compartment of MGM containing 10 ng/ml of PDGF or 0.01 M HCl (control) (n= 2).

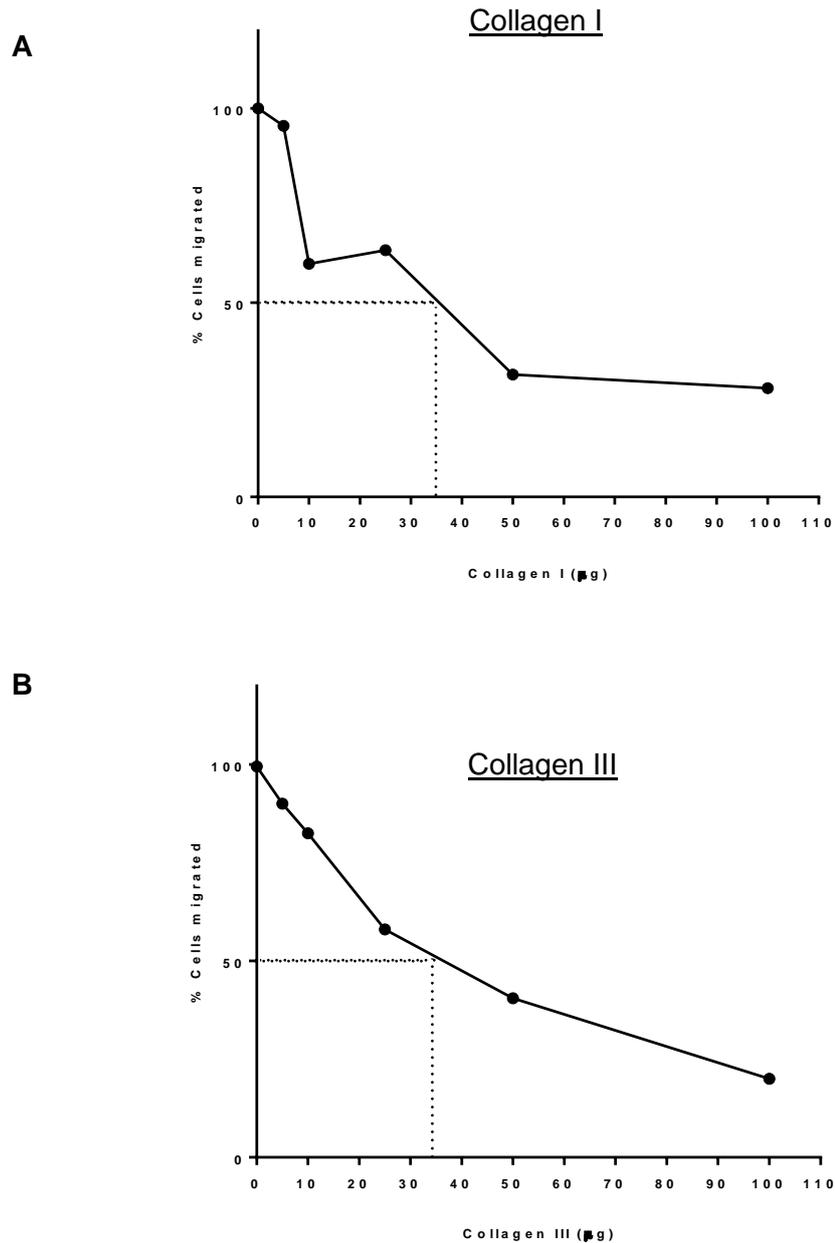


Figure 5.3: Normalised curves quantifying half maximum inhibitory concentration of collagen I and III versus % cells migrated.

Calculation of a suitable concentration of collagen that inhibits SV-SMC invasion by approximately 50% through collagen I (A) or collagen III (B) ($n=2$). The concentration that inhibited 50% (IC_{50}) of cell invasion was 35 μg for each collagen type.

5.4. Time course of miR-21 upregulation in SV-SMC

Before commencing any functional assay, it was necessary to investigate whether the upregulation of miR-21 following transfection was maintained for sufficient duration to perform the migration experiments. A time course study was conducted to measure miR-21 expression in SV-SMC between 24 – 96 h post transfection with either 30 nM premiR-21 or premiR-negative. Cell pellets were collected each day and used for RNA extraction and RT-PCR. Within 24 h premiR-21 transfection showed a marked 5600-fold increase in miR-21 expression which fell rapidly, but was still significantly upregulated to 550-fold at 96 h. This confirmed that miR-21 overexpression would be maintained over the required period of time following transfection (n= 6, Figure 5.4, P< 0.001).

5.5. Effect of miR-21 overexpression or knockdown on SV-SMC invasion through collagen I or III coated Boyden chambers

To explore the role of miR-21 overexpression or knockdown on the invasion and migration capacity of SV-SMC from five different donors, Boyden chamber membranes were coated with either 35 µg collagen I or III before being loaded with mock, premiR-21 and antimiR-21 transfected cells together with their negative controls (transfection method is described in Section 2.2.2). The lower chambers were loaded with either MGM as a control or MGM containing 10 ng/ml PDGF-BB. Transfected SV-SMC were incubated in MGM for 48 h then 1×10^5 cells were seeded into the coated chambers in duplicate wells for 24 h to allow collagen degradation and invasion of cells through the coated membranes. Conditioned medium from the upper chambers was collected to measure secreted MMP-1 and invaded cells were counted in 10 different fields under high power as described in Section 2.2.6. All cells migrated towards PDGF, regardless of their transfection type, such that a significant 3-fold increase in cell invasion was observed relative to control (Figure 5.5). Unexpectedly, overexpression of miR-21 did not augment the invasion capacity of SV-SMC through either collagen I or collagen III (Figure 5.5A). Moreover, miR-21 knockdown in SV-SMC did not affect the invasive capacity of cells through either collagen type (Figure 5.5B).

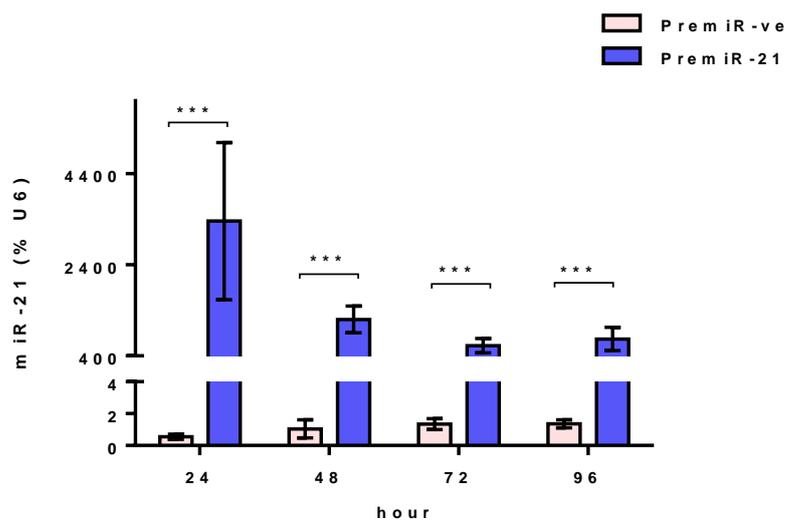


Figure 5.4: Time course of miR-21 expression for 96 h post transfection in SV-SMC.

RT-PCR of miR-21 expression in ND SV-SMC transfected with 30 nM premiR-21 or premiR-negative (control) over a time course of 24-96 h (n= 6, ***P< 0.001; One Way ANOVA, with Newman Keuls post-hoc test).

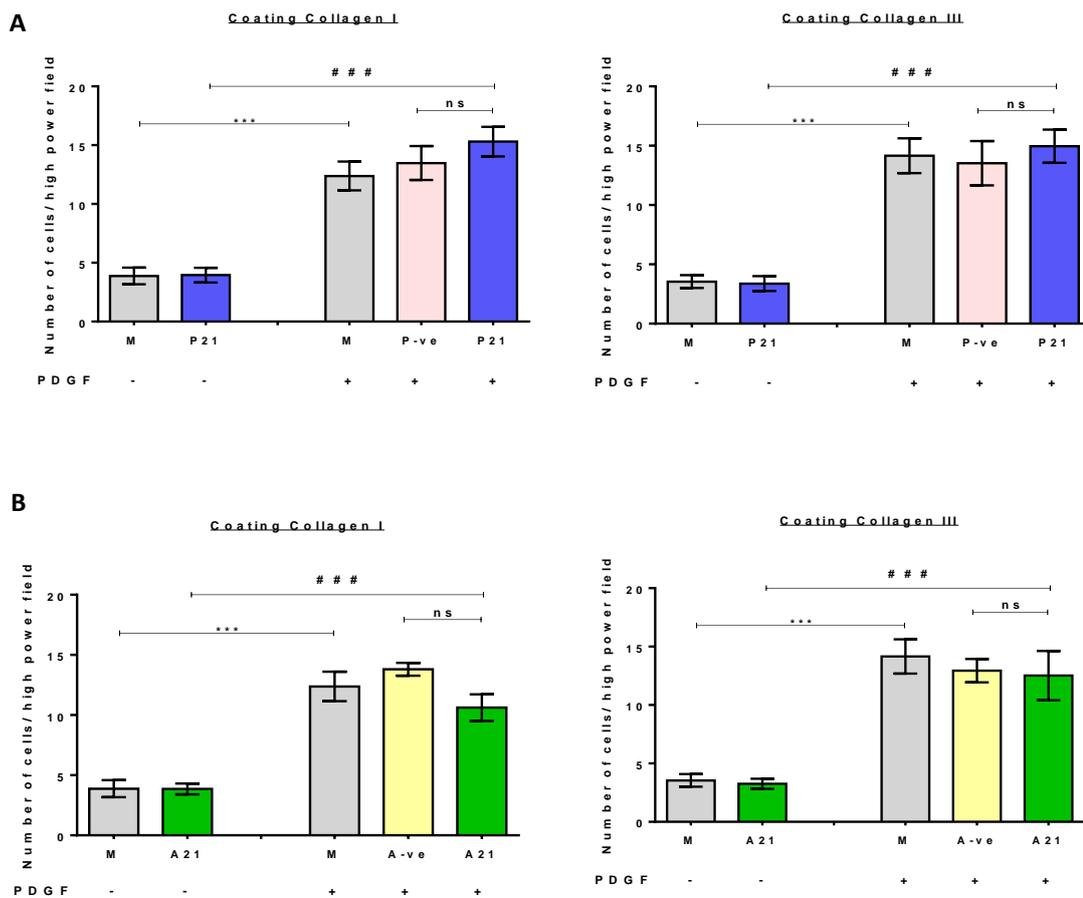


Figure 5.5: Effect of overexpression or knock down of miR-21 on SV-SMC invasion through collagen I or III.

Number of invaded SV-SMC following transfection with either 30 nM premiR-21 or premiR-negative (A) or 100 nM antimiR-21 or antimiR-negative (B) compared to mock transfected (lipofectamine only) cells. Cells invaded through Boyden chambers coated with 35 μ g collagen I or collagen III and allowed to migrate for 24 h towards MGM (control) or 10 ng/ml of PDGF-containing MGM. (Each group n=5, *** and ### P< 0.001, One way ANOVA, with Newman Keuls post-hoc test), ns= not significant.

5.6. Pro-MMP-1 secretion by transfected SV-SMC during invasion through collagen I and II coated Boyden chambers

In order for SV-SMC to be capable of migrating through collagen coated Boyden chambers towards the chemoattractant PDGF-BB they must secrete MMPs. In particular, MMP-1, by cleaving collagen, has emerged as a key contributor to vascular remodelling and atherosclerosis (Sukhova et al., 1999). The conditioned medium collected from the upper chamber of the Boyden chambers was therefore utilised to analyse the secretion of pro-MMP1 (precursor of MMP-1) by the invaded cells, using an ELISA kit as described in 2.2.7A. PremiR-21 transfected cells migrating towards PDGF through both collagen types showed a modest increase in pro-MMP-1 secretion in comparison to premiR-negative (control) cells (Figure 5.6A). Additionally, the highest increase in pro-MMP1 secretion was observed in the mock transfected group migrating through both collagen I and III towards PDGF-BB (n= 5, Figure 5.6A). However, conditioned medium from antimiR-21 transfected SV-SMC showed no significance difference in pro-MMP-1 secretion compared with its antimiR-negative control group (n= 5, Figure 5.6B).

5.7. Correlation between SV-SMC invasion through collagen I and III and pro-MMP-1 secretion

To determine whether there was a correlation between the number of invaded transfected SV-SMC and pro-MMP-1 secretion into the conditioned medium, the number of cells invaded through collagen was plotted against the amount of pro-MMP-1 secretion. There was no correlation between the increase in cell invasion through collagen I ($R^2 = 0.04$, Figure 5.7A) or collagen III ($R^2 = 0.011$, Figure 5.7B) with the secreted amount of pro-MMP-1 (n= 40).

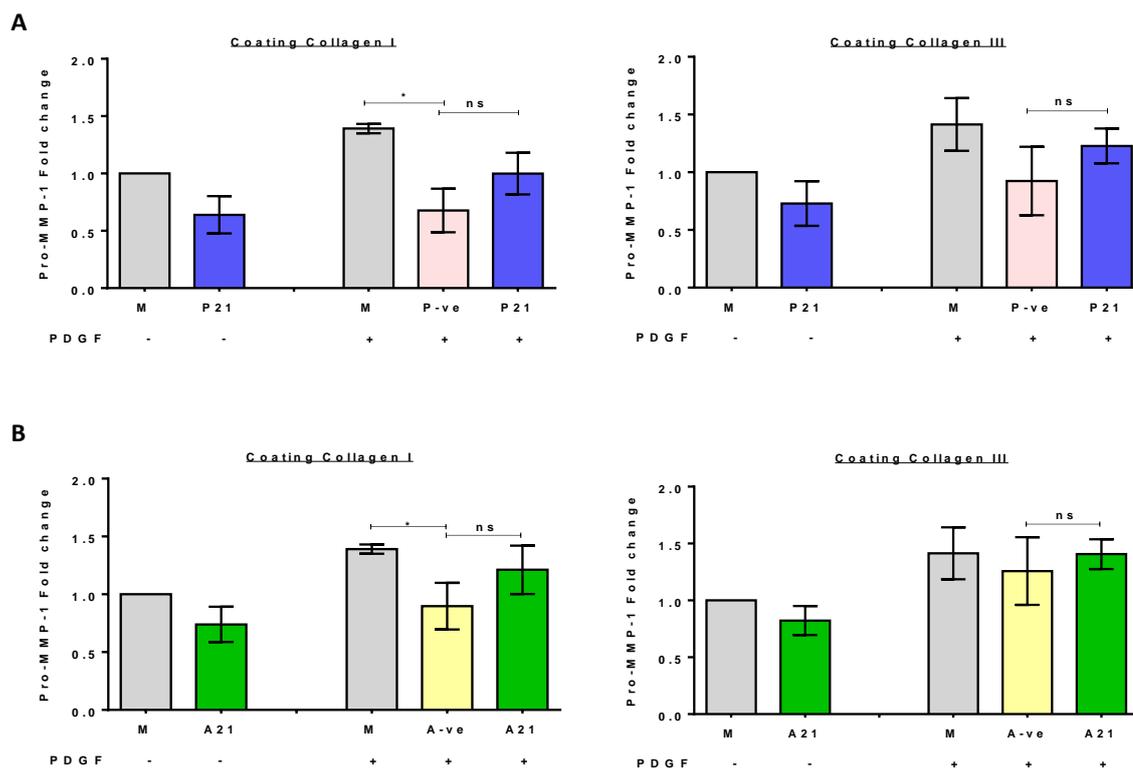


Figure 5.6: Measurement of pro-MMP-1 secretion into conditioned media collected from premiR-21 and antimiR-21 transfected SV-SMC invading through collagen I or III coated Boyden Chambers.

Pro-MMP-1 secretion in the CM of invaded SV-SMC transfected with either 30 nM premiR-21 or premiR-negative (A), 100 nM antimiR-21 or antimiR-negative (B) compared to mock transfected (lipofectamine only) cells using ELISA. Cells were allowed to migrate through Boyden chambers coated with 35 μ g collagen I or collagen III for 24 h towards MGM (control) or 10 ng/ml PDGF-containing MGM. The actual ELISA measurement of MMP-1 concentration secreted by invaded SV-SMC through collagen type I and III was 0.09 – 5.12 ng/ml and 0.04 - 5.2 ng/ml; respectively (n= 5, *P< 0.05, One way ANOVA, with Newman Keuls post-hoc test), ns= not significant.

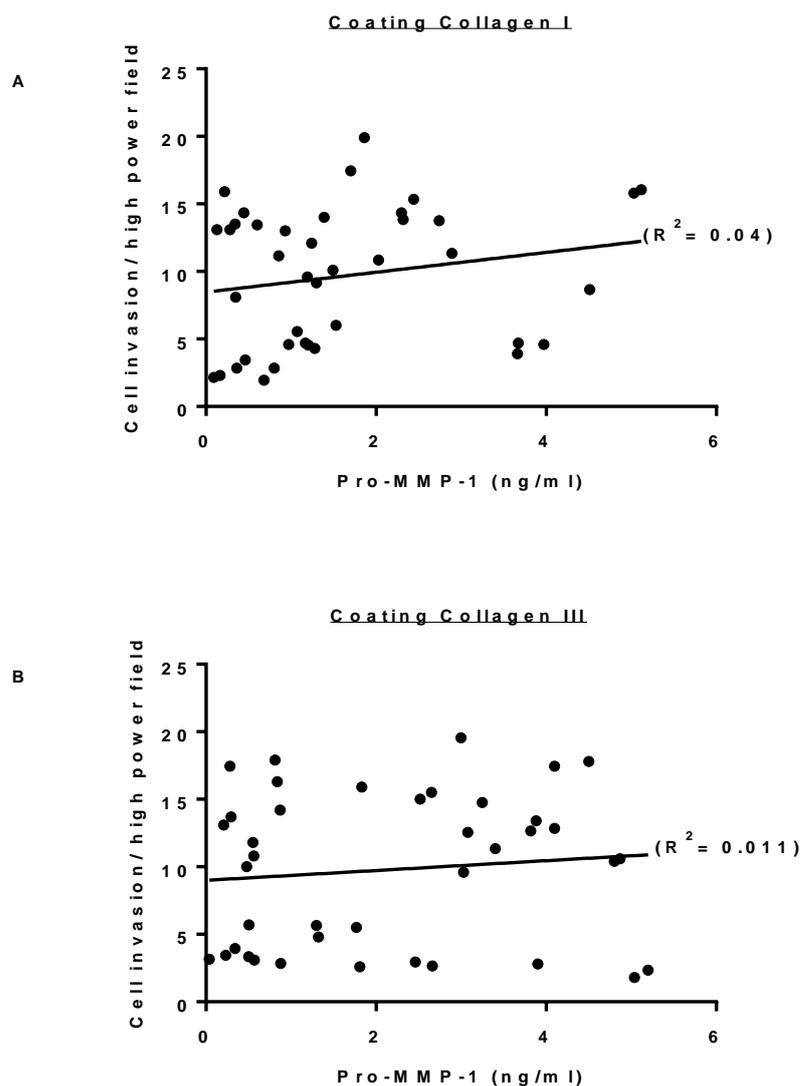


Figure 5.7: Correlation between the number of cells invaded through collagens and pro-MMP-1 secretion.

Correlation between SV-SMC invasion through Boyden chambers coated with 35 μ g collagen I (A) or collagen III (B) and pro-MMP-1 secretion in the CM measured using ELISA. Cells were transfected with 30 nM premiR-negative or premiR-21, 100 nM antimiR-negative and antimiR-21 or mock (lipofectamine only) towards MGM (control) or 10 ng/ml of PDGF-containing MGM (n= 40).

5.8. Measuring pro- and active MMP-1 secretion in premiR-21 and anti-miR-21 transfected SV-SMC

Although we observed that the secretion of pro-MMP-1 was not significantly increased by miR-21 overexpression, this did not rule out a change in MMP-1 activity and it remains possible that the ratio between pro-MMP-1 and active MMP-1 was varied. In another set of experiments, the effect of miR-21 overexpression or knockdown on pro-MMP-1 expression and activity was measured in transfected unstimulated SV-SMC that were collected 3 days after transfection. Conditioned medium was then collected to analyse pro-MMP-1 and active MMP-1 using two different ELISA kits (described in Section 2.2.7A/B). PremiR-21 transfected cells did not show a significant difference in pro-MMP-1 secretion relative to control cells nor did anti-miR-21 transfected cells (n= 4, Figure 5.8A). Measuring the active MMP-1 secretion in another three different sets of transfected cells also showed no significant difference between premiR-21 or anti-miR-21 treated cells relative to their negative controls (n= 3, Figure 5.8B).

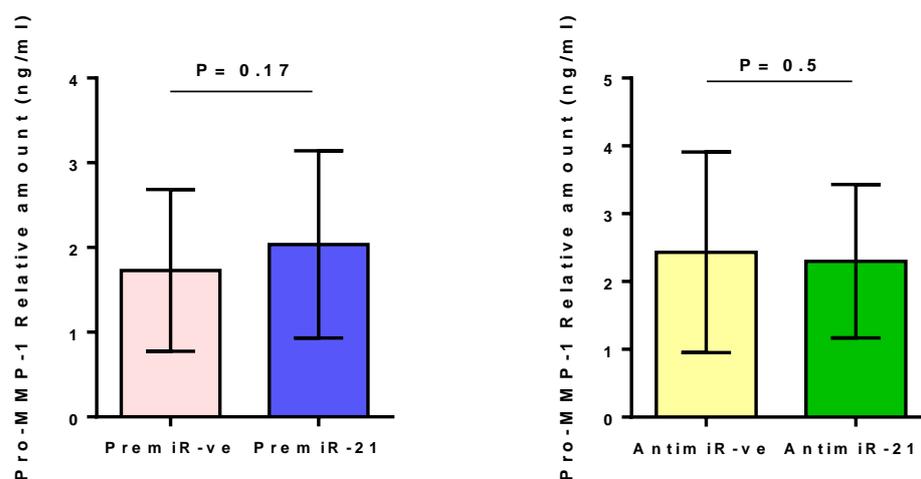
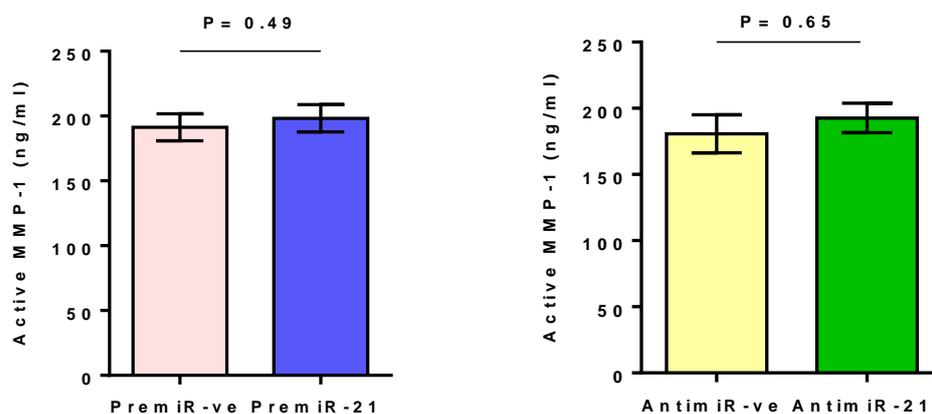
A- Pro-MMP-1**B- Active MMP-1**

Figure 5.8: Measurement of pro-MMP-1 and active MMP-1 in conditioned media of premiR-21 and anti-miR-21 transfected (non-stimulated) SV-SMC using ELISA.

Measuring pro-MMP-1 (A, n= 4) and active MMP-1 (B, n= 3) secretion in CM of SV-SMC transfected with 30 nM premiR-negative (PremiR-ve) or premiR-21, 100 nM anti-miR-negative (anti-miR-ve) or anti-miR-21 for 72 h using ELISA (ratio paired t-test).

5.9. Discussion

The data in this chapter have validated the efficient chemoattractant activity of PDGF-BB and also confirmed that SV-SMC invasion through collagen I or III coated membranes was inversely proportional to the concentration of collagen. Overexpression of miR-21 in SV-SMC following transfection with 30 nM premiR-21 was demonstrated to still be significantly upregulated for 96 h. Surprisingly, the overexpression and knockdown of miR-21 did not modulate the invasive capacity of cells through either collagen I or III. Neither the invaded nor unstimulated premiR-21 transfected cells mediated a distinguishable effect on the levels of secreted pro-MMP1 and active MMP-1.

PDGF-BB is known to be an important chemoattractant and mitogenic agent for normal angiogenesis and wound healing. In terms of vascular injury, PDGF activates SMC migration from the media and accumulation of ECM into the intima which leads to intimal hyperplasia, atherosclerosis and coronary occlusion (Newby and Zaltsman, 2000). In the present study, the main focus was on the regulation of SV-SMC migration and invasion by miR-21 manipulation in the presence of 10 ng/ml PDGF-BB. As expected, this study showed a significant 2-fold increase in SV-SMC migration towards PDGF when compared to cells migrating towards MGM alone (Figure 5.1), confirming the chemotactic effect of this agent. We confirmed miR-21 overexpression reached the maximum significant 5600-fold increase 24 h after premiR-21 transfection, followed by a reduction to 550 fold increase, which was maintained at 96 h post transfection (Figure 5.4).

Having previously shown that MMP-1 mRNA expression levels were enhanced by miR-21 overexpression in SV-SMC, it was hypothesised that miR-21 could enhance cell invasion and migration through Boyden chamber membranes coated with MMP-1 substrates. MMP-1 activity was reported to correlate well with ECM breakdown, cell migration and invasion (Shi et al., 2009). We employed a transwell invasion system by coating the membranes of a double well chamber with collagen I or collagen III. Therefore, the SV-SMC were encouraged to secrete MMP-1, degrade the coating and finally invade through the membrane towards PDGF. MMP-1 is an important interstitial collagenase, capable of degrading collagen type I, II, and the highest affinity to cleave collagen type III in the adventitial layer of the vascular wall, thus facilitating cell migration through the matrix barrier (Lemaitre and D'Armiento, 2006, Cooley, 2013). Treating a mouse carotid artery injury model with MMP-1 signalling inhibitor significantly reduced cell migration and intimal hyperplasia formation (Austin et al., 2013b). It has been upregulated with other MMPs following femoral artery injury in

mice (Lijnen et al., 1999) and MMP-1 upregulation has been reported in a monolayer of vascular SMC line (Rb-1 cells) in response to mechanical injury (James et al., 1993).

In our study, the number of cells that migrated to the lower chamber was inversely proportional to increasing collagen concentration (Figure 5.2). After analysis, the approximate amount of collagen that prevented 50% of cell invasion (IC_{50}) was 35 μ g for both collagen I and III. This was applied separately to occlude the pores in the Boyden chamber membranes and provide a “window” to see the functional effect for the premiR-21 and antimiR-21 transfected cells.

Following loading the transfected SV-SMC in parallel to their controls in the upper compartment of the Boyden insert, significant migration was seen in all cells exposed to PDGF. Surprisingly, overexpressing miR-21 did not increase the invasive capacity of cells above the negative control treatment. Multiple reasons could explain the lack of response in cells overexpressing miR-21 (Figure 5.5). Firstly, having observed that miR-21 overexpression enhanced MMP1 mRNA expression, this suggests that MMP-1 is an indirect target of miR-21 in SV-SMC. MiR-21 may affect the expression of gene(s) that negatively regulate MMP-1 gene expression and activity such as TIMPs. Secondly, studies have shown that MMP-1, when upregulated, can activate other cytokines such as IL-1 β (Schonbeck et al., 1998), and in the previous chapter, IL-1 α showed a negative regulation of miR-21 expression. This indicates that the secreted cytokines which might accompany MMP-1 secretion could mask the possible effect by miR-21 in inducing SMC migration. This could be confirmed by knocking down IL-1 α and comparing the effect of premiR-21 transfection on cell invasion both in the presence and absence of IL-1 α . Thirdly, the result may also suggest that cells reached the maximum migratory capacity in response to PDGF and no further effect, if produced, by miR-21 and MMP-1 was noticeable. Repeating the invasion assay using PDGF concentrations less than 10 ng/ml would be helpful to identify whether a suitable lower submaximal concentration of PDGF would allow miR-21 manipulation to modulate the invasive capacity of SV-SMC.

Cell invasion and migration is a complex process that is mainly initiated by adhesion, locomotion, invasion and chemotaxis (Ross, 1993). For the vascular SMC to migrate, cell surface receptors are stimulated that lead to series of coordinated remodelling events which in turn change the cytoskeleton structure. This starts with actin polymerisation which directs the cells toward the chemoattractant agent. All organelles of the cells are then carried along with the remodelling cytoskeleton by adaptor and motor proteins to support cell transfer with the cytoskeleton. Additionally,

reactive oxygen species secretion and activation of MAPK/ERK signaling pathway are mediators usually stimulated in response to PDGF chemoattractant activity (Xi et al., 1999, Schroeder, 2014).

The role of miR-21 in inducing cell migration and invasion has been reported in the majority of cancer cells (Selcuklu et al., 2009a), however, little is known about its role in vascular SMC migration. MiR-21 overexpression increased migration of hepatocellular carcinoma through targeting PTEN, PDCD4 and RECK (Zhou et al., 2013) and miR-21 inhibition decreased hepatocellular cell proliferation and migration (Meng et al., 2007b). MiR-21 has been reported to induce tumour invasion and metastasis by targeting multiple tumours and metastasis suppressor genes including PTEN, PDCD4, and tropomyosin 1 (Zhu et al., 2008, Liu et al., 2013) and therefore, miR-21 knockdown may provide a useful anticancer therapeutic target. MiR-21 overexpression was reported to increase cell migration and invasion of esophageal squamous cell carcinoma by suppression of PDCD4 gene (Liu et al., 2014), gastric cells by inhibiting PTEN (Zhang et al., 2012), bladder cell carcinoma through targeting PTEN (Lei et al., 2015), breast cancer by suppressing smad7 (Han et al., 2016). Additionally, miR-21 overexpression showed a significant increase of airway SMC migration using transwell assay and that was through targeting PTEN (Liu et al., 2015).

Numerous studies have also reported the ability of miRs to regulate multiple components involved in the cellular adhesion process (Valastyan and Weinberg, 2011). In particular, tropomyosin 1 is widely distributed in different cell types and it is an important actin cytoskeletal regulatory protein which stabilises microfilaments (Perry, 2001). Repression of tropomyosin 1 mRNA and protein levels by miR-21 could play an important role in enhancing cell migration (Zhu et al., 2007).

In line with cell migration, it is documented that under physiological and pathological conditions, MMPs work in coordination to facilitate cell invasion (Aoyagi et al., 1998). ECM breakdown, and perhaps other MMPs if secreted, would not help to increase invasion through collagen I or III coating. Furthermore, the exact mechanism underlying MMP activation that occurs either at the intracellular or extracellular levels is still unclear. Typically, activators cause a conformational change in the molecule that separates the Cys residue of pro-MMP from the zinc atom to generate a functionally active site. This might be through proteolytic activation by cellular endoproteases, oxidative stress, phosphorylation and via extracellular enzymes such as plasmin, thrombin or trypsin (Cha et al., 1996, Gaffney et al., 2015). Owing to the

lack of contact with other environmental cues that help in MMPs activation, thus, our study provided an incomplete picture of the ECM degradation and cell invasion.

During vascular remodelling, various factors such as inflammatory mediators increase MMP secretion and steps to modulate cell migration appear to function as a network. Therefore, using *in vitro* SMC in culture fails to accurately recreate the precise environment which leads to MMP-1 secretion *in vivo*. Performing an *in vivo* study using intact SV could help to elucidate the signalling pathways and other mediators involved in the stimulation of cell invasion.

It would have been interesting to measure TIMPs secretion, particularly TIMP1, TIMP2 and TIMP3 which are the main inhibitors of MMP-1 (Flannelly et al., 2002) in the CM collected. They can reduce MMP-1 activity, which in turn would affect SMC migration capacity through the coated membranes. Lastly, after 72 h of transfection, it may be too early to detect the functional effect of miR-21 overexpression on MMP-1 protein activity and further time points might be studied to detect changes in SMC invasive phenotype. On the other hand, knockdown of miR-21 in SV-SMC did not change or reverse the invasive capacity of the cells through either collagen types. Taken together, understanding this previously mentioned scenario would help to give a broader overview of the condition and factors involved during SMC migration to conclude the role of miR-21 through MMP-1 expression in inducing SMC migration and invasion.

This was followed by measuring pro-MMP-1, the precursor of MMP-1 which indicates the total amount of MMP-1 secreted in the CM of invaded SV-SMC. MMP-1 is a member of a family of zinc-dependent proteases whose major function is the breakdown of ECM polymers (Newby, 2012). Given the ability of MMP-1 to affect the invasive capacity of the cells through collagen I and III, it was surprising that the secreted MMP-1 that we measured did not increase cell invasion in a discernible manner. There was a modest increase in total MMP-1 secretion in the premiR-21 transfected cells which is consistent with the increased MMP-1 mRNA in the previous chapter, but it was not significant (Figure 5.6). This indicated that the MMP-1 secretion was not sufficient to induce an increase in cell invasion. Alternatively, other mediators that are regulated by miR-21 overexpression could have been secreted into the CM and antagonised the effect of miR-21 on MMP-1 upregulation. Likewise, there was no significant difference in total MMP-1 secreted by the anti-miR-21 relative to anti-miR-negative transfected cells which migrated through collagen towards PDGF. An important study demonstrating the important role of miR-21 in cell migration was performed by Sarkar et al. (2010). They found that miR-21 was 3-fold increased when

the pulmonary SMC were exposed to hypoxia. Additionally, miR-21 targeted PDCD4 and SPRY2 and knocking down miR-21 was able to reduce 50% of cell migration induced by hypoxia. Although they did not measure the secreted MMPs by hypoxic cells, it could be that the cells should be exposed to a pathological insult like a vascular injury to be triggered to simulate the clinical pathological situation.

Although the secretion of MMP-1 is low in unstimulated cells, another set of experiments was carried out to assess the role of miR-21 manipulation on MMP1 secretion in the absence of the chemoattractant PDGF. Total and active MMP-1 secretion after premiR-21 or anti-miR-21 transfection of SV-SMC showed that there was no significant difference in MMP-1 secretion in both groups. In the presence of an increased interstitial shear flow the increased cell migration correlated with the increased activity of MMP-1 (Shi et al., 2009). This indicates that the lack of the pathological stimuli would not trigger the static cells to secrete MMP-1 and also perhaps suggests that the inactive zymogen (MMP-1) remained in the latent stage due to the lack of sufficient trigger to activate the proteolytic activity in the unstimulated cells. It could be that miR-21 manipulation is unlikely to have an impact on active or pro-MMP-1 secretion at the tested time point (72 h). Lastly, there was no correlation between pro-MMP1 secretion and the number of migrated cells, through either collagen I or III in any of the transfected cells groups, indicating that other MMPs may be more important in mediating the migration of cells through collagen I and II than MMP-1.

In summary, MMPs including MMP-1 play a crucial role in vascular remodelling and cell migration. The results of this study demonstrated the inability of miR-21 overexpression to induce a further increase in cell migration; nevertheless, the secretion of pro-MMP-1 was moderately increased in the premiR-21 transfected cells.

Chapter 6

The role of miR-21 on SV-SMC proliferation and exploring downstream target genes

6.1. Introduction

In the normal healthy vessel wall, SMC have an extremely low rate of proliferation, however following vascular injury, these cells display high plasticity and can adopt a dedifferentiated phenotype (proliferative and migratory). They remain susceptible to environmental factors; such as ECM components (laminin and collagens), reactive oxygen species, thrombin, TGF- β , shear stress and many other neuronal or mechanical mediators (Owens et al., 2004). PDGF-BB is a particularly important growth factor secreted by platelets, activated macrophages, vascular SMC and endothelial cells upon vascular injury. It is one of the major mitogenic factors which is also involved in SMC proliferation and motility by activating the MAPK signalling pathway in vascular physiological conditions (Kawai-Kowase and Owens, 2007).

Recent reports have documented the important role miRs play controlling vascular SMC proliferation and differentiation in vascular diseases (Jamaluddin et al., 2011). In response to the wound healing process, proliferative and synthetic genes are also modulated by miRs. For example, suppression of the Smad1 protein by miR-24 resulted in a synthetic human pulmonary SMC phenotype (Chan et al., 2010). MiR-21 is involved in cell proliferation, migration, and invasion both in human and murine cancer and cardiovascular diseases (Rossi et al., 2007, Jazbutyte and Thum, 2010). Additionally, evidence suggests that the target proteins of miR-21 differ according to cell and tissue type (Bronnum et al., 2013).

The most commonly reported target genes for miR-21 that showed a strong association with cell proliferation, migration, apoptosis and invasion are Phosphatase and Tensin Homolog (PTEN), Programmed Cell Death 4 (PDCD4), Sprouty-1 (Spry-1) and Reversion-Inducing Cysteine-rich protein with Kazal motifs (RECK). Therefore, they were chosen to be investigated in this chapter in the context of SV-SMC remodelling.

PTEN is known to regulate the cell cycle and inhibit phosphoinositide-3-kinase (PI3K). It is the most documented target gene affected by miR-21 in different cancer and cell types such as breast (Meng et al., 2006), hepatocellular cancer (Bao et al.,

2013) and vascular SMC of rat carotid artery (Maegdefessel et al., 2015). In a key study, upregulation of miR-21 accelerated rat carotid artery SMC proliferation, whilst downregulation of the aberrantly overexpressed miR-21 in SMC inhibited neointimal formation following vascular injury, and this was through targeting PTEN (Ji et al., 2007). Following vascular injury, it was reported that PTEN was downregulated by miR-21 leading to increased SMC proliferation (Horita et al., 2011).

PDCD4 is a tumour suppressor protein, which inhibits cell proliferation, migration and invasion and has been shown to block protein synthesis by inhibiting translation (Zhen et al., 2013). MiR-21 induces translational inhibition of PDCD4 protein synthesis and targets PDCD4 mRNA post-transcriptionally (Asangani et al., 2008). Thus, the upregulated miR-21 in a variety of human cancers and inflammation have been associated with the loss of PDCD4 protein in these conditions (Lu et al., 2008b). Additionally, miR-21 mediated cell proliferation and migration through targeting PDCD4 in human pulmonary SMC (Sarkar et al., 2010). MiR-21 also protected cardiac myocytes in rats from ischaemia reperfusion injury through targeting PDCD4 (Cheng et al., 2010).

Another gene known to be a target for the actions of miR-21 is SPRY-1, a member of a protein family that negatively regulate receptor tyrosine kinase signalling by suppression of the activation of MAPK/ERK (Jazbutyte and Thum, 2010). In a study by Thum et al. (2008), upregulated miR-21 inhibited Spry-1 resulting in activation of MAPK that led to cardiac fibrosis and dysfunction in mice. Finally, both PDCD4 and Spry-1 were found to be involved in miR-21 mediated rat cardiac fibrosis (Bronnum et al., 2013).

RECK is a membrane-anchored glycoprotein and a novel regulator of MMPs. Its main function is to protect the ECM by negatively regulating expression and activity of MMP-2, MMP-9 and MT1-MMP (Oh et al., 2001, Takahashi et al., 1998). RECK is another tumour suppressor gene that prevents angiogenesis, invasion and metastasis and is documented to be a target of repression by miR-21 in different tissues including lung and gastric cancers, glioma cells and many others (Gabriely et al., 2008). Current studies have shown that the reduction or inactivation of RECK by miR-21 overexpression is strongly associated with increased cell proliferation, invasion, and apoptosis (Wu et al., 2014). Although these four genes have a strong presence in the literature and their roles have been explored in various tissues, the regulation of these genes by miR-21 in SV-SMC has not been investigated.

In addition to these four genes, miR-21 regulates several other genes in different human and animal tissues and these are listed in Table 1.4.

The aims of this chapter were therefore

- To explore the role of miR-21 in human SV-SMC proliferation.
- To identify the major targets of miR-21 and signalling pathways mediating its function in SV-SMC phenotypic changes, cell proliferation, and vascular remodelling.

6.2. Effect of PDGF-BB on SV-SMC proliferation

Having previously observed the significant upregulation of miR-21 mediated by PDGF-BB (1 and 10 ng/ml) in chapter 4, we proceeded to investigate the link between the PDGF-induced changes in cell proliferation and miR-21 overexpression in SV-SMC. To determine a suitable concentration of the mitogenic growth factor PDGF-BB for inducing cell proliferation, SV-SMC were seeded at a density of 1×10^4 cells per well in FGM in 24-well plates. Cells were incubated overnight at 37 °C in 5% CO₂ to enable attachment, then quiesced in MGM for 72 h. At this point cell counts were performed on day 0 and the culture medium was replenished on the remaining cells with MGM containing either 1, 3, 5, or 10 ng/ml of PDGF. For each condition, cells were counted in triplicate and parallel wells of cells incubated in MGM or FGM acted as negative and positive controls, respectively. All culture medium was replenished with the same treatment at 48 h and cells were then counted 48 h later (Day 4). Cell proliferation showed a proportional increase in response to PDGF concentration from 1-10 ng/ml (n= 2, Figure 6.1). The highest concentration of PDGF (10 ng/ml) showed an approximately equal proliferation rate to FGM which is the positive control. A concentration of 3 ng/ml was chosen as a suitable submaximal mitogenic concentration that showed approximately 50% increase in cell count relative to MGM and was used in the subsequent proliferation experiments.

6.3. Effect of miR-21 overexpression/knockdown on SV-SMC proliferation

SMC proliferation is a key feature of neointimal hyperplasia and vascular remodelling, therefore the effect of miR-21 on cell proliferation was investigated. To examine the association of miR-21 with PDGF-related proliferation in ND SV-SMC, cells were transfected with premiR-21, antimiR-21 or their respective negative controls (premiR-ve and antimiR-ve) as described in Section 2.2.2. Briefly, 1×10^4 cells were seeded in triplicate in MGM in 24-well plates after being transfected and quiesced for 48 h. Following 24 h, cells from the four groups were counted at day 0. The remaining wells were treated with 3 ng/ml PDGF in MGM to stimulate cell proliferation at a submaximal level, as the cell cycle is growth factor-dependent. The medium was replenished on day 2 and cells quantified on day 4.

When data from five patients were pooled together, there was a trend of increased cell number at day 4 in the premiR-21 transfected cells relative to their controls, indicating an increase in cell proliferation (Figure 6.2A). However, knockdown of miR-21 in SV-SMC did not induce changes in proliferation rate from day 0 to 4 relative to antimiR-negative transfected cells ($n=5$, Figure 6.2B). Following measurement of the individual fold change in cell count for each group, the cells overexpressing miR-21 in SV-SMC exhibited a significant 1.8 fold increase in cell number at day 4 relative to premiR-negative transfected cells ($P < 0.01$), whereas the antimiR-21 transfected cells were comparable to control (Figure 6.2C), suggesting that miR-21 knockdown was not sufficient to reverse the PDGF effect on cell proliferation.

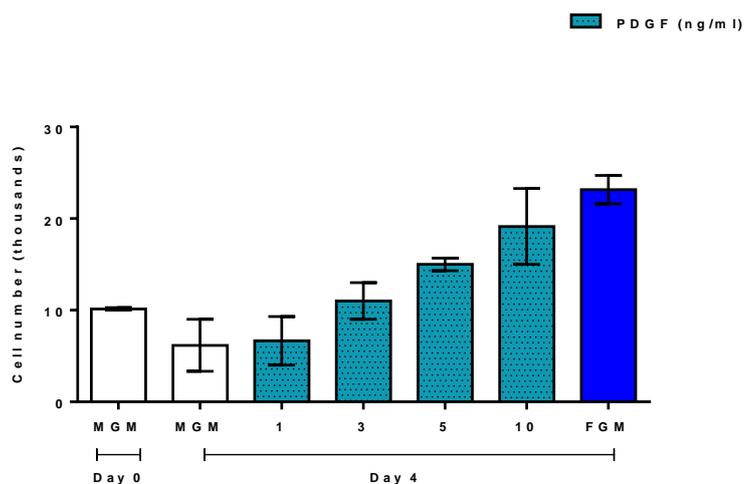


Figure 6.1: Concentration-dependent SV-SMC proliferation in response to PDGF-BB.

SV-SMC proliferation in response to PDGF-BB stimulation (1-10 ng/ml) relative to MGM (negative) and FGM (positive) controls. Cells were quantified at day 0 and 4 following PDGF treatment (n= 2).

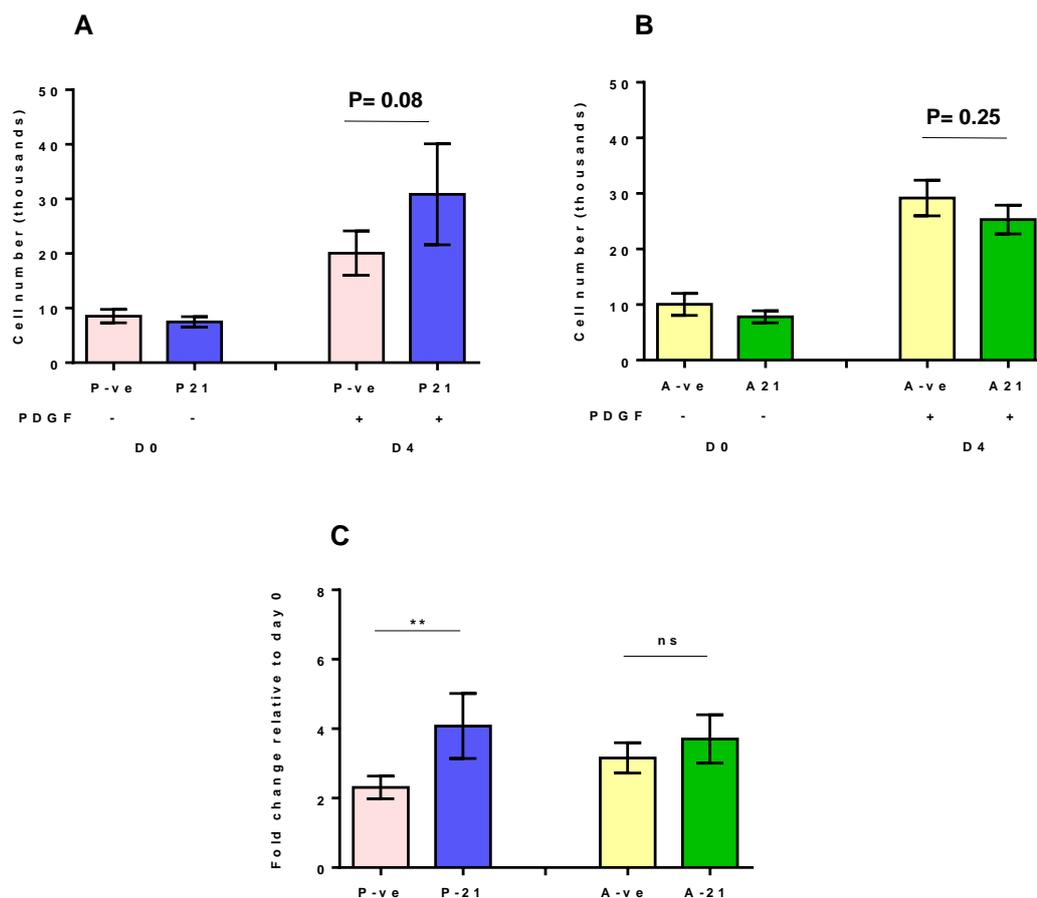


Figure 6.2: Effect of miR-21 upregulation/knockdown on SV-SMC proliferation. Proliferation rate of SV-SMC transfected with (A) 30 nM premiR-negative (P-ve) or premiR-21 (P-21), (B) 100 nM antimiR-negative (A-ve) or antimiR-21 (A-21), from day 0 - 4 in the presence of 3 ng/ml of PDGF. (C) Measurement of fold change in the transfected cell proliferation at day 4 relative to day 0 (** $P < 0.01$, ns = $P > 0.05$, paired t-test, $n = 5$).

6.4. Exploration of potential target genes involved in SV-SMC proliferation following miR-21 manipulation

Having observed a significant increase in cell number induced by miR-21 overexpression in SV-SMC, we proceeded to examine the downstream signalling pathway involved in the functional effects mediated by miR-21; particularly on cell proliferation.

Western blotting was conducted as described in Section 2.2.11 after miR-21 manipulation as in Section 2.2.2 for the four different genes (PTEN, PDCD4, SPRY-1 and RECK). These genes were chosen as they are documented as potentially negatively regulated targets of miR-21 and highly associated with SMC proliferation and migration (detailed in 6.1).

A. Effect of miR-21 modulation on PTEN, PDCD4, and SPRY-1 protein expression

SV-SMC were transfected with premiR-21 or anti-miR-21 for 6 h followed by 72 h incubation in MGM. Cell lysates were prepared and the effect of miR-21 manipulation on PTEN, PDCD4, and SPRY-1 proteins was measured using western blotting. Densitometry measurements for each patient lysate were normalised relative to β -actin as a loading control.

As shown in the representative blots there was no difference in PTEN (A), PDCD4 (B) and SPRY-1 (C) protein levels between the premiR-21 and anti-miR-21 transfected cells and their negative controls (Figure 6.5). The average densitometry of different patients samples also showed that PTEN (n= 6), PDCD4 (n= 4) and SPRY-1 (n= 3) protein expression was not affected by miR-21 manipulation in SV-SMC after 72 h of transfection (Figure 6.3).

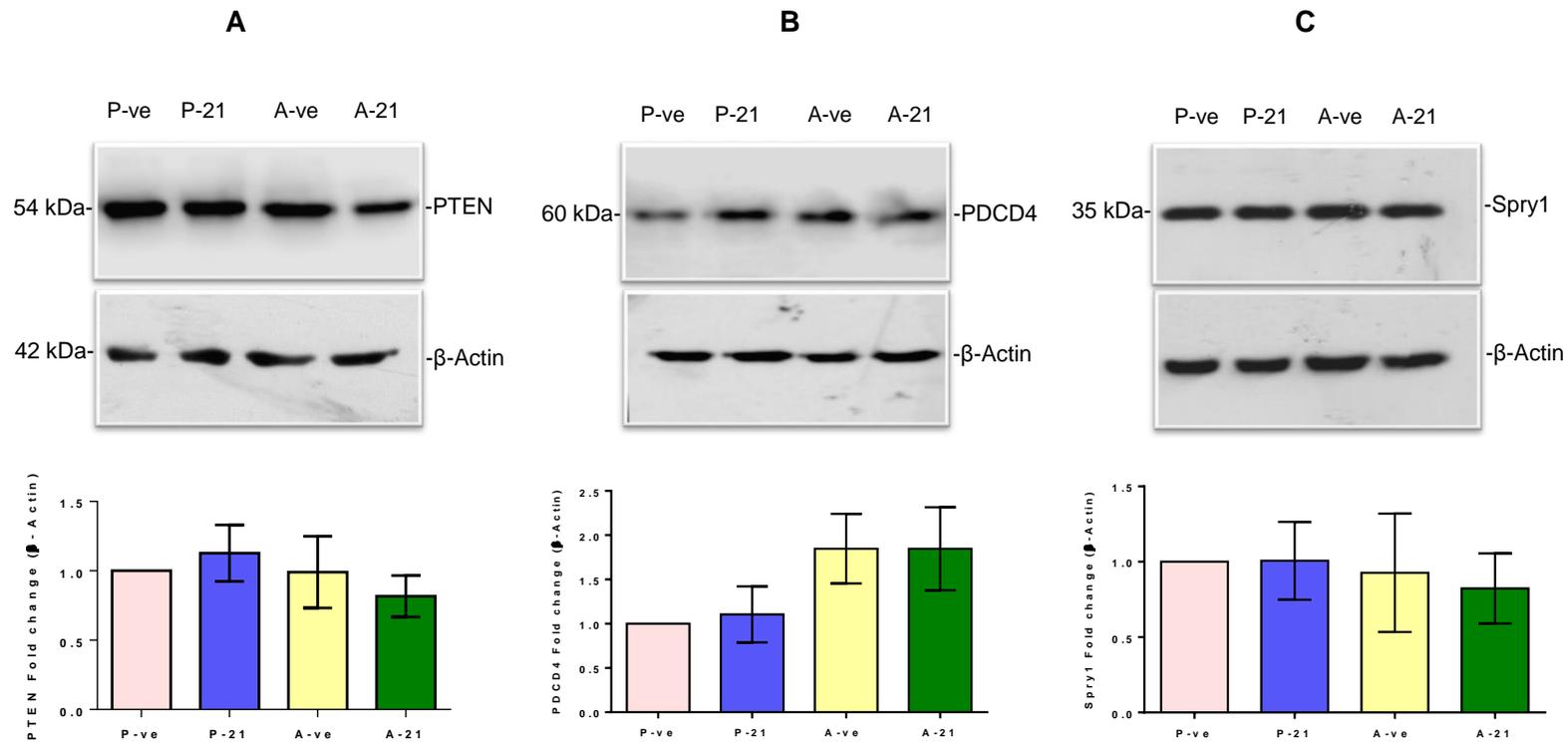


Figure 6.3: Quantification of PTEN, PDCD4, and SPRY1 protein after 72 h of premiR-21 and anti-miR-21 transfection in SV-SMC.

SV-SMC were transfected with 30 nM premiR-ve (P-ve) control, premiR-21 (P-21) or 100 nM anti-miR-negative (A-ve) control or anti-miR-21 (A-21). Representative immunoblots of PTEN (A, n= 6), PDCD4 (B, n= 4) and SPRY-1 (C, n= 3) protein levels 72 h following transfection are shown. All densitometry measurements were normalised to β-actin and the pooled densitometry data are displayed in the lower panel showing the average response of miR-21 overexpression or knockdown on the three proteins relative to their respective negative controls (all were non-significant, One way ANOVA with Newman Keuls post-hoc test).

B. Effect of miR-21 modulation on RECK mRNA expression and protein levels in SV-SMC

Recent studies have shown that miR-21 modulates cell growth and invasion through downregulating RECK in many cancer cell types (Wu et al., 2014). To test the effect of miR-21 on RECK expression in SMC, miR-21 was overexpressed and knocked down in SV-SMC. Following 72 h of transfection, cell pellets were collected for RNA extraction and RT-PCR. MiR-21 overexpression led to approximately 50% reduction in RECK mRNA expression ($P < 0.001$, $n = 8$, Figure 6.4A). However, miR-21 knockdown did not change RECK mRNA expression ($n = 4$, Figure 6.4B).

As shown in the previous chapter in Figure 5.4, transfecting SV-SMC with premiR-21 significantly maintained miR-21 overexpression up to 96 h following transfection. Having observed the inhibitory effect on RECK mRNA induced by the overexpression of miR-21, we next sought to examine the effect on RECK protein levels. Cell lysates of premiR-negative and premiR-21 transfected SV-SMC were collected for western blotting over a time course from 24 to 96 h. However, over this time interval, no significant changes in RECK protein were detected, as represented in the blot (Figure 6.5A) and the quantitative measurement of RECK protein ($n = 7$, Figure 6.5B). This indicates that the effect of miR-21 overexpression on RECK might not reach the translational effect at this relatively early time point.

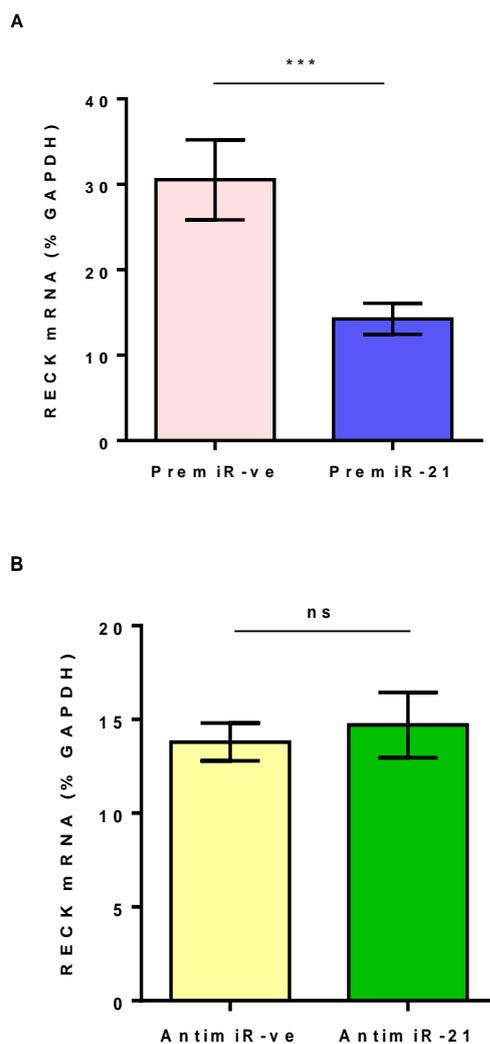


Figure 6.4: Effect of miR-21 overexpression/knockdown on RECK mRNA expression in SV-SMC 72 h following transfection.

RT-PCR of RECK mRNA of SV-SMC transfected with 30 nM premiR-negative or premiR-21 (A, n= 8) or 100 nM antimiR-negative or antimiR-21 for 72 h (B, n= 4). (***) $P < 0.001$, ns= not significant; ratio paired t-test). Gene expression was normalised to the endogenous control GAPDH and error bars denote SEM.

Whilst RECK protein levels appeared unresponsive to miR-21 overexpression when measured over 24-96 h post transfection, this raises the possibility that the turnover rate of RECK is low and the response to miR-21 is delayed until a later time point. Therefore, we then sought to determine whether an effect on RECK protein expression in SV-SMC could be observed at the later time point of days 8 and 10 post miR-21 transfection.

As shown in a representative immunoblot RECK protein was downregulated after day 8 in cells overexpressing miR-21 (Figure 6.6A). The quantitative measurement of RECK bands in five different samples consistently showed approximately a 40% reduction in RECK protein in the premiR-21 relative to premiR-negative transfected SV-SMC at day 8 (Figure 6.6B, $P < 0.001$) and to a lesser extent at day 10 (Figure 6.6C, $P < 0.05$).

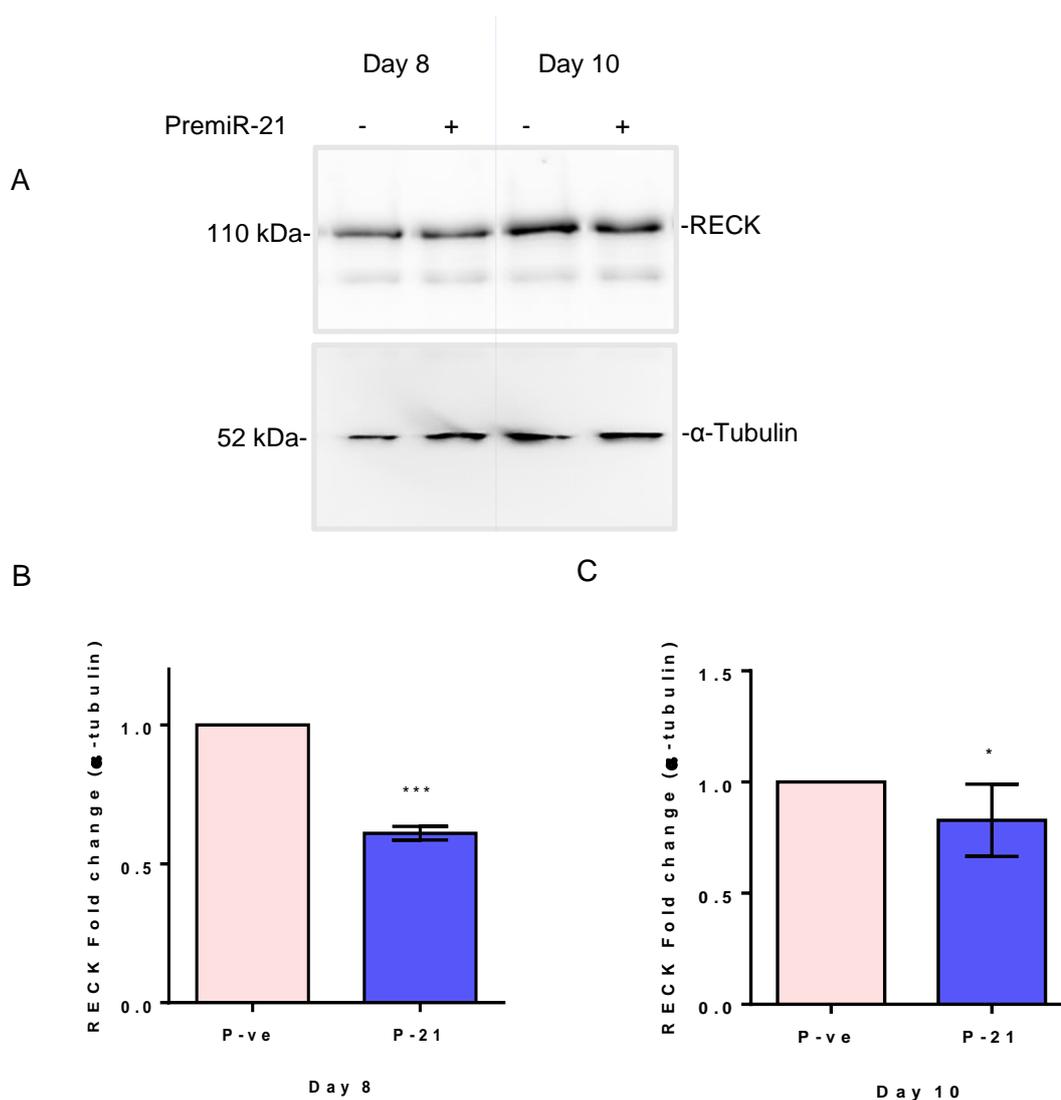


Figure 6.6: Quantification of RECK protein expression in SV-SMC following miR-21 overexpression at day 8 and 10.

SV-SMC were transfected with 30 nM premiR-negative (P-ve) or premiR-21 (P-21). Representative blot shows the effect of miR-21 overexpression on RECK protein at day 8 and 10 (A). Pooled densitometry data of the effect of miR-21 overexpression on RECK protein level in SV-SMC at day 8 (B) and day 10 (C) (***) $P < 0.001$ and *) $P < 0.05$, ratio paired t-test). Both $n = 5$, RECK bands were normalised to α -tubulin as a loading control.

6.5. Discussion

The present study demonstrated the role of miR-21 in significantly enhancing SV-SMC proliferation. In addition to this, four target genes of miR-21 were investigated. Interestingly, whilst miR-21 overexpression in SV-SMC did not appear to modulate PTEN, PDCD4, and SPRY1, it did downregulate RECK mRNA and protein levels, although the protein changes were not evident until 8-10 days following transfection.

The focus in this chapter was on SMC proliferation, which is a key cellular contributor of intimal hyperplasia. Vascular SMC are inherently stable in healthy vessels, however in response to injury, ECM degradation and mitogen secretion activate SMC phenotypic switching into a proliferative phenotype (Bennett et al., 2016). Additionally, upon injury, platelet adhesion and PDGF secretion into the vascular wall also stimulate SMC proliferation and migration into the intima (Marx et al., 2011). The effect of the mitogenic agent PDGF-BB on SV-SMC is of particular interest as the SV graft is a widely used conduit in CABG. The data in this chapter further confirmed, as expected the mitogenic effect of PDGF-BB, whereby PDGF-BB-induced SV-SMC proliferation in a concentration-dependent manner.

MiR-21 plays a critical role in cardiovascular diseases, as discussed in the earlier chapters, and was found to be significantly upregulated in different proliferative CVDs (Ji et al., 2007). In the present study, we investigated the regulation of SV-SMC proliferative phenotype by miR-21 modulation. The proliferation assay was performed in the presence of PDGF-BB which is an important growth factor that triggers cell cycle transition from G0 into G1 phase. To determine the potential role of miR-21 in modulating SV-SMC proliferation, a submaximal concentration of PDGF-BB (3 ng/ml) was introduced into the premiR-21 and anti-miR-21 transfected cells. In all five patients' samples, there was a noticeable increase in cell proliferation in the premiR-21 transfected cells at day 4 relative to premiR-negative control. However, the absolute cell count was variable and this was most likely due to the inherent differences in proliferation rate between cells from different donors. This inherent variation in SV-SMC function was demonstrated in Figure 3.4A and has been previously reported by our group (Madi et al., 2009). However, after normalising to fold change, cell proliferation at day 4 was found to be significantly increased in the premiR-21 transfected cells.

Thus, the result of increased SMC proliferation by miR-21 overexpression in our study was consistent with previous work performed by Ji et al., 2007. They induced injury in rat carotid artery that resulted in miR-21 upregulation. They also demonstrated the proliferative effect of miR-21 on cultured SMC and knocking down

miR-21 significantly reduced SMC proliferation. This suggests the important role of miR-21 in promoting cell proliferation and perhaps involvement in the process of intimal hyperplasia. On the contrary, in our study, knockdown of miR-21 did not show a reversal or change in SV-SMC proliferation at day 4 relative to control. The results in our study may indicate that the premiR-21 pathway to facilitate cell proliferation is more sensitive to miR-21 overexpression. Whilst anti-miR-21 transfection did not show a change in proliferation this suggests other miRs or transcription factors are more important and thus proliferation of the cells was persistent. As we showed previously that anti-miR-21 transfection was able to knockdown the overexpressed miR-21 (Figure 3.8), it would be useful to combine anti-miR-21 with premiR-21 to test the effect of miR-21 knockdown after miR-21 overexpression on modulating cell proliferation. This would be consistent with the study performed by Ji et al., 2007 as they induced injury to the artery following angioplasty to induce miR-21 aberrant upregulation, then they knocked down miR-21 (Ji et al., 2007). This potentially models the clinical situation while our cultured cells were not exposed to the pathological insult that normally upregulates miR-21 in SMC.

Overexpression of a single miR, such as miR-21 in our study, can repress more than 100 mRNAs, which then lead to a reduction in a large fraction of protein-coding genes (Miranda et al., 2006). Moreover, several miRs can act on the same target gene. The next logical step was therefore to identify the potential downstream target genes of miR-21 that may mediate the augmented response in SV-SMC proliferation. Four of the documented target genes (PTEN, PDCD4, SPRY1 and RECK) were studied at the protein level 72 h after overexpressing or knocking down miR-21.

Several cancer studies have confirmed the negative regulation of PTEN by miR-21 overexpression in a number of tissues such as liver (Meng et al., 2007a), breast (Han et al., 2012) and lung (Zhang et al., 2010). PTEN has consistently been demonstrated to be a target of repression by miR-21 leading to cell proliferation, migration and invasion in cancer cells, however, little is known about its role in vascular SMC (Jazbutyte and Thum, 2010). Additionally, the study performed by Ji et al. (2007) showed that miR-21 is involved in the neointimal formation and cell proliferation and the resultant *in vitro* reduction of proliferation in SMC treated with anti-miR-21 was associated with an upregulation of PTEN protein expression. In contrast to this work, we were unable to detect an inverse relationship between miR-21 and PTEN protein levels after 72 h of premiR-21 transfection and instead found there to be no correlation between the premiR-21 or anti-miR-21 transfection and PTEN protein levels relative to control. The discrepancy between the findings of Ji et al. (2007) and our recent data

is probably due to differences between species and tissues utilised. PTEN protein was measured in our present study at an early time-point (72 h post miR-21 transfection) and perhaps the effect of miR-21 modulation on its translation process was not yet reached, whilst in the study by Ji et al (2007), their PTEN measurement was conducted 14 days after angioplasty. Finally, it could be that PTEN is not a downstream target for miR-21 in SV-SMC. Our unexpected result is also supported by recent work performed by Hermansen and colleagues who demonstrated no correlation between miR-21 and PTEN using human glioblastoma cells (Hermansen et al., 2016). They suggested that this could be down to an absence of miR-21 binding sites on PTEN mRNA.

The other two documented target genes of miR-21 which are known to be involved in cell proliferation and migration were also tested by western blotting. PDCD4 is an important miR-21 target mediating an anti-apoptotic effect on vascular SMC and rat cardiac myocytes (Cheng and Zhang, 2010, Cheng et al., 2009b). Additionally, miR-21 downregulated Spry-1 in mouse model which is known to regulate cardiac fibroblast growth (Thum et al., 2008). In our study, both PDCD4 and SPRY-1 protein levels were measured 72 h after SV-SMC transfection with premiR-21 and anti-miR-21. The result showed no significant difference between premiR-21 and anti-miR-21 relative to their negative controls for both genes. This is perhaps due to the previously mentioned reasons about the lack of changes to miR-21 modulation obtained with PTEN, including the early time of measurement or these genes are not targets of miR-21 in SV-SMC.

RECK is an important negative regulator of MMPs (particularly, MMP-2 and MMP-9) that degrade ECM and are involved in regulation of several human physiological and pathological conditions. Different studies documented that RECK is negatively regulated by miR-21 in glioma cells (Gabriely et al., 2008), lung and gastric cancers (Zhang et al., 2008, Wu et al., 2014). Various studies have also shown that RECK plays a crucial role in cell proliferation (Li et al., 2012, Wu et al., 2014). RECK was also a target of repression by miR-21 in different types of cancer-mediated cell proliferation such as in oesophageal squamous cell carcinoma (Wang et al., 2013a), keratinised oral cancer (Jung et al., 2012) and prostate cancer (Reis et al., 2012).

Studies have demonstrated that factors stimulating vascular SMC proliferation, for example, growth factors, act through activation of the Rac1-Skp2-P27^{Kip} signaling pathway (Matozaki et al., 2000). Additionally, the normal cell cycle is regulated by cyclin-dependent kinases, which are inactivated by P27^{Kip} (Wu et al., 2008). SKP2 (S-phase kinase-associated protein 2) is important for P27^{Kip} degradation resulting in

increased cell proliferation. The effect of RECK on suppressing cell proliferation is mainly mediated by inhibiting SKP2 (Pan et al., 2015) as illustrated in Figure 6.7. Thus, this suggests that the suppression of RECK by miR-21 can facilitate cell proliferation by removing the suppressive effect on cell progression mediated by RECK as illustrated in Figure 6.8.

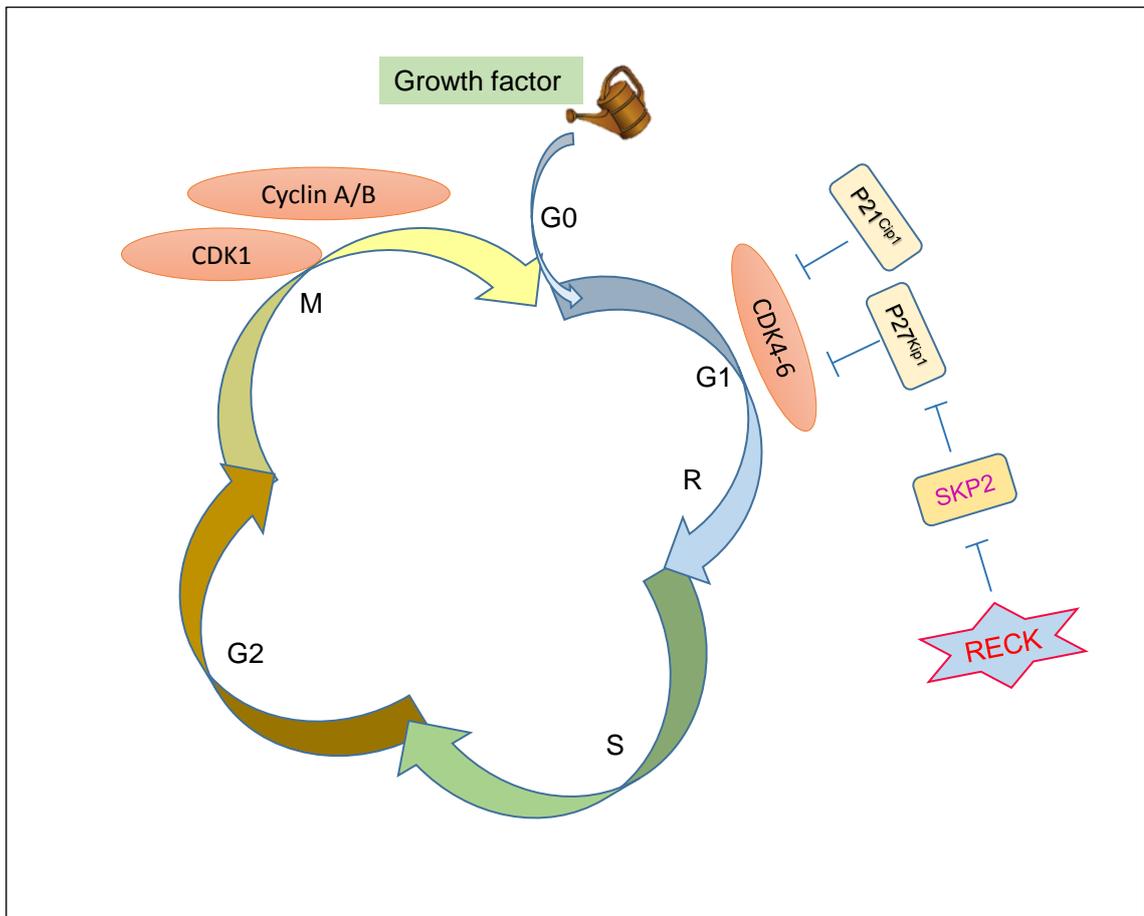


Figure 6.7: Schematic diagram shows the effect of RECK on cell cycle.

Cell cycle progression is dependent on the regulated expression and activation of a set of kinases (cyclin-dependent kinases (CDK) and cyclins which phosphorylate proteins that regulate cell growth. CDK inhibitors P21^{Cip1} and P27^{Kip1} delay cell cycle progression by inhibiting CDK activity. RECK can suppress cell cycle by downregulation of SKP2 and consequent upregulation of P27^{Kip1}.

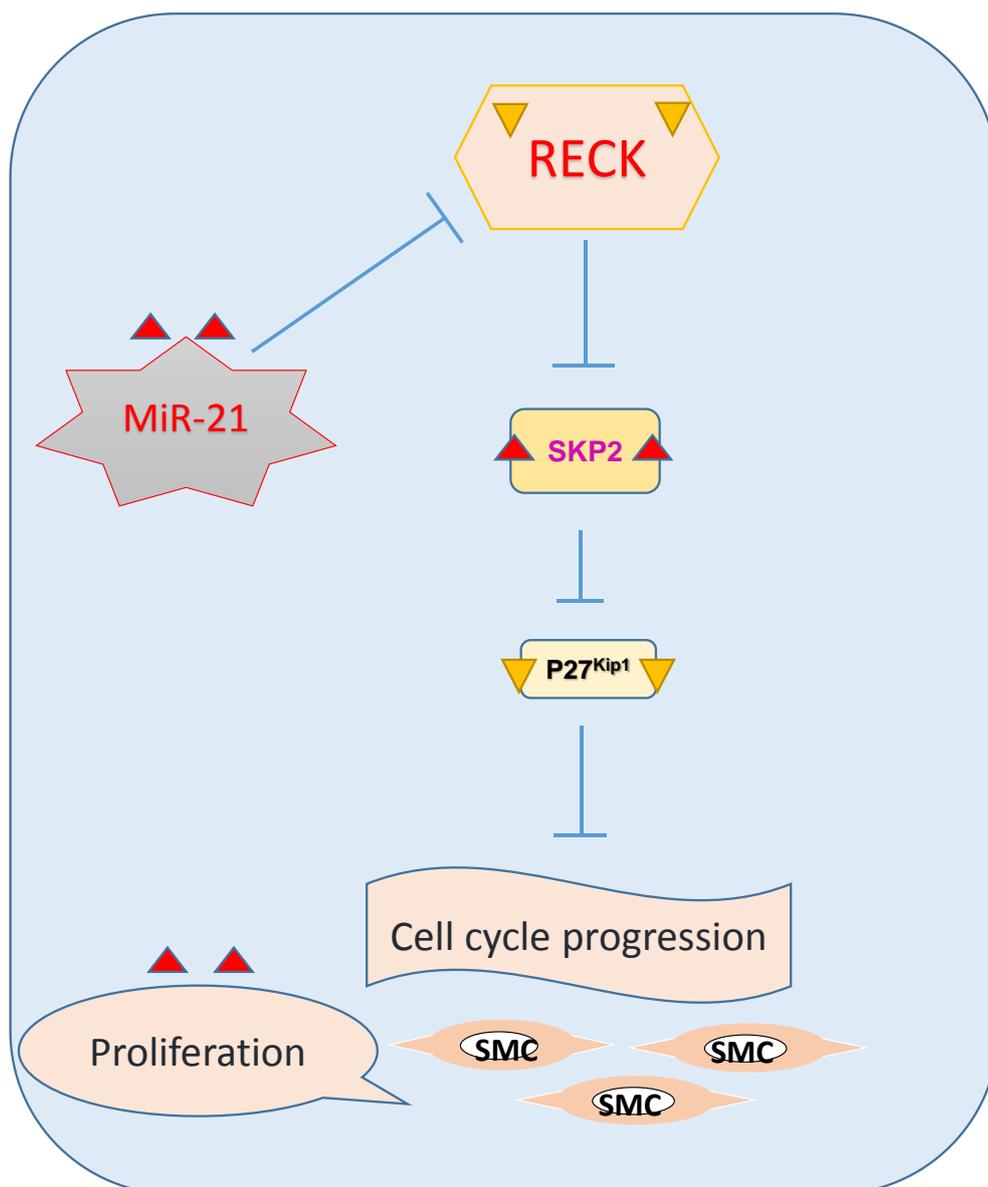


Figure 6.8 Diagram demonstrating the mechanism of action of RECK and its relation to miR-21 and SV-SMC proliferation.

A model for RECK targeted by miR-21 in relation to cell cycle regulating factors and SMC proliferation. Overexpression of miR-21 negatively regulates RECK expression, consequently triggering SKP2 activation and P27^{Kip1} degradation. This will remove the repression effect on cell cycle progression and eventually increase cell proliferation. SKP2 (S-phase kinase-associated protein 2) and P27^{Kip1} (also known as cyclin-dependent kinase inhibitor 1B).

In the present study, using RNA samples of SV-SMC from eight different donors, overexpressing miR-21 induced a significant reduction in RECK mRNA 72 h after transfection. In contrast, the anti-miR-21 transfected cells did not show any significant difference in RECK mRNA expression relative to control, indicating that the impact of miR-21 on RECK expression was most apparent when levels of miR-21 were increased by premiR-21. The SV-SMC that were utilised in all the functional assays were not compromised by the pathological stimulus which aberrantly upregulates miR-21 as would occur in pathological conditions to be able to knock it down. Therefore, this probably makes it difficult to detect any effect in anti-miR-21 experiments. Moreover, whilst miR-21 is knocked down, other miRs are still active and can modulate cell proliferation through other target genes.

MiRs downregulate their target mRNA levels, mediating degradation and repression of protein synthesis (Dangwal et al., 2012). Therefore, it was important to validate that the achieved reduction in RECK mRNA was translated into a reduction in RECK protein. The effect of miR-21 overexpression on RECK protein was analysed by western blotting. However, unexpectedly, changes in RECK protein were not detectable at any time points up to 96 h. Different proteins have different turnover rates and the lack of response to miR-21 overexpression at earlier time points suggested that the turnover rate of RECK is relatively slow.

To check whether this was the case, RECK protein measurement was performed at day 8 and 10 following miR-21 overexpression. The result showed that miR-21 overexpression significantly reduced RECK protein levels in the SV-SMC of five different donors at both time points. This also confirms that the turnover rate of RECK protein is low and thus the changes in its level are not detectable shortly after transfection.

Finally, the effect of miR-21 upregulation on increasing SV-SMC proliferation was a key finding in this study. Therefore, it is important to follow up this observation and map the expression of RECK mRNA and protein levels for miR-21 functional effects, particularly in cell proliferation. Following up future work would identify whether miR-21 is involved in mediating functional or phenotypic changes through RECK gene repression.

Chapter 7
GENERAL DISCUSSION AND CONCLUSIONS

Chapter 7

General discussion and conclusions

Coronary artery disease is a chronic disorder which remains the major cause of death worldwide. When coronary blood flow is compromised, CABG surgery using the IMA and SV is mandatory to re-route the coronary circulation. However, neointimal hyperplasia and graft restenosis remain important clinical problems and are considered a major limitation following the use of SV in CABG surgery. T2DM is one of the major metabolic disorders which increases the patients' susceptibility to cardiovascular diseases. Moreover, T2DM patients are prone to higher rates of CABG surgery and associated with worse consequences following SV graft surgery (Berry et al., 2007). Although a large amount of research is directed to targeting intimal hyperplasia and prolonging graft patency, none of the available therapies treat the original factor(s) involved in this process and robust therapeutics are lacking. Therefore, further investigation of the molecular mechanisms of intimal hyperplasia is mandatory to improve the patency of SV after CABG.

Recent research highlighted the important role of miRs in epigenetic regulation of the vasculature (Alexander and Owens, 2012) and in particular, vascular remodelling (Albinsson and Sessa, 2011). The aberrant expression of miRs is highly associated with cardiovascular abnormalities and therefore, these miRs could be possible novel therapeutic targets in regulating vascular remodelling. Distinct miRs are also implicated in T2DM and its related cardiovascular diseases including neointimal lesion formation following vein grafting (Alexandru et al., 2016, Riches et al., 2014).

MiR-21 is one of the most highly expressed miRs in the vasculature and is strongly associated with increased cell migration and proliferation (Wang et al., 2015a). However, relatively few studies have linked miR-21 to vascular remodelling and SV graft failure. Vascular SMC play a vital role in neointima formation and vascular remodelling following SV grafting owing to their plasticity and phenotypic switching into a synthetic and migratory phenotype (Alexander and Owens, 2012). Neointima formation is highly associated with excessive SMC proliferation and migration; therefore we decided to explore the role of miR-21 in vascular remodelling, particularly with respect to human SV-SMC phenotypic changes.

SV-SMC from T2DM patients exhibited a distinct phenotype that was previously reported by our group (Madi et al., 2009). The diabetic SV-SMC were characterised by their rhomboidal shape and slower proliferation rate in comparison to cells from non-diabetic counterparts. Therefore, the central research question was whether miR-21 was aberrantly expressed in native T2DM SV-SMC and was responsible for cellular dysfunction that conceivably contributes to the poor outcomes of SV grafting in T2DM patients. Whilst the results of this study emphasised that SV-SMC from T2DM patients exhibited distinct morphological and functional characteristics from ND counterparts, the level of miR-21 was comparable between T2DM and ND SV-SMC. Therefore, we proceeded to investigate the role of miR-21 expression levels in regulating SV-SMC phenotype and function independent of T2DM.

SMC are the principal cellular component of the vascular wall and their plasticity is very important for both normal adaptation to the arterial circulation and vein remodelling. When this remodelling becomes abnormally excessive in the conduit, adverse remodelling subsequently leads to intimal hyperplasia. Thus, miR-21 was artificially overexpressed or knocked down in native SV-SMC to ascertain which phenotypic and functional changes were mediated by miR-21 modulation. SV-SMC were treated with 'diabetic factors' and various mediators involved in vascular remodelling to identify which physiological and pathological factor(s) could modulate miR-21 expression. Whilst "diabetic" stimuli had no significant effect on miR-21 expression, PDGF-BB demonstrated a robust miR-21 overexpression in SV-SMC and therefore, it was utilised in the subsequent functional assays. A broad range of downstream target genes of miR-21 that are related to vascular remodelling were investigated using a focussed 'atherosclerosis' RT-PCR array. Out of the 84 array candidate genes, a follow up RT-PCR showed significant changes induced by miR-21 overexpression on MMP-1 and IL-1A mRNA expression.

To address our research question and to further achieve the goals of the research, MMP-1 was followed up to test the effect of miR-21 in inducing SV-SMC invasion through MMP-1 modulation. The regulation of miR-21 on SV-SMC proliferation, the second principal factor of intimal hyperplasia, was also explored. Finally, the findings obtained in this thesis indicate that miR-21 undoubtedly plays an important role in the regulation of the SMC phenotype and hence can be implicated in intimal hyperplasia and vascular remodelling. The main findings of this study are summarised in the following section.

7.1. Principal findings

7.1.1. Distinct phenotypic differences between T2DM and ND SV-SMC that were not associated with miR-21 overexpression

Both SV-SMC obtained from T2DM and ND patients co-expressed the characteristic SMC marker genes α -SMA and SM-MHC, identifying them as SMC. Consistent with previous studies obtained by our group (Riches et al., 2014), SV-SMC cultured from T2DM exhibited a predominantly rhomboidal phenotype, increased average cell area, and slower proliferation rate. In contrast, the ND SV-SMC exhibited mainly classical spindle-shaped elongated SMC morphology, with higher proliferation rate. These distinct phenotypes probably play an important role in increasing the prevalence of cardiovascular diseases and impaired vascular remodelling in T2DM compared to ND counterparts. Owing to their slow proliferation rate, this may underlie an impaired adaptation of diabetic cells within SV grafts used to bypass the arterial circulation. A rhomboidal dedifferentiated SMC phenotype was also reported to be a prevalent phenotype in atherosclerotic lesions and intimal hyperplasia (Hao et al., 2003). The characteristic larger cell area could predispose the diabetic SV graft to earlier neointimal lesion formation. Perhaps with poor glycaemic control, loss of the associated intercellular interaction or a higher apoptotic propensity of the expanded cell size results in accelerated intimal occlusion in diabetic patients. This can therefore explain, at least in part, the higher tendency to graft failure in diabetic patients following SV graft surgery (Gao et al., 2015).

Whilst miR-21 expression was elevated in SV-SMC of a small number of five T2DM patients relative to non-diabetic counterparts, this difference was not reproducible when the number of samples was increased to 16. A subsequent power calculation revealed that a sample number of 11 was required for each group to obtain 80% power of detecting a 50% difference between the two groups. Therefore, the initial assumption that was built on the elevated miR-21 in five diabetic samples was underpowered to make robust conclusions. In contrast, the comparable level of miR-21 expression obtained from the 16 different samples was powered enough to draw the conclusion that miR-21 levels were not associated with diabetic status and thus subsequent experiments of the project were carried out on SV-SMC of non-diabetic patients.

7.1.2. PDGF-BB significantly upregulated miR-21 in SV-SMC through activation of Akt and ERK pathways

The results of our study clearly showed a significant upregulation of miR-21 expression by 1-10 ng/ml PDGF in SV-SMC (Figure 7.1A). We also showed that blocking either one of the two main signalling pathways of PDGF (Akt or ERK) abrogated the PDGF-induced miR-21 overexpression, indicating that both pathways are linked to each other and both pathways are involved in miR-21 upregulation as inhibiting either pathway abolished miR-21 upregulation.

PDGF-BB is an important chemoattractant and mitogenic factor known to be activated in response to vascular injury. PDGF-BB exerts its biological action through binding and phosphorylation of protein tyrosine kinase receptors (PDGFR α and PDGFR β). Dimerisation of the activated PDGF receptors mediates activation of several downstream intracellular pathways through PI3K, Src, Ras, Raf and PIP2 pathways which eventually lead to phosphorylation of ERK/MAP kinase and Akt pathways within the target cell (Farooqi and Siddik, 2015, Lopatina et al., 2014).

The genomic location of pri-miR-21 is reported to be on chromosome 17q23.2., in the 10th intron where miR-21 overlaps with the protein-coding gene, TMEM49 (Patel and Nouredine, 2012). Regulation of miR-21 expression is conserved and controlled by its own promoter sequence (Fujita et al., 2008). The promoter of miR-21 can be controlled independently of the overlapping gene. MiR-21 transcription can be activated by activation protein 1 (AP-1) and by other multiple transcription factors and hormones including signal transducer and activator of transcription 3 (STAT3), oestrogen and the androgen receptor (Jazbutyte and Thum, 2010).

The transcription factor AP-1 complex is composed of Fos (c-fos, FosB, Fra1 and Fra2) and Jun (c-jun, JunB and JunD) proteins (Eferl and Wagner, 2003). ERK is reported to be an upstream regulator of Fra2 and JunB (Reich et al., 2010, Cevik et al., 2008). Additionally, phosphorylation of PI3K/Akt by PDGF is reported to induce selective modulation of c-jun and Fra1 which are major components of AP-1 (Ramachandran et al., 2010). Therefore, the underlying mechanism of miR-21 overexpression could be a dual activation of Fra2/JunB by ERK and c-jun/Fra1 by Akt signalling pathways.

PDGF has previously been shown to modulate the expression of several other miRs, mediating an increase in cell proliferation and migration (Yu and Li, 2014). Overexpression of miR-31 accompanied by increased SMC proliferation was reported in rat carotid artery treated with 20 ng/ml of PDGF. Interestingly, the selective MAPK/ERK pathway inhibitor PD98059 significantly reduced miR-31 expression in

SMC (Liu et al., 2011). PDGF also induced miR-221 expression alongside the PDGF-BB-mediated increase in pulmonary artery SMC proliferation and migration (Davis et al., 2009). Knocking down these two miRs (miR-31 and miR-221) partially reduced PDGF-mediated SMC dedifferentiation. Conversely, other miRs were downregulated by PDGF-BB including miR-30, miR-198 and miR-216 in different types of cancer cells (Shao et al., 2011).

A relatively recent study has shown that treating human hepatic stellate cells with 5 ng/ml PDGF significantly upregulated miR-21 and collagen α 1 expression (Wei et al., 2013) resulting in increased liver cell fibrosis. The positive association between PDGF-BB and miR-21 in SV-SMC suggests that the regulatory effect of PDGF on SMC proliferation and migration could possibly be mediated through miR-21 upregulation.

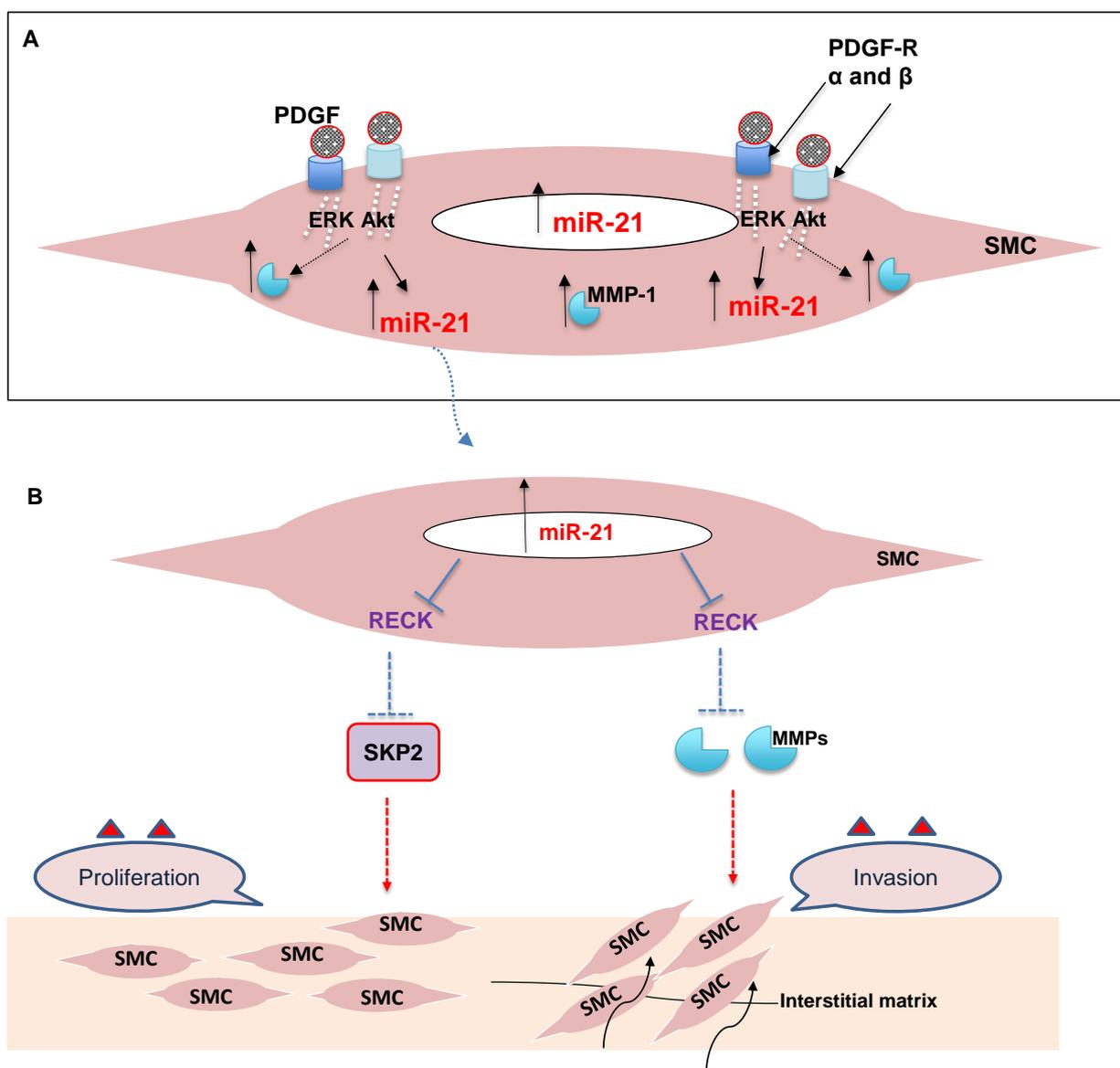


Figure 7.1: Schematic illustration showing the effect of PDGF-BB on miR-21 and the upregulation of miR-21 on SV-SMC function.

Diagram illustrating PDGF-BB-induced activation of PDGF-Rs and upregulation of miR-21 and MMP-1 expression in SV-SMC (A). Overexpression of miR-21 downregulated RECK that could be responsible for the increased SMC proliferation via inhibiting SKP2 and migration through inhibiting MMPs (B). Solid lines represent experimental results and dashed lines indicate an unconfirmed mechanism.

7.1.3. Overexpression of miR-21 induced MMP-1 mRNA expression

MiR-21 is reportedly elevated 4.6-fold in human atherosclerotic plaque (Raitoharju et al., 2011). For the first time to be documented, our human 'atherosclerosis' microarray and RT-PCR data showed that miR-21 overexpression induced upregulation of MMP-1 mRNA expression. Owing to the natural negative effect of miRs on gene expression, the upregulation of MMP-1 by miR-21 is almost certainly indirect, which makes this relationship challenging to uncover. This indicates that miR-21 may inhibit a gene that normally acts as a negative regulator for MMP-1 such as TGF- β 1 (Uria et al., 1998). The response of MMP-1 to different miRs is variable, as one would expect. Overexpression of miR-155 reduced the expression of MMP-1 whereas miR-203 overexpression showed an indirect increase of MMP-1 expression in rheumatoid arthritis synovial fibroblasts and this was highly correlated with chronic inflammation of the disease (Stanczyk et al., 2008).

Our study also showed that the upregulation of MMP-1 expression was observed in PDGF-treated SV-SMC, indicating a positive relationship between PDGF, miR-21, and MMP-1 (Figure 7.1). As described earlier the activation of PDGF receptors by PDGF leads to Akt and ERK phosphorylation which further stimulate downstream intracellular signalling pathways. Studies also reported that AP-1, which is an activating transcription factor for miR-21, directly activates MMP-1 transcription (Wang et al., 2012). Therefore, the precise signalling mechanism behind MMP-1 upregulation by PDGF, and whether miR-21 upregulation is involved in this pathway, would be interesting to delineate. It is reported that PDGF can upregulate MMP1 expression through activation of the ERK1/2 signalling pathway (Ito et al., 2009), therefore it would be pertinent to block the ERK pathway in the PDGF-treated SV-SMC and measure whether MMP1 and miR-21 are regulated.

Both PDGF and MMP-1 are important mediators involved in SMC phenotypic switching and vascular injury (Metz et al., 2012). MMP-1 is a collagenase that preferentially degrades type I and III collagen; facilitating SMC migration and invasion in the vessel wall. MMP-1 plays an important role in vascular remodelling and clinical studies have also reported that high serum levels of MMP-1 are a useful predictor of human atherosclerosis (Lehrke et al., 2009). In an experimental mouse model, blocking MMP-1 with FN-439 (a selective MMP-1 inhibitor) following carotid injury significantly attenuated neointima formation inside the artery (Austin et al., 2013a). Therefore, by further understanding the mechanistic link between miR-21 and MMP-1 upregulation, antimiR-21 therapy could potentially provide a protective therapeutic strategy for human intimal hyperplasia.

7.1.4. Overexpression of miR-21 increased SV-SMC area and proliferation

A number of studies reported that miR-21 expression was dysregulated in various proliferative and migratory cancer and cardiovascular diseases (reviewed in Bonci (2010)). In our study, overexpression of miR-21 in SV-SMC altered the typical spindle SMC morphology, resulting in increased average cell area. Therefore, miR-21 was able to drive phenotypic changes in SMC that are characteristic of those observed in both native T2DM and dedifferentiated SMC phenotypes in general. These dedifferentiated phenotypes that are also reported to be involved in atherosclerotic lesion may cause enzymatic changes and enhanced fibrosis inside the intima (Hao et al., 2003, Campos et al., 2016) and thus, the upregulation of miR-21 in SV-SMC can be implicated in atherosclerosis and neointimal occlusion.

A key finding in our study was that overexpression of miR-21 significantly increased SV-SMC proliferation. Whilst my project was in progress, an important study was published by McDonald et al. (2013). They reported a significant upregulation of miR-21 expression in *ex vivo* models of porcine, mouse and human SV graft models, mediating neointima formation in comparison to the ungrafted SV. When they blocked miR-21 in the human *ex vivo* SV graft with 5000 nM antimiR-21 or using miR-21 ablated mice, the neointima was significantly reduced in the engrafted SV. Therefore, the increased miR-21 was implicated in increasing SMC and fibroblast proliferation and thus increased neointima formation.

Our finding is highly important as increased SMC proliferation is an important risk factor for proliferative cardiovascular diseases. The characteristic dedifferentiated and proliferative phenotype of SMC is positively correlated with neointima formation following SV graft surgery (Sur et al., 2014). Numerous other studies have documented increased tumour growth induced by miR-21 via inhibition of tumour suppressor genes including PTEN and PDCD4 (Luo et al., 2015, Bao et al., 2013). Moreover, RECK has an anti-proliferative effect by suppressing cell cycle progression as illustrated in Figure 6.7. RECK was validated as a target of repression by miR-21 that induced lung cancer cell line growth and proliferation (Xu et al., 2014). However, the target genes by which miR-21 regulates SV-SMC proliferation are not yet identified and this is a worthy area for further investigation (Figure 7.1).

7.1.5. RECK was identified as a novel target of miR-21 overexpression

Various studies have demonstrated the vital role of RECK in relation to angiogenesis and cell migration (Takahashi et al., 1998, Yoon et al., 2003). RECK was shown to be a target of repression by miR-21 in different types of cancer cells. For example, miR-21 was reported to increase proliferation and invasion of gastric cancer (Zhang et al., 2008), glioma (Gabriely et al., 2008) and lung cancer cell lines through direct inhibition of RECK (Xu et al., 2014). However, to the best of our knowledge, RECK expression and its modulation by miR-21 has not been investigated with respect to neointima formation and vein graft failure.

Our preliminary finding demonstrated a novel finding by identifying RECK as a target gene of miR-21 in SV-SMC (Figure 7.1B). Overexpression of miR-21 significantly downregulated RECK mRNA at the earlier time point (at day 3) and a subsequent reduction of protein levels was detected at a later time point (day 8-10). Therefore, targeting RECK by miR-21 would be implicated in the phenotypic changes mediated by miR-21 overexpression.

RECK is a membrane-anchored protein that protects ECM and regulates basement membrane remodelling; mainly by suppressing MMP-2, MMP-7, MMP-9 and MT1-MMP (Oh et al., 2001, Miki et al., 2007). It suppresses MMP secretion from the cell and competitively inhibits MMP protease enzymatic activity (Welm et al., 2002). In addition to its anti-migratory effect, RECK has an anti-proliferative effect that is mainly mediated by inhibiting SKP2. SKP2 is an important stimulus for cell proliferation by mediating P27^{KIP} degradation which usually blocks cyclin-dependent kinases (Figure 6.7) (Pan et al., 2015). This therefore suggests that the suppression of RECK by miR-21 can facilitate cell proliferation by removing the negative effect on cell progression mediated by RECK as illustrated in Figure 7.1B.

7.2. Strengths and limitations

Owing to the high propensity of SV graft failure, the use of SMC from human SV samples which are harvested for CABG surgery strongly links the findings to the clinical problem. Therefore, a major strength of this thesis is the use of SMC explanted from different patients allowing us to study the effect of miR-21 on this specific cell type. Another strength of this study was the use of multiple patients' samples in each experiment to reflect inter-individual variation from a wider population. In this study, SV-SMC were cultured from a total of 136 patients recruited between August 2012 and January 2016. Although the majority of CABG patients are T2DM patients (Riches

et al., 2014), we deliberately selected ND patients in chapters 4-6, therefore the majority of SV-SMC were from ND patients (78%). The mean \pm S.E.M. age of the patients was 64 years \pm 0.9. Most of them were males (88%), reflecting the high prevalence of coronary artery disease and CABG in male patients.

The walls of blood vessels normally experience a dynamic environment that is subjected to forces mediated by pressure variations and haemodynamic factors such as shear stress and the vasoactive property of the wall itself. It is worth mentioning that all of the functional experimental studies in this thesis were conducted by using *in vitro* SMC under static culture conditions and importantly in the absence of a defined vascular injury. The lack of an interaction with the haemodynamic environment and the contribution of other cell types and matrix molecules in our cell culture models are obvious limitations. In addition there was a high degree of variability in the response of cells obtained from different patient's samples and a large number of samples were needed to achieve sufficient statistical power.

7.3. Future directions:

7.3.1. Investigate the role of miR-1260 and miR-1280

An initial screening was performed to identify the levels of miR expression in SV-SMC between 6 T2DM samples relative to 6 ND samples. The results obtained from a High Throughput Sequencing approach (Vertis Biotechnologie AG) were presented in Table 3.1. Relatively more is known about miR-145 and miR-21 which have been studied widely in cancer and cardiovascular fields, indicating their important roles in physiological and pathological conditions. Although there was a high fold increase of miR-1260 and miR-1280 in T2DM relative to ND, there are only two studies implicating miR-1280 in metastasis of bladder and pancreatic cancer cells (Majid et al., 2012, Piepoli et al., 2012), but nothing in the cardiovascular field. Similarly there are no existing studies suggesting high expression of miR-1260 in cardiovascular diseases or involvement of this miR in any other type of human disease which make them challenging to undertake in this thesis. Studying miR-1280 and miR-1260 as an extension to the current project might be attractive to investigate their role in cardiovascular diseases.

7.3.2. Silencing RECK gene and test the effect of miR-21 modulation on SV-SMC function

It is known that increased SMC proliferation and migration from the media into the intima plays a major role in neointimal hyperplasia and vein graft occlusion. Having obtained a significant increase in SV-SMC proliferation induced by miR-21 and given that miR-21 overexpression suppressed the RECK gene, it would be a worthwhile aim to investigate and confirm whether the effect of miR-21 on SMC migration and proliferation is mediated through RECK inhibition (Figure 7.1).

The increased SV-SMC proliferation induced by miR-21 was observed 7 days following transfection which is equivalent to the time at which miR-21 overexpression showed a significant suppression of RECK protein levels. As an extension of the increased SMC proliferation effect induced by miR-21, it would be an attractive strategy to use siRNA to knock down RECK, then the effect of miR-21 on SMC proliferation could be measured in the presence and absence of RECK gene silencing. If silencing RECK showed a reduction in cell proliferation in the cells overexpressing miR-21, this would provide novel evidence for RECK in miR-21-induced SMC proliferation. RECK has been reported to antagonise cell proliferation by inhibiting SKP2 (Figure 6.8), which activates cyclin-dependent kinase in the G1 phase of the cell cycle and therefore its suppression promoted cancer cell proliferation (Pan et al., 2015).

Flow cytometry, or fluorescence-activated cell sorting (FACS), is a widely used method to stain DNA and assess cell cycle progression. FACS analysis could be used to test the effect of RECK modulation on cell cycle progression. The FACS technique is able to identify and quantify each phase of the cell cycle affected by miR-21-induced RECK modulation, namely G0 (resting phase), G1 (metabolic changes), S (DNA synthesis), G2 (protein synthesis) and M (cell division) (Kim et al., 2012). SMC would be seeded in a 6-well plate, transfected with premiR-21 or antimiR-21 and incubated for 7-8 days. Cells could then be harvested and the effect of RECK on cell cycle progression measured by FACS.

Another important property of RECK that could regulate SV-SMC function is its role in inhibiting cell migration. MMP-2 and MMP-9 are the two most affected proteases inhibited by RECK, so these MMPs could be measured to test their contribution in SMC migration and invasion. Conditioned media could be collected from miR-21 transfected migrated cells and gelatin zymography and/or ELISA used to measure the proteolytic activity of MMPs. Using this approach we could measure the secreted MMP-2 and MMP-9 and determine whether their activity is enhanced by suppressing

their negative modulator (RECK) by miR-21 overexpression. Similarly, RECK siRNA could be utilised to investigate the effect of miR-21 on regulating MMP-2 and MMP-9 activity and SMC migration in the presence and absence of RECK gene silencing.

The unexpected negative result obtained in chapter 5 (when miR-21 overexpression did not increase SV-SMC invasion through collagen-coated chambers) does not rule out an enhanced migratory effect of miR-21 *per se*, as miR-21 overexpression has been implicated in increasing cell migration and tumour cell metastasis by direct suppression of PTEN, PDCD4 and RECK (Zhou et al., 2013). It would be relevant to confirm the positive effect of miR-21 on SMC migration by repeating the Boyden chamber assay in the absence of collagen coating and loading premiR-21 and anti-miR-21 transfected SV-SMC in the upper chamber. This assay would allow us to test the effect of miR-21 on cell migration (i.e. motility) without the need for matrix degradation, and at the same time all of the MMPs and TIMPs secreted in the conditioned media could be quantified using ELISA.

7.3.3. Identify the effect of miR-21 on intact SV neointima thickness

Intimal hyperplasia has important clinical implications in SV graft failure following CABG surgery. To test the effect of miR-21 on neointimal hyperplasia, an intact *ex vivo* human SV organ culture system has previously been validated as a promising model that partially mimics the clinical situation of *in vivo* SV responses to environmental changes.

Whilst under normal conditions the level of miR-21 is expected not to be elevated, it would therefore be important to induce vascular injury to increase miR-21 overexpression and measure the subsequent effect of miR-21 knockdown on the regulation of SV remodelling. To investigate the effect of miR-21 on neointimal thickness and vascular remodelling, a high concentration of anti-miR-21 could be added to the culture media to knock down miR-21 in the SV; a method utilised by McDonald et al 2013. Alternatively, the delivery of a modified anti-miR-21 to the target cells could be performed using a viral vector such as lentivirus or adenovirus (Chen et al., 2015). Other studies have demonstrated the efficacy of using exosome nanoparticles to encapsulate the target miR-21 and deliver it into the target cells (Zomer et al., 2010). At the end of the experimental period, neointimal thickness would be measured and SMC isolated by collagenase digestion to test the identified target genes of miR-21 (such as RECK) and this could be measured by RT-PCR. Additionally, serial sectioning of SV across various time points could be performed

followed by immunohistochemistry for visualising the vascular SMC marker genes α -SMA and SM-MHC to test the differentiation state of SMC.

A drawback of the organ culture model is lack of flow and pressure, however using a suitable bioreactor machine can mimic normal arterial blood flow. This model would provide clinical relevance as it maintains the cell-cell and cell-matrix interactions of different vascular cell types (e.g. endothelial cells and adventitial fibroblasts) in wound healing, following vascular injury. An isolated sample of SV could be maintained in tissue culture under optimised flow conditions and the level of miR-21 could be measured in different sections of SV at different time intervals, before and after introducing a catheter which induces tissue injury. Where elevated levels of miR-21 are observed, the intimal thickness could be evaluated by performing cross-sectioning of the SV segment and making micromorphometric measurements.

Haematoxylin and Eosin stains are utilised to visualise the vein sections for photomicrographs (imaging) and morphometric (shape) analysis of the injured SV segment and compared with a healthy uninjured SV. The size of the intimal thickness of the vascular wall can be quantified by tracing the stained sections and drawing out the boundaries around the cells. Additionally, SMC proliferation towards the intima can be quantified using Bromodeoxyuridine (BrdU), a useful assay to label replicating DNA and measure cell proliferation (Uemura et al., 2015, Porter et al., 1996) or immunostaining with proliferation cell nuclear antigen (PCNA). The proliferation rate is measured by counting BrdU/PCNA-positive cells that selectively highlight nuclei within S-phase (Castronuovo et al., 2002).

A well-known approach for gene delivery is the use of adenovirus vectors, however, an efficient delivery of gene therapy remains to be identified for the human SV organ culture model. The local and direct administration of gene therapy into the vein would also retain the therapy at the area of administration and minimise the risk of it reaching the systemic circulation (Southerland et al., 2013). The time period of miR knockdown should be temporary to stabilise the initial secretion of mediators and suppress genes involved in graft failure, at least until the SV graft adapts to the new arterial circulation to prevent early vascular remodelling. In an *ex vivo* porcine SV model experiment, an isolated SV was treated with adenovirus gene therapy of TIMP3 and grafted into the arterial circulation. A maintained reduction in neointima formation was effectively obtained up to 9 weeks following the surgery (George et al., 2011). A relatively recent study has shown a protective effect by using adenovirus-mediated delivery of sarco/endoplasmic reticulum calcium ATPase (SERCA) 2a isoform gene. This gene is important for normalising calcium levels and it has an important role for normal

cardiac function and an inhibitory effect on SMC proliferation. Transforming this gene into a human IMA *ex vivo* organ culture model antagonised neointima formation and in-stent restenosis that occurred following coronary intervention (Lipskaia et al., 2013). Clinically, targeting the selected gene in SV is easily accessible during harvesting the SV and before implanting into coronary circulation and this potentially minimises the risks of systemic effects. Therefore, utilising an *in vivo* model of vascular injury and targeting miR-21 in SV would provide a potentially challenging, yet useful therapeutic tool to prevent vital aspects of vascular remodelling caused by SMC proliferation.

Using an intact SV in the *ex vivo* model would also permit the study of changes in all of the 3 main parts of the blood vessel; the endothelial injury level, the media and the adventitia. The adventitial layer plays an important role in controlling blood vessel homeostasis and it also contains fibroblasts and collagen to provide structural support to the wall (Stenmark et al., 2013). The accumulation and migration of the adventitial cell components into the media and the intimal layer also contribute to intimal hyperplasia (Chen et al., 2013b). Therefore, this would also give a broader overview of the involvement of each vascular layer together with their cellular content in the development of neointimal hyperplasia.

7.3.4. Measuring circulating miR-21 in plasma of patients before and after CABG and precision medicine

Recent studies are highlighting the important role of miRs as critical biomarkers for different pathological diseases and survival rate. MiRs can be secreted into biological fluid and blood in microparticles or exosomes to resist degradation by RNase (Beltrami et al., 2015). Therefore, measuring the aberrantly expressed miRs in blood could reveal competent biomarkers for early detection of cardiovascular diseases and many other life-threatening conditions. The significantly high level of miR-21 in blood was measured as an indication of disease prognosis and survival rate of patients with late-stage pancreatic cancer (Bauer et al., 2012), hepatocellular carcinoma (Tomimaru et al., 2012), colon cancer (Li et al., 2013), gastric cancer (Wu et al., 2015), prostatic cancer (Huang et al., 2015) and breast cancer (Yang et al., 2015). Measuring miR-21 along with HbA1c in plasma of CAD patients undergoing CABG surgery might provide a useful tool as a biomarker of CVDs and SV graft failure. To our knowledge, no studies in the literature have reported miR-21 measurement in body fluid as a biomarker for cardiovascular diseases or T2DM. This could be a novel area of

research if miR-21 was measured as a marker to investigate the SV graft status following CABG.

As cardiovascular and diabetic patients exhibit a broad degree of heterogeneity, a future promising approach will be that of personalised medicine. Patients would first be stratified into diabetic and non-diabetic groups. Their future treatment could then be tailored according to the phenotypic characteristics of the individual. This is what is called 'precision medicine' which takes into account every individual's variability (Coote and Joyner, 2015). This includes analysing a patient's inherited genomic characterisation (such as proteomics, metabolomics and genomics) together with studying the epigenetic status of each individual which results from the interaction with environmental cues. As 50% of SV grafts fail within 10 years in the UK (Deb et al., 2012), research exploring the epigenetic factors involved in genetic regulation and disease pathogenesis could be a future therapeutic strategy. Therefore, it is worth classifying the patients and studying their epigenetic characteristics to try to determine the similarities and variabilities between each individual and to implement personalised therapy that helps prolong graft patency and prevent pathological vascular remodelling.

7.4. Conclusion

In summary, the results presented in this thesis suggest that miR-21 plays a vital role in modulating SMC function that leads to the development of intimal hyperplasia. This was concluded after demonstrating the ability of miR-21 overexpression to significantly increase SV-SMC area and cell proliferation. A focused array and follow-up TaqMan RT-PCR data showed that overexpression of miR-21 in SV-SMC upregulated MMP-1 expression. PDGF-BB, as one of the important factors upregulated in response to vascular injury, was positively correlated with the increase of miR-21 and MMP-1 expression. Moreover, this is the first study to reveal that the overexpression of miR-21 significantly increased SV-SMC proliferation and reduced RECK mRNA and protein levels. Further understanding of miR-21's downstream signalling and mechanistic effects would determine the promising role of miR-21 on SV-SMC function and provide a novel therapeutic target for the prevention of vein graft failure.

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