Does altered insulin signalling modulate vascular regeneration?

Dr Ben Nigel Mercer

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds
School of Medicine
July 2014

Statement on authorship of publications arising from this work

The candidate confirms that the work submitted is his/her own, except where work which has formed as part of jointly authored publications has been included. The contribution of the candidate and the other authors to 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.

The work in Chapter 5 has appeared in publication as follows:

Restoring Akt1 activity in outgrowth endothelial cells from South Asian men rescues vascular reparative potential. Cubbon RM, Yuldasheva NY, Viswambharan H, Mercer BN, Baliga V, Stephen SL, Askham J, Sukumar P, Skromna A, Mughal RS, Walker AM, Bruns A, Bailey MA, Galloway S, Imrie H, Gage MC, Rakobowchuk M, Li J, Porter KE, Ponnambalam S, Wheatcroft SB, Beech DJ, Kearney MT. Stem Cells. 2014 Jun 10. doi: 10.1002/stem.1766. [Epub ahead of print]

I was responsible for the results that have been presented in this thesis, where assistance has been given, it has been explicitly indicated.

Acknowledgements

The research included in this thesis is my own, though, where the assistance of other investigators has contributed, this is explicitly indicated.

I would like to thank my supervisors, Dr Richard Cubbon and Professor Mark Kearney for their insightful guidance, support and encouragement throughout my studies. I would like to offer particular thanks to Dr Nadira Yuldasheva, whose specialist surgical skills have been vital to this project.

This project would not have been possible without the financial support of the British Heart Foundation, and I thank them for funding my clinical research training fellowship. Next, I would like to thank all the researchers and technicians that have offered their assistance over the years. I am grateful for the help offered to me by: Mrs Stacey Galloway, Dr Hema Viswambharan, Dr Helen Imrie, Dr Anshu Sengupta, Dr Romana Mughal, Mrs Jessica Smith, Miss Anna Skromna, Dr Matthew Gage, Dr Kate Gatenby, Dr Andrew Walker, Dr Amir Aziz, Dr Piruthivi Sukumar, Dr Guy Gheradi and Dr Noman Ali. I am also grateful to all of the volunteers that have given up their time to participate in the project.

Finally, I would like to thank my family. Without the collective, unwavering support over the years from my parents and my wife, Kirsty, I would not have been in the position to carry out my studies and write this thesis. My two children Charlie and Gracie also deserve a mention, who have provided me with many, necessary distractions.

Abstract

The prevalence of insulin resistant syndromes is rising worldwide. Affected individuals are at increased risk of morbidity and premature mortality, much of which is driven by cardiovascular disease (CVD). Enhancement of vascular regeneration, using pharmacological or cell-based therapies, has been suggested as a strategy to help address these issues. Although many pathophysiological processes associated with insulin resistant syndromes are likely to impair vascular regeneration, the effect of insulin resistance *per se* is not established.

South Asian (SA) ethnicity is associated with increased risk of CVD, and insulin resistance is thought to be a major contributor to this. We compared the angiogenic capacity of late outgrowth endothelial progenitor cells (LEPCs) from young SA men, with those from a matched group white European (WE) men. LEPC have previously been shown to offer potential as an autologous cell therapy in preclinical models of ischaemic CVD. Both groups were well matched, and free of classical cardiovascular risk factors, but the SA group were relatively insulin resistant. SA LEPCs did not augment vascular regeneration in a murine model of limb ischaemia, in contrast with WE LEPC. Akt activity, a critical modulator of angiogenesis, was reduced in SA LEPC, and we were able to rescue SA LEPC dysfunction by enhancing Akt activity.

We then established the impact of insulin resistance *per se* on vascular regeneration, using insulin receptor haploinsufficient mice (IRKO). Indices of angiogenesis were reduced in isolated endothelial cells, aortic ring

segments, and ischaemic hind limb muscle. Moreover, this was associated with functional resistance to vascular endothelial growth factor (VEGF), which may have mechanistically contributed to our observations.

Together, these data provide insight into how insulin resistance may promote the development of premature CVD, and show that by manipulating key growth factor signalling nodes, we can rescue impaired vascular regeneration. Furthermore, we have established that insulin resistance negatively impacts on the functional response to VEGF, and it will be important to explore the mechanisms underlying this phenomenon in future studies. It is hoped that these findings will help lead to the development of strategies to mitigate the effects of CVD in individuals with insulin resistance.

Table of Contents

Table of Figures

Chapter 1

Chapter 4

Chapter 5

Chapter 6

Abbreviations

Akt	Protein kinase B
ATP	Adenosine triphosphate
BAECs	Bovine aortic endothelial cells
BMI	Body mass index
BP	Blood pressure
CAC	Circulating angiogenic cell
CAD	Coronary artery disease
CFU	Colony forming unit
CPC	Circulating progenitor cell
CVD	Cardiovascular disease
CXCR4	Chemokine X receptor-4 (SDF receptor)
Dil	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
EBM	Endothelial basal medium
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGM	Endothelial growth media
ELISA	Enzyme linked immune-sorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FMD	Flow mediated vasodilatation

GSK	Glycogen synthase kinase
HIF-1a	Hypoxia inducible factor-1alpha
HbA1c	Glycosylated haemoglobin
HOMA-IR	Homeostasis assessment of insulin resistance
HRP	Horse radish peroxidise
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHD	Ischaemic heart disease
IR	Insulin receptor
IRKO	Insulin receptor knockout
IRS-1	Insulin receptor substrate-1
KDR	Kinase domain receptor (or VEGF receptor-2)
LDL	Low density lipoprotein
LNMMA	Levo-N-monomethyl arginine
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
NADPH	Nicotine adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PEC	Pulmonary endothelial cell
PI3-K	Phosphatidyl Inositol-3 Kinase
ROS	Reactive oxygen species
SA	South Asian
SDF	Stromal cell-derived factor

SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Short inhibitory ribonucleic acid
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VENIRKO	Vascular endothelial insulin receptor knockout
WE	White European
WT	Wild type

Publication list

Publications arising from this project:

Cubbon RM, Yuldasheva NY, Viswambharan H, **Mercer BN** *et al.* Restoring Akt1 activity in outgrowth endothelial cells from South Asian men rescues vascular reparative potential. Stem Cells. 2014 Jun 10. doi: 10.1002/stem.1766. [Epub ahead of print]

Cubbon RM, **Mercer BN**, Sengupta A, Kearney MT. Importance of insulin resistance to vascular repair and regeneration. Free Radic Biol Med. 2013 Jul;60:246-63 (Review)

Mercer BN, Morais S, Cubbon RM, Kearney MT. Diabetes mellitus and the heart. Int J Clin Pract. 2012 Jul;66(7):640-7 (Review)

Other publications arising during this project:

Cubbon RM, Woolston A, Adams B, Gale CP, Gilthorpe MS, Baxter PD, Kearney LC, **Mercer B**, Rajwani A, Batin PD, Kahn M, Sapsford RJ, Witte KK, Kearney MT. Prospective development and validation of a model to predict heart failure hospitalisation.Heart. 2014 Jun;100(12):923-9

Cubbon RM, Adams B, Rajwani A, **Mercer BN**, Patel PA, Gherardi G, Gale CP, Batin PD, Ajjan R, Kearney L, Wheatcroft SB, Sapsford RJ, Witte KK, Kearney MT. Diabetes mellitus is associated with adverse prognosis in chronic heart failure of ischaemic and non-ischaemic aetiology. Diab Vasc Dis Res. 2013 Jul;10(4):330-6.

Kahn MB, Cubbon RM, **Mercer B**, Wheatcroft AC, Gherardi G, Aziz A, Baliga V, Blaxill JM, McLenachan JM, Blackman DJ, Greenwood JP, Wheatcroft SB. Association of diabetes with increased all-cause mortality following primary percutaneous coronary intervention for ST-segment elevation myocardial infarction in the contemporary era. Diab Vasc Dis Res. 2012 Jan;9(1):3-9

Abstracts arising from this project:

British Cardiovascular Society June 2014: Vascular Regeneration is Impaired in the Setting of Systemic Insulin Resistance.

British Cardiovascular Society June 2013: Restoring akt activity in late outgrowth endothelial progenitor cells from humans at high cardiovascular risk rescues their angiogenic capacity.

Chapter 1. Introduction

‘Insulin resistance’ is the impaired response to the metabolic actions of the glucose lowering hormone, insulin, and plays a central role in a number of overlapping clinical syndromes including obesity, pre-diabetes and diabetes mellitus (DM). DM represents a major global health burden with an estimated 170 million individuals affected worldwide in the year 2000. There has been a rapid rise in the prevalence of this disorder and estimates suggest that increasing levels of obesity, coupled with an aging population, mean that the number of people affected will continue to rise, and at least double by 2030 [1]. DM and its complications cause significant morbidity, with the potential for patients to experience debilitating eye disease, peripheral vascular disease (PVD), chronic kidney disease (CKD) and coronary artery disease (CAD). Besides the complications that can significantly impact on a patient’s quality of life, it has been established that DM is associated with premature death. Population studies have demonstrated an increased risk of mortality for nearly all causes of death in patients with DM, and there is therefore a significant reduction in life expectancy [2, 3].

Cardiovascular disease is responsible for much of this excess mortality, with major vascular events occurring in patients with DM approximately 15 years prior to those without DM [2, 4]. In patients with established cardiovascular disease, DM is an important independent risk factor for adverse outcomes [5]. Preceding the onset of DM (heralded by sustained hyperglycaemia), there is a well recognised ‘pre-diabetic’ period. This is typically characterised by insulin resistance that can manifest clinically as impaired fasting

glycaemia (IFG) or impaired glucose tolerance (IGT). Importantly, patients exhibiting IFG or IGT have also been shown to experience increased cardiovascular mortality [6]. Abnormal glucose handling (IGT, IFG and DM) is associated, and commonly occurs in conjunction with, a cluster of classical cardiovascular (CV) risk factors. Despite advances in the treatment for the conditions that occur as part of this insulin resistant phenotype, such as hypertension and dyslipidaemia, mortality remains disproportionately high in patients with IFG, IGT and DM [7, 8]. Observations of trends in CV disease in clinical practice show that the proportion of patients who experience CV events with DM is increasing and these patients have double the mortality of those without DM [5, 9]. Insulin resistance, and potentially treatments for this, have been mooted as both the explanation for this increased risk, and also a possible avenue by which CV mortality discrepancies between patients with and without DM could be addressed.

The principle factor underlying the increased vascular risk associated with pre-diabetes and DM is atherosclerosis. Atherosclerosis is a condition characterised by arterial inflammation and lipid deposition, which over time will progress, causing luminal obstruction of the affected vessel. These atherosclerotic lesions are covered by a fibrous cap, which partitions the thrombogenic material contained within from the circulation. In the case of ‘plaque rupture’ this fibrous cap is disrupted, triggering a cascade of platelet aggregation, thrombus formation and acute luminal obstruction that ultimately leads to major cardiovascular events. These events are defined by the site of vascular compromise; in the cerebral circulation these processes

may result in a stroke, in the heart it may cause myocardial infarction, and in the peripheral arterial circulation there may be limb ischaemia. A key initiating step in the development of atherosclerosis is endothelial dysfunction, which is manifested as a reduction in the bioavailability of nitric oxide (NO). NO, which has a portfolio of anti-atherosclerotic properties [10], is produced in the vascular endothelium and plays a crucial homeostatic role. Human studies have demonstrated a correlation between insulin resistance and endothelial dysfunction [11, 12]. NO production in the endothelium can be triggered by the actions of insulin via signalling cascades similar to those that result in glucose uptake in metabolic tissues. This therefore provides a mechanistic link between endothelial dysfunction and metabolic insulin resistance [13].

Endogenous vascular repair and regeneration is a process by which the effects of endothelial injury and atherosclerosis may be mitigated. Considering the prevalence of ischaemic disorders in patients with insulin resistant disorders, advancing our understanding of how insulin resistance impacts upon these autologous repair mechanisms is important. Ultimately, this may lead to the identification of novel therapeutic targets, with the potential to retard disease progression or revascularise ischaemic tissue. This thesis will investigate how modified insulin signalling, and insulin resistance, impact upon vascular regeneration.

1.1 Insulin signalling

Insulin signals via binding its cognate tyrosine kinase receptor (IR). This is an atypical tyrosine kinase in the sense that it exists as a homodimer, and binds its ligand to induce autophosphorylation, as opposed to typical tyrosine kinase receptors in which ligand binding triggers dimerisation and activation [14]. Insulin receptor phosphorylation brings about recruitment and subsequent tyrosine phosphorylation of docking proteins, including insulin receptor substrates-1 and 2 (IRS-1 and IRS-2). Insulin signal transduction is a complex process activating many cascades, with multiple regulatory feedback loops, and cross talk with other receptor signalling pathways. A number of downstream molecules have been shown to bind to IRS in response to insulin stimulation, but for the sake of simplicity it is helpful to consider the two principle signalling cascades: the PI3-kinase pathway and the ERK MAP-kinase pathway.

Phosphorylated IRS binds the regulatory p85 subunit of PI3-kinase via its SH2 domain, allowing activation of the p110 catalytic subunit. This results in increased phosphatidylinositol 3-phosphate (PIP_3) production, which is localised in the plasma membrane. Akt then localises to the plasma membrane, via interaction of its plekstrin homology domain with PIP_3, and is partially activated by threonine 308 phosphorylation by phosphoinositide dependant protein kinase 1 (PDK1), which is itself activated by PIP_3. Full activation of Akt occurs after additional serine 473 phosphorylation by the mTORC2 complex. In the classical metabolic tissues this cascade then results in GLUT4 translocation to the cell membrane, consequently

increasing glucose disposal into the cell. In vascular endothelium it results in endothelial nitric oxide synthase (eNOS) activation, and nitric oxide (NO) production [15]. Other signal transducing proteins interact with IRS, including Grb2. This leads to a signalling cascade resulting in MAP kinase activation and this pathway mediates the mitogenic actions of insulin [16].

1.1.1 Mechanisms of insulin resistance

The epidemic of insulin resistant syndromes, such as the metabolic syndrome and type 2 DM are clearly linked with obesity, and indeed some of the mechanisms responsible for insulin resistance are thought to relate to adiposity and effects that this can have on intracellular lipids, their metabolites, as well the accompanying environment of hyperglycaemia and chronic, low grade inflammation. Murine models have helped elucidate the effect that accumulation of lipid has in certain tissues. Mice with tissue specific (muscle and liver) over-expression of lipoprotein lipase have increased muscle and liver triglyceride content and consequently are insulin resistant [17]. For instance, in skeletal muscle, where insulin resistance ultimately leads to impaired glucose disposal, it has been shown that diacylglycerols (DAG), a breakdown product of triglycerides, activate protein kinase C isoforms. This in turn leads to serine phosphorylation of the insulin receptor substrate 1 (IRS-1), which may have an inhibitory effect on signal transduction by interfering with the tyrosine phosphorylation of IRS-1, which in turn reduces PI3-kinase activity, and its downstream effects [18-20]. It has also been shown that intramuscular levels of diacylglycerol (DAG) are higher

in obese and diabetic subjects compared with lean individuals. There is also a correlation between intramyocellular DAG and insulin resistance [21, 22].

Insulin resistance progresses after the onset of frank diabetes and this can be partially attributed to the effects of sustained hyperglycaemia. This occurs due to the molecular effects of advanced glycation end products (AGEs) and oxidative stress, both of which are manifestations of hyperglycaemia. Although precise mechanisms are not yet clear, hyperglycaemia has been shown to increase reactive oxygen species (ROS) production, which in turn can cause insulin resistance. The mechanisms by which ROS induce insulin resistance are multiple and include the activation of serine/threonine kinases c-Jun NH 2-terminal kinase (JNK), PKCs, and IκB kinase complex ß (IKKß). These kinases induce serine phosphorylation of IRS-1, which reduces the downstream activity of PI3-K/Akt and ultimately reduces GLUT4 translocation [23-25]. This oxidative stress also leads to the activation of nuclear factor (NF)-κB [26] and this complex of transcription factors regulates a host of inflammatory signalling pathways, most of which have been shown to induce insulin resistance [27]. Hyperglycaemia increases the production of advanced glycation end products (AGEs) [26], which induce insulin resistance by inducing inhibitory phosphorylation of IRS-1 and IRS-2, thereby reducing Akt/PI3-k signalling [28]. AGEs also increase oxidative stress [29], which as outlined, induces insulin resistance. Hyperinsulinaemia has also been shown to exacerbate insulin resistance and *in vitro* studies have shown that insulin reduces IRS1 and IRS2 abundance via PI3-K/Akt signalling [30].

Substances released from adipose tissue, including adipokines and inflammatory mediators, have also been implicated in insulin resistance. For example, tumour necrosis factor (TNF)-alpha, is released from visceral adipose deposits, and studies in leptin deficient *ob/ob* mice crossed with TNF-alpha deficient mice highlight this role for TNF-alpha in insulin resistance. These leptin deficient/TNF-alpha deficient mice had rescued insulin sensitivity in muscle and fat tissues [31]. Mechanistic insight is provided by further studies in leptin deficient mice, which demonstrate that increased levels of TNF-alpha result in up regulation of suppressor of cytokine signalling proteins (SOCS), which interferes with the association of IRS-1 and the p85 subunit of PI3-kinase by reducing IRS-1 tyrosine phosphorylation.

This brief discussion highlights some of the mechanisms responsible for insulin resistance and also serves to demonstrate that the pathways involved are numerous and complex. It also highlights pathways and signalling nodes that are commonly implicated, such as the insulin receptor and its substrates, Akt and PI3-K. Although the balance of evidence suggest that defects at the level of these critical signalling nodes are likely to be significant with regards insulin resistance, it is important to note that conflicting data exists. For example, experiments utilising *in vitro* models of insulin resistance to mimic conditions described above suggest that aberrant signalling at the IR/IRS level may not be so critical. Hoehn *et al* experimentally mimicked insulin resistance *in vitro* using lipotoxicity, inflammation and hyperinsulinaemia models, and found that platelet derived growth factor (PDGF) signalling was

also impaired. This an important observation as PDGF bypasses the IRS node, and therefore suggests that modifications to the insulin signalling cascade below the IRS node are more important [32].

The origins of cellular insulin resistance have also been challenged in other ways, with some authors suggesting that insulin resistance is, in fact, necessary to protect the cell from further oxidative damage when nutrients are in oversupply [33].

The mechanisms that result in perturbed insulin signalling and insulin resistance remain debated, and the preceding explanation offers just a few putative mechanisms; unfortunately, a complete review is beyond the scope of this introduction. However, it does highlight that there is unlikely to be one pathophysiological defect in insulin signalling responsible for the insulin resistance seen clinically, but rather multi-level signalling defects.

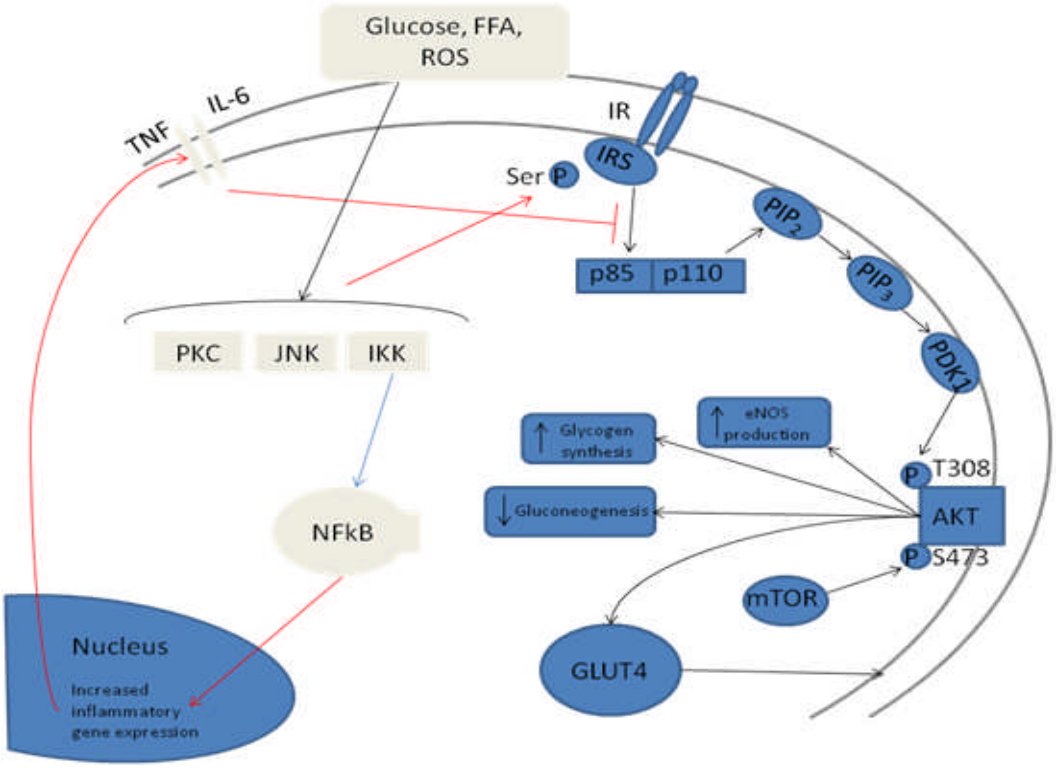

Figure 1-1. Multiple facets of the metabolic syndrome have been implicated in the underlying mechanism of insulin resistance. Dyslipidaemia and elevation in FFAs has been shown to induce a number of deleterious effects on insulin signalling. Elevated levels of DAG leads to activation of PKC, serine phosphorylation of IRS and decreased PI3-K activity. Hyperglycaemia results in increased AGE and ROS production, which activate PKC, JNK, IKKß and NFkB. This causes inhibitory phosphorylation of IRS and promotes pro-inflammatory gene expression. Inflammation, a common feature of the metabolic syndrome, is characterised by increased levels of cytokines such as TNF, IL-6. These cause insulin resistance via SOCS3 and JNK. FFA indicates free fatty acid; ROS, Reactive oxygen species; TNF, Tumour necrosis factor; PKC, protein kinase C; JNK, c-Jun amino-terminal kinase; IKK, IkB-kinase; NFkB, Nuclear factor kB; IR, Insulin receptor; IRS, insulin receptor substrate; PIP_2, Phosphatidylinositol 4,5-bisphosphate; PIP_3,

Phosphatidylinositol 3, 4,5-triphosphate; mTOR, mammalian target of Rapamycin; IL, interleukin; PDK1, phosphoinositide dependent protein kinase-1; GLUT4, glucose transporter type 4.

1.2 Vascular regeneration

1.2.1 Vasculogenesis

The process by which blood vessels form *de novo* in the embryo is termed vasculogenesis. Endothelial progenitor cells, angioblasts, arising from embryonic mesoderm coalesce to form the vasculature [34]. Vasculogenesis is principally restricted to the early stages of vascular development. However, there is evidence that circulating progenitor cells may enhance vascular regeneration and these cells have been shown to incorporate into neovessels, highlighting the potential for post-natal vasculogenesis. The notion that, beyond embryogenesis, new blood vessel formation is an angiogenesis dependent process was initially challenged by the work of Shi *et al.* These authors observed endothelialisation in non-marginal regions of Dacron grafts that had been implanted in the thoracic aortic region of dogs. This was an interesting observation as, if endothelialisation is indeed entirely dependant on local endothelial cell migration, re-endothelialisation should occur from the margins inward. Shi *et al* concluded therefore, that circulating cells must have contributed [35]. Following this, Asahara *et al* published seminal work providing further support for circulating cells contributing to postnatal vasculogenesis [36]. However, controversy remains, and there is work from other groups that contest these and subsequently published data. For example, work from Hagensen *et* al, demonstrates that flanking

endothelial cells, rather than bone marrow derived cells, contribute to re-endothelialisation in the setting of atherosclerosis, vessel injury and allograft vasculopathy respectively [37-39]. The putative circulating vasculogenic cells proposed to contribute to post-natal vasculogenesis will be discussed later in greater detail.

1.2.2 Angiogenesis

Angiogenesis is a term commonly used to denote new vessel growth, but actually describes the process by which vessels sprout or intussuscept from the existing vasculature [40]. It is a complex process, requiring the orchestration of numerous processes including pericyte detachment, endothelial cell migration, proliferation, tubule formation, anastamosis, and subsequent vessel maturation with the recruitment of mural cells. Disordered angiogenesis is central to the pathogenesis of a number of diseases. Inadequate vessel formation is implicated in ischaemic disorders such as myocardial infarction, stroke and peripheral vascular disease. Excessive or unregulated vessel growth is a hallmark of tumour growth, diabetic retinopathy and age-related ‘wet’ maculopathy.

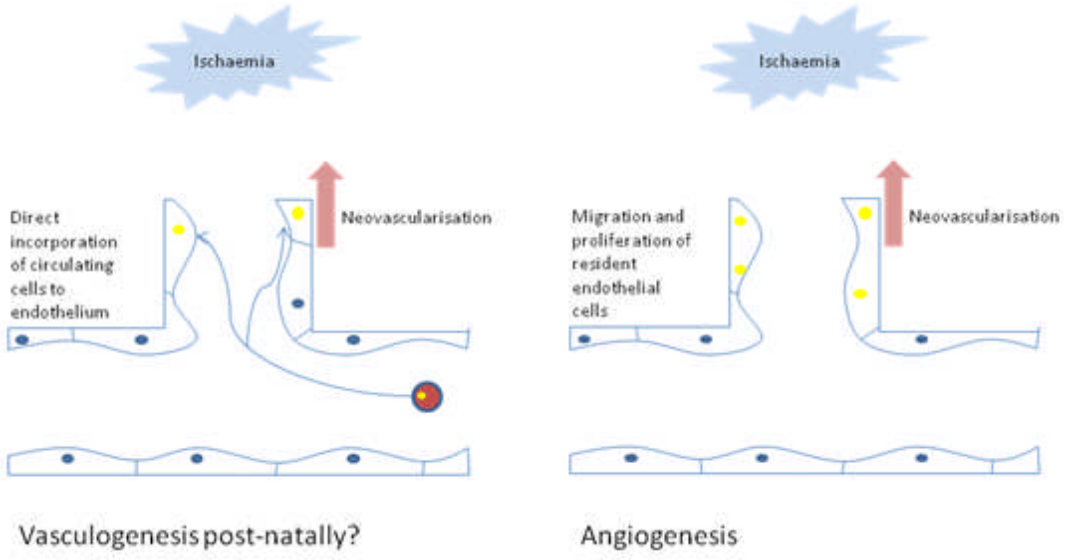

Figure 1-2. Schema demonstrating involvement of resident endothelial cells and circulating angiogenic cells in proposed models of neovascularisation.

1.2.2.1 Sprouting angiogenesis

Hypoxic tissues release growth factors and chemokines that initiate the formation of new vessels from the existing plexus in an effort to restore supply and demand back to equilibrium. In the quiescent state, endothelial cells line blood vessels in a monolayer and are surrounded by a supporting network of pericytes and vascular smooth muscle cells. These pericytes are multifunctional and help maintain quiescence by suppressing endothelial proliferation and producing molecules that aid endothelial cell survival. This provides stability, and together the two layers of cells share a basement membrane forming a sleeve that encloses endothelial tubules, thereby preventing endothelial cell migration [41]. In the presence of abundant oxygen, hypoxia inducible factors (HIFs) are regulated by the oxygen sensing prolyl hydroxylase domain 1-3 (PHD1-3) proteins. In physiological conditions, these PHDs hydroxylate HIFs and thereby target them for

preoteolytic degradation. In hypoxic situations, however, levels of HIF rise and production of the prototypical angiogenic molecule, VEGF, is stimulated [42]. In response to increased VEGF production, pericytes detach from the endothelial tube and the basement membrane is degraded by matrix metalloproteases. VEGF also makes endothelial junctions ‘leaky’ allowing extravasation of plasma proteins and the establishment of an extracellular matrix and pro-angiogenic milieu [40]. Once this environment has developed, one tip cell is ‘selected’ to lead the newly forming sprout. Endothelial cells at the angiogenic front have an advantage in terms of differentiation to tip cells as they are exposed to higher concentrations of VEGFA, which up regulates cell surface Delta-like 4 (DLL4) expression, then triggering Notch activity in neighbouring cells. Studies have recently demonstrated an important role for the DLL4/Notch signalling pathway in the differentiation of endothelial cells into either tip or stalk cells, with blockade of DLL4 or Notch leading to excessive sprouting and tip cell formation [43]. This suggests that the tip cell phenotype is the default endothelial response to angiogenic stimuli. Notch signalling in cells neighbouring those at the tip alters gene expression, promoting the proliferative stalk cell phenotype, thereby leading to elongation of the endothelial sprout [40]. Tip cells, under the influence of VEGF, produce long, dynamic filopodia that have within them an abundance of VEGFR2, and are capable of responding to directional cues. Stalk cells do not produce filopodia and proliferate in response to VEGFA [44]. These processes will continue until a vascular network is formed allowing the flow of blood and consequently the resolution of hypoxia. It is important to note that a properly functioning vessel requires not only a perfused vascular tube, but

also a plethora of supporting mural cells, including pericytes and vascular smooth muscle cells. Endothelial cells actively partaking in angiogenesis release platelet derived growth factor B (PDGF-B) and this attracts PDGF receptor B expressing (PDGF-B^{+}) pericytes [45, 46]. The importance of this process to vascular development is highlighted in animal studies, with genetic ablation of PDGF-B in mice leading to vessel leakage, micro-aneurysm formation and lethal bleeding in the late embryonic stage [46].

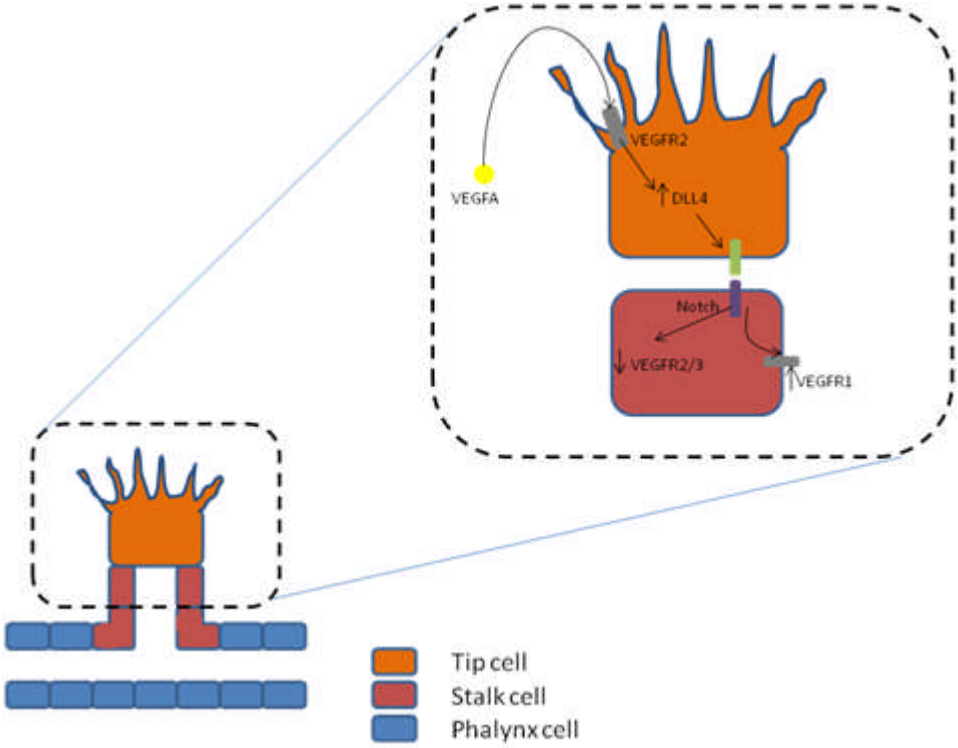

Figure 1-3. Schema highlighting tip/stalk cell selection. Filopodia at the angiogenic front are studded with VEGFR2. Cells exposed to higher levels of VEGFA, have higher levels of VEGFR2 activity, thereby upregulating DLL4 and inducing Notch signalling in neighbouring cells. Notch signalling results in a number of transcriptional changes, including upregulation of VEGFR1 and downregulation of VEFR2/3, and this assigns the cell to a stalk cell fate. Tip cells are characterised by high levels of DLL4 production and low levels of Notch signalling. Stalk cells have lower levels of DLL4 production and

higher levels of Notch signalling. Tip/stalk cell fate are dynamic however and alter according to changes in the described signalling pathways.

1.2.3 VEGF

As highlighted in the preceding discussion, vascular endothelial growth factor (VEGF) plays a central role in the processes required to bring about angiogenesis. The following discussion will expand on VEGF and its actions in relation to neovascularisation. The VEGF family consists of 5 related growth factors: VEGFA or vascular permeability factor (VPF), VEGFB, VEGFC, VEGFD and placental growth factor (PlGF) [47-51]. All 5 of the human VEGF proteins can be spliced to form multiple isoforms. VEGFA, the first to be discovered, is thought to be the principal growth factor involved in angiogenesis. It's production is sensitive to hypoxia inducible factor (HIF) and can be produced by almost any cell, including endothelial cells in both healthy and disease states [52, 53].

The family of VEGFs exert their effects via binding to their cognate tyrosine kinase receptors. Principally, VEGFR1 (or FLT1), VEFGR2 (or FLK1 in the mouse, and KDR in the human) and VEGFR3 (or FLT4) [54-56]. VEGFA, -B and PlGF bind to VEGFR1, VEGFA binds to VEGFR2, and VEGFC and D bind to VEGFR3 [57]. The effects of VEGFs are multiple and complex. They share many similar regulatory functions such as cell survival, proliferation and migration with other growth factors, including platelet derived growth factor (PDGF) and epidermal growth factor (EGF) [57]. In other respects however, they are unique, as they are able to transmit signals that bring

about angiogenesis and regulate vascular permeability. VEGFR1 is a regulator of macrophage and monocyte migration; its role in the endothelium is more controversial, as is discussed later. VEGFR2 is important in all aspects of endothelial cell biology, and VEGFR3 is important in lymphatic and endothelial cell development, along with tip cell activity in all sprouting endothelial cells [57, 58].

The main biological actions of VEGFA are its ability to stimulate endothelial cell migration and proliferation [59-61], and to increase vascular permeability [47, 62]. VEGFA exists in at least 9 nine different isoforms that are brought about by alternate splicing of the VEGFA gene. These are $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165}$b, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ [63, 64]. $VEGF_{165b}$ is an endogenous isoform that exerts an inhibitory effect on VEGFR2 and does not trigger the intracellular signalling cascade. This splicing affects the ability of certain isoforms to bind to heparan sulphate and neuropilins (discussed later), and also dictates their solubility and temporo-spatial distribution [65].

1.2.4 VEGF signalling

There are three structurally related VEGF receptor tyrosine kinases found in humans - VEGFR1, VEGFR2 and VEGFR3. These VEGF receptors all share a similar structure with an extracellular, ligand-binding domain composed of immunoglobulin-like loops, a transmembrane domain, a juxtamembrane domain, a split tyrosine kinase domain, and a c-terminal tail [66]. Binding of VEGF to its receptor tyrosine kinase occurs at the N-terminal part of the

extracellular domain. This ligand binding can occur by freely diffusible VEGF, and also by the presentation of VEGF whilst bound to a co-receptor [66]. These co-receptors, chiefly Heparan Sulphate (HS) and Neuropilins 1 and 2 (NRP1, NRP2), can modulate VEGF action in several ways. For instance, HS binding to various VEGFA isoforms impacts on solubility, signalling amplitude and duration as wells as acting as a 'reservoir' for VEGFA and can aid in the creation of VEGF gradients, important to processes such as endothelial cell migration. NRP1 modulates VEGFR signalling and enhances endothelial cell migration and survival. Although molecular mechanisms are not fully understood, it is clear that these co-receptors play a crucial role in vascular development. Murine models with over- and under-expression of NRP1 are not viable, with embryonic lethality due to vascular abnormality observed at E12.5-13.5 [67, 68]. The knockout of NRP2 seems to have greater impact on small capillary and lymphatic development, whereas arterial and venous development is preserved [69].

1.2.4.1 VEGFR1

VEGFR1 expression is abundant in vascular endothelial cells, monocytes, macrophages, vascular smooth cells and many other cell types. VEGFR1 binds VEGFA with higher affinity than VEGFR2, but this induces weak tyrosine kinase activity only [66]. The exact role for VEGFR1 in endothelial cells is disputed, but animal models have helped elucidate its role. Mice with holoinsufficiency of VEGFR1 (*VEGFR1*$^{-/-}$) experience embryonic lethality at E9. This is due to increased proliferation of endothelial cells, and severe disorganisation of the vascular system [70]. However, targeted deletion of

the intracellular domain of VEGFR1, rather than the whole receptor, is compatible with vascular development [70]. This supports the notion that VEGFR1 acts as a ‘reservoir’ to reduce VEGFA activity, and plays an important regulatory role in tip cell selection via its Notch dependent upregulation in stalk cells [71]. In addition to this negative regulatory role in angiogenesis, VEGFR1 is important in mounting immune responses and inflammatory angiogenesis. Mice expressing tyrosine kinase deficient VEGFR1 (VEGFR $TK^{-/-}$) exhibit impaired angiogenesis in a number of pathological conditions, including malignancy [72] and cerebral ischaemia [73].

1.2.4.2 VEGFR2

VEGFR2, is also known as the kinase insert domain receptor (KDR) in humans, and foetal liver kinase-1 (Flk-1) in mice. Although primarily expressed in vascular endothelial cells, this is not exclusive and it is also found in pancreatic duct cells, retinal progenitor cells, megakaryocytes and haematopoietic cells. Its expression is greatest during periods of active angiogenesis, such as during development, in the uterus during the menstrual cycle, and in tumour cells during cancer growth. The majority of its angiogenic actions are as a result of binding with VEGFA, but it also binds processed forms of VEGFC and VEGFD[66]. It binds VEGFA with 10 fold less affinity compared with VEGFR1. VEGFR2 signal transduction mediates all the processes required for vascular development and regeneration, such as proliferation, migration, survival and permeability. Signalling cascades activated by VEGFR2 are numerous and complex. Upon binding of VEGFA,

secondary signalling molecules attach at their respective tyrosine phosphorylation sites on VEGFR2 and this results in the activation of downstream mediators, which include phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) [66]. The importance of VEGFR2 in angiogenesis and vascular development has been highlighted in animal models. Mice holoinsufficient for Flk-1 (Flk-$1^{-/-}$) die at E8.5-9.5 due to a failure of development of vasculature and haematopoietic progenitors, therefore indicating the crucial role of VEGFR2 in vasculogenesis [74]. Other models with more targeted manipulation of VEGFR2 reveal important tyrosine phosphorylation sites; for example, mice with a phosphorylation resistant mutation affecting tyrosine 1173 have a similar phenotype to Flk-$1^{-/-}$ mice [75]. A complete review of the VEGFR2 action and signalling is beyond the scope of this introduction; for an excellent review see Koch *et al* [66].

1.2.4.3 VEGFR3

VEGFR3, or Flt4 in the mouse, seems to be important in lymphatic cell development and this is highlighted in patients with hereditary lymphoedema, who carry mutations in VEGFR3. A point mutation in VEGFR3 rendering the kinase inactive results in the Chy mouse, which develops chylous ascites [66]. It also appears to play a crucial role in blood vessel development however, perhaps via heterodimerisation with VEGFR2, or binding of VEGFC and thereby modulating VEGFR2. This role has been revealed, again, via genetic studies. Mice holoinsufficient for VEGFR3 (Flt4$^{-/-}$) die at E10.5 due to failure of vascular development [76], whereas mice with knockout of VEGFC

and VEGFD, the main ligands for VEGFR3, retain normal blood vessel development [77]. The importance of VEGFR3 to the angiogenic process has been reinforced with recent work suggesting a critical role in tip cell function, independent of its canonical ligand binding function [58].

1.3 Endothelial progenitor cells

Although, vascular regeneration is the primary concern of this project, there is a complex overlap with the process of vascular repair. Vascular repair is a term generally used to denote the process by which re-endothelialisation of injured vessels occurs. Important in maintaining vascular homeostasis, vascular repair occurs following endothelial injury from direct trauma incurred during clinically relevant procedures such as arterial stenting, and also injury from pathological processes, including atherosclerosis. Until recently it has been generally accepted that all endothelial repair occurs via the migration of adjacent, resident healthy endothelial cells, and indeed there is a body of work that would appear to corroborate this [37-39]. This paradigm, however, is not entirely accepted and there is data to suggest that bone marrow derived progenitor cells do incorporate into areas of injured vascular endothelium [78]. Work published by Asahara *et al* gave new weight to the consideration that vascular repair could be aided by circulating endothelial precursors in a process akin to postnatal vasculogenesis. They described ‘putative progenitor cells’, which were derived after *ex vivo* expansion of human peripheral blood mononuclear cells expressing the haematopoietic stem cell marker CD34. These cells, or those expressing the VEGF receptor 2 (KDR/Flk-1), augmented angiogenesis in rabbit and murine models of

hindlimb ischaemia. Furthermore, these infused cells were found to incorporate into capillary like structures when tissue was harvested after six weeks [36]. These data have been extrapolated to suggest that such circulating cells, co-expressing CD34 and KDR, represent 'endothelial progenitor cells' capable of augmenting vascular repair and regeneration [79]. However, it is known that mature endothelial cells, which might also be expected to express these markers, can be shed from the vessel wall in to the circulation. In light of this, circulating CD133+/CD34+/KDR+ have been proposed to more accurately represent endothelial progenitor/precursor populations [80]. Although there is no doubt that the relative abundance of these cells correlates independently with vascular risk [81], it is not clear exactly what role these cells have *in vivo*. For these reasons, this population of cells are better referred to as 'circulating progenitor cells' or CPCs.

1.3.1 CPC biology

Until recently, it has been the generally accepted dogma that blood vessel development occurs via distinct mechanisms, depending on the stage of development. *In utero*, new blood vessels are derived via a process termed vasculogenesis, which involves the coalescing of angioblasts to form new vessels. After birth, to meet the demands of growth and pathological processes, it has long been thought that native endothelial cells divide and migrate to form new vessels. The seminal work of Asahara has led to a paradigm shift and the consideration of a role for circulating progenitor cells in post-natal vasculogenesis. It is thought that these bone marrow derived cells respond to stimuli that trigger mobilisation from the bone marrow and

migrate to sites of vascular injury, hypoxia and inflammation. Here they may have a pro-angiogenic action via the secretion of mediators that promote neovascularisation, as well as acting as ‘building blocks’ to form new endothelial cells, aiding vessel growth.

The mobilisation of CPCs is triggered by processes such as ischaemia and inflammation. Growth factors, including vascular endothelial growth factor (VEGF) and stromal derived cell factor (SDF), released by ischaemic tissue, lead to up-regulation of eNOS activity in bone marrow stromal cells [82], which in turn increase MMP-9 activity and results in the cleavage of c-kit ligand, which releases CPCs from the osteoblastic region to the vascular zone [83]. The CPCs then respond to chemotactic stimuli, via receptors such as VEGFR2 and CXCR-4, and home to sites of ischaemia [84] where they facilitate vascular repair and regeneration [85, 86] possibly by direct incorporation into the endothelium [78], or via paracrine effects.

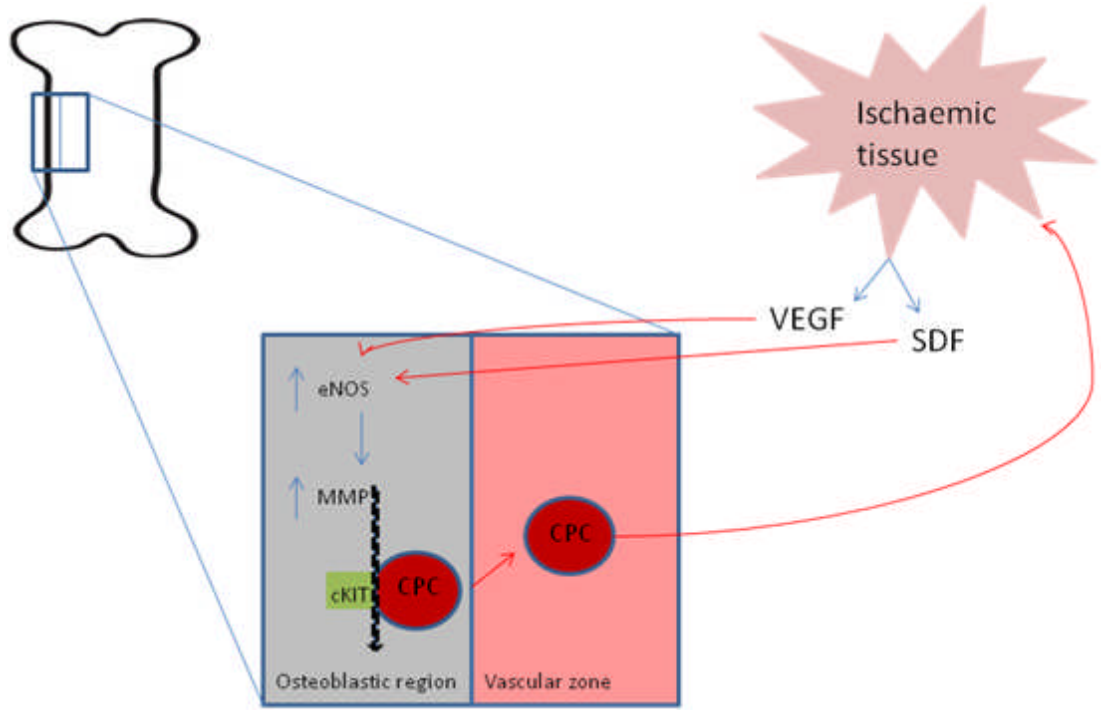

Figure 1-4. Schema demonstrating mobilisation of CPCs from bone marrow. Ischaemic tissue produces growth factors such as VEGF and SDF, which upregulates eNOS activity in the bone marrow. In turn, this induces MMP activity and the cleavage of cKit ligand. This releases the CPC to the vascular zone, and from here it localises to the area of ischaemia. Abbreviations: VEGF; Vascular endothelial growth factor, SDF; Stromal derived factor, MMP; Matrix metalloproteinase, CPC; Circulating progenitor cell.

1.3.2 EPC classification

The in vivo study of circulating progenitor cells is difficult due to their rarity, and there is still no universally accepted definition of what constitutes a circulating endothelial progenitor cell, or even whether such a lineage exists. Based on extrapolation of Asahara's initial work, circulating 'EPCs' were initially defined as cells co-expressing endothelial and haematopoietic cell

surface markers, namely CD34, CD133 and VEGFR2. The problem with this definition is that none of these markers are restricted to such ‘EPCs’ and are variously expressed by mature endothelium, and haematopoietic stem/progenitor cells [87]. Because of these difficulties, and the low abundance of circulating progenitors, studies of these cells with regards their effect on vascular repair, relies on *ex vivo* expansion of circulating cells. This results in an entirely different population, generally referred to as EPCs.

The study of EPCs involves cell culture based manipulation of circulating peripheral blood mononuclear cells (PBMCs). The term EPC can refer to more than one cell type, and by culturing PBMCs in specific cell culture media, three distinct cell types have been described. ‘Early outgrowth endothelial progenitor cells’ or EEPCs refers to two cell populations. If PBMCs are plated and cultured for approximately four days this gives rise to so called ‘circulating angiogenic cells’ or CACs. These spindle shaped cells are capable of augmenting vascular repair and angiogenesis, but have a limited proliferative capacity and do not incorporate directly into vascular structures [88]. Methods to identify and define these cells include assessment of the capacity of cultured cells to absorb acylated LDL and bind Ulex europaeus agglutinin-1 lectin (UEA-1). This method is fraught with limitations due to the fact that monocytes (present abundantly in PBMCs) also absorb acylated LDL and bind UEA-1. Furthermore, platelets contaminating the mononuclear fraction release platelet fragments expressing non-specific endothelial markers, such as CD31, which can be phagocytosed (and then pseudo-expressed) by monocytes [89]. Flow

cytometric analysis can also be used to identify cell surface markers (such as CD31) but many of these are not endothelial-specific [87]. It is now widely accepted that this subset of EEPCs are indeed of monocytic lineage. Nevertheless, much work has shown that these cells promote vascular regeneration, for example in murine models of critical limb ischaemia [90]. Any effect they have on vascular repair and regeneration is considered to be as a result of paracrine action [87], consistent with data demonstrating that EEPCs release an array of pro-angiogenic growth factors, including VEGFA, VEGFB, IGF-1 and SDF-1 [91].

If initially non-adherent PBMCs are cultured for approximately four to nine days, a population of cells form colonies. These cells are called ‘colony forming unit – Hill cells or CFU-Hill cells. Although CFU-Hill cells express endothelial markers, and the abundance of these colonies has been to shown to correlate with Framingham risk score [92], and they can enhance *in vitro* angiogenesis[93], it has been demonstrated that these cells are in fact T-lymphocytes [94, 95]. These CFU clusters have a gene expression profile indistinguishable from CACs [87], do not directly form blood vessels *in vivo,* and in keeping with their leukocytic lineage, possess non-endothelial functional characteristics [96].

If PBMCs are subjected to more prolonged culture over seven to twenty-one days, colonies of ‘late outgrowth endothelial progenitor cells’ (LEPCs), also referred to as outgrowth endothelial cells, are seen to develop [93]. These cells from tubules on Matrigel and directly contribute to blood vessels when

implanted *in vivo* in collagen/fibronectin matrices [96]. They have also been shown to incorporate into vascular endothelium in murine models of vascular disease [93]. When considering their proliferative capacity, it has been shown that cells within a LEPC colony display a heterogeneous mix of proliferative capacity. Some single cells have low proliferative potential and do not form secondary colonies when re-plated; others form small aggregates of 2-50 cells, whilst others have high proliferative potential forming colonies of >2000 cells [97]. These cells express endothelial markers (CD31, CD141, CD105, CD146, CD144, VWF, Flk-1), but not leukocyte markers (e.g. CD45 or CD14). They therefore possess the functional features one may expect from an endothelial cell, together with a proliferative capacity indicative of progenitor hierarchy. Therefore, LEPC possess many of the properties one might expect an EPC to possess, and as such have been mooted to have therapeutic potential [98].

Although the role and classification of ‘EPCs’ is still debated, experimental data over the last 10-20 years have provided insight. It is generally accepted that ‘early outgrowth EPCs’ do not incorporate long term into injured or newly developing endothelium. They do, however, promote vascular repair and regeneration and exert their pro-angiogenic effect in a local paracrine fashion. The cell that most closely resembles the originally conceived ‘EPC’ is probably the late outgrowth EPC. These cells have been shown to incorporate into vascular endothelium and are in fact almost indistinguishable endothelial cells [99], although it is important to note that it remains unclear whether they form spontaneously *in vivo*.

1.4 Vascular regeneration and insulin resistance

1.4.1 Metabolic syndrome (MetS)

Insulin resistance is present in a number of related disorders including diabetes, pre-diabetes and obesity. It is well established that diabetes is associated with impaired vascular regeneration, with animal studies demonstrating defects in angiogenesis using wound healing and hindlimb ischaemia models [100, 101]. Human studies also correlate diabetes with impaired collateral vessel formation in ischaemic myocardium [102]. Diabetes is also associated with defects in progenitor function and abnormal progenitor production and mobilisation have also been described [103]. As mentioned, a cluster of conditions tend to occur along with diabetes and other insulin resistant states. This heterogenous group of disorders occur with varying degrees of severity and depending on the levels of abnormality found, and the guidelines that are applied, this cluster of conditions is termed the metabolic syndrome [104]. The metabolic syndrome is essentially defined as the presence of varying combinations of central obesity plus hypertension, dyslipidaemia and evidence of impaired glucose handling [104]. Although many of the facets of the metabolic syndrome are associated with reduced indices of vascular regeneration, the impact insulin resistance *per se* has is less established. The following discussion will focus on the impact that each facet of the metabolic syndrome may have on vascular regeneration.

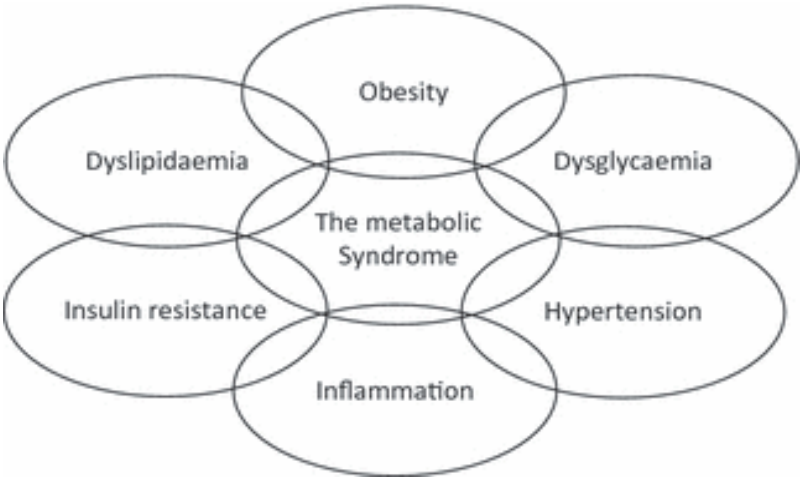

Figure 1-5. The clustering of cardiovascular risk factors in the metabolic syndrome. From: Mercer *et al* Int J Clin Pract. 2012 Jul;66(7):640-7

1.4.2 Hypertension

Rodent studies have demonstrated a reduction in the number of circulating progenitor cells in hypertensive animals, in addition to impaired EPC function, such as migration [105]. Oxidative stress was implicated mechanistically in this progenitor dysfunction. This correlation between hypertension and impaired progenitor function is paralleled in human studies. Blood from hypertensive men yields fewer early EPC (EEPC) colonies [106]. EEPCs from men with essential hypertension exhibit impaired migration and adhesion compared with those derived from healthy volunteers. Moreover, these cells augmented vascular repair to a lesser degree when used in a murine model of vascular injury [107]. Drawing conclusions on the isolated impact of hypertension in these studies is complicated by differences in baseline profiles of the two groups with non-significantly higher levels of LDL-cholesterol, triglycerides and lower levels of HDL-cholesterol. All these changes are also associated with perturbations in aspects of progenitor

function. Hypertensive rats have also been shown to exhibit impaired reperfusion in response to hindlimb ischaemia compared with normotensive animals. Perfusion was augmented by the infusion of EEPC from control animals to a greater degree than was observed with EEPCs from the hypertensive littermates [108]. Increased oxidative stress from the endothelin A (ET(A)) / Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway was implicated in this progenitor cell dysfunction. Current data from both human and animal studies suggests that hypertension has a deleterious effect on both progenitor function and markers of angiogenesis, with oxidative stress playing a key role (see later). There is conflicting evidence however, with a key mediator of hypertension, aldosterone, being shown to enhance vascular regeneration in murine hindlimb ischaemia studies and co-treatment with spironolactone (aldosterone receptor antagonist) or valsartan (Angiotensin-2 receptor antagonist) abrogating this pro-angiogenic effect [109].

1.4.3 Dyslipidaemia

The characteristic lipid profile of elevated triglycerides, elevated small dense low density lipoprotein (LDL) cholesterol, reduced high density lipoprotein (HDL) cholesterol and elevated apolipoprotein (apo)-B is often found in patients with MetS [110]. Increased levels of LDL cholesterol correlate with reduced abundance of EPCs, measured by formation of colony forming units [111]. Oxidised LDL (OxLDL), an important cardiovascular risk factor, has been shown to significantly impair *in vitro* EEPC function, with impaired survival, adhesion, migration, proliferation and tube forming capacity seen in

cells treated with OxLDL [112, 113]. OxLDL treatment of EEPCs led to a reduction in Akt phosphorylation and eNOS mRNA expression [112]. Animal models of hypercholesterolaemia have demonstrated a negative impact on vascular regeneration [114-116]. This impairment is associated with reduced T-lymphocyte infiltration, increased oxidative stress and reduced EPC numbers. The reduced vascular repair seen in hypercholesterolaemic mice is reversed in NADPH oxidase isoform (Nox)$2^{-/-}$ mice, together with an increase in EPC number, implicating oxidative stress as a key molecular mechanism [116].

Lower levels of HDL cholesterol are associated with a reduction in abundance of EPCs [111, 117]. HDL treatment of EPC *in vitro* has been shown to increase eNOS expression and reduce apoptosis, potentially explaining the relationship between circulating HDL and EPC abundance [117]. EPC function is also improved with HDL treatment, with enhanced adhesion and proliferation being demonstrated [118]. Furthermore, HDL treatment can enhance both *in vitro* and *in vivo* angiogenesis. Improved limb perfusion is seen in murine models of hindlimb ischaemia following HDL administration. This beneficial effect was abrogated by the concomitant administration of a PI3K inhibitor, or in eNOS knockout mice [119]. HDL increases EPC tube formation and can also reverse the deleterious effects of LDL on their survival [120].

1.4.4 Inflammation

Obesity is associated with a chronic, indolent inflammatory state, evident both on a systemic and tissue level, with increased circulating levels of pro-inflammatory cytokines [121]. Increases in the abundance and activation state of inflammatory cells are also noted [121]. Inflammation denotes a wide variety of processes, and notably 'inflammatory' cells also play an important role in hypoxia induced angiogenesis and vascular renewal by releasing proangiogenic molecules, which increase vascular permeability and degrade the basement membrane [122]. Animal studies have shown that acute inflammatory stimuli are associated with enhanced EPC mobilisation [123]. Human studies seem to concur with this finding, for example, enhanced EPC mobilisation is observed in response to acute myocardial infarction [124]. In conflict with this is the apparent detrimental effect of chronic systemic inflammation, with *in vitro* studies demonstrating decreased EPC number and increased apoptosis after exposure to TNF alpha and C-reactive protein exposure respectively [125, 126]. Conditions associated with chronic inflammation in humans, such as rheumatoid arthritis and heart failure, are associated with a reduction in circulating progenitors [127, 128]. The evidence-base suggests that although inflammation and the resulting cellular responses are required for normal vascular renewal, chronic low grade inflammation much like that seen in obese states is associated with deleterious effects on progenitor cells, and may be detrimental to vascular repair.

1.4.5 Adipokines

It is accepted that adipose tissue does not merely serve as storage depot for excess lipids, but also acts much like an endocrine organ. A long list of signalling molecules produced in adipose tissue (adipokines) have been found to be important in maintaining energy homeostasis by affecting signalling cascades in diverse target tissues [129]. As is the case with dyslipidaemia in the metabolic syndrome, there is a typical derangement in the levels of the adipokines seen. The vast majority of these are found in greater levels with increasing adiposity, with the most notable exception being adiponectin [129]. This insulin sensitising factor is found in reduced concentrations in the metabolic syndrome, and has been reported to induce anti-inflammatory, anti-apoptotic and pro-angiogenic changes [129, 130]. The majority of other adipokines exhibit a positive correlation with indices of adiposity, and these molecules have been shown to induce inflammation and inhibit adiponectin signalling [129].

With regard to adipokines and vascular regeneration, adiponectin is probably the most studied. Data show that adiponectin augments angiogenesis in animal models[131, 132] via mechanisms including promotion of AMP-activated protein kinase signalling [131, 132] and increased PI3K/Akt/eNOS signalling [132]. Adiponectin has also been shown to exert positive effects on both EPC number and function [133, 134].

Leptin has been shown to have beneficial effects on human EPC function at low concentration, but paradoxically impairs function at higher

concentrations, with reduced tube formation and migration noted [135]. Animal studies have also mirrored this effect, with pre-incubation of human EPC with leptin resulting in enhanced adhesion and vascular repair [136]. Leptin has also been shown to have a positive effect on vascular regeneration [137, 138].

Resistin is found in higher concentrations with increasing adiposity and has been shown to augment *in vitro* tubule formation, endothelial cell proliferation and aortic sprouting [139] and [140]. PI3K/Akt signalling and NF-kB activity has been implicated in this pro-angiogenic effect [140].

Visfatin has been shown to have detrimental effects on EPC function and its levels are increased in obesity and insulin resistant states [141], but has also been shown to augment angiogenesis and vascular regeneration [142]. Other data show that visfatin enhances endothelial cell function (HUVECs) and augments *in vitro* and *in vivo* angiogenesis [143].

It is difficult to comment on the net effect that the adipokine profile seen in obese and insulin resistant states has on vascular regeneration. The available data suggest that some features of the obese adipokine profile will have negative effects on angiogenesis whilst others could have beneficial impact on EPC function and re-vascularisation.

1.4.6 Hyperglycaemia

The insulin resistance seen in the metabolic syndrome generally progresses, and is accompanied by compensatory hyperinsulinaemia, but eventually pancreatic beta cell failure ensues. These processes lead to sustained hyperglycaemia, and are central to the diagnosis of diabetes. This section will look at the effect of hyperglycaemia on EPC function and vascular regeneration. EPC are grown less readily from patients with type 1 diabetes; moreover these EPC have reduced indices of function, with the cells less able promote endothelial tubule formation [144]. Similar findings are observed in studies of patients with type 2 diabetes [145]. It is difficult to dissect out and quantify the impact of hyperglycaemia versus insulin resistance on EPC function in these studies. It is clear however, that type 1 diabetes is associated with a lesser degree of insulin resistance than type 2 diabetes. Also, EPC number has been shown to inversely correlate with glycosolated haemoglobin levels in patients with type 2 diabetes. It is therefore reasonable to surmise that hyperglycaemia has a significant impact on EPC biology. Data from *in vitro* studies support this conclusion, and highlight the negative impact hyperglycaemia has on both EPC number and function. Incubation in a high glucose environment leads to reduced early and late outgrowth EPC number, increased senescence, decreased migration and decreased tubule formation. This was independent of any osmotic effect and mechanistically, reduced PI3K/Akt signalling and NO bioavailability was implicated [146].

In vitro studies have demonstrated that hyperglycaemia results in abnormal angiogenesis. Spheroid assays utilising endothelial and smooth muscle cells cultured in control and high glucose environment revealed an increase in fragile, thin endothelial sprouts that was a result of increase Jag1 activity, and resultant down regulation of Notch signalling [147].

Animal models of type 1 diabetes mellitus include mice with streptozotocin induced pancreatic injury. These animals exhibit impaired limb perfusion recovery in hindlimb ischaemia studies [148] and this has been associated with a reduction in progenitor cell number, and attributed to an increase in reactive oxygen species (ROS) [149]. This defect in limb perfusion recovery was reversed by the administration of an antioxidant, or reducing the production of ROS via genetic manipulation of NADPH oxidase [149].

Impaired vascular regeneration is also seen in murine models of type 2 diabetes. Leptin deficient mice are obese, with fasting hyperglycaemia and hyperinsulinaeima [150]. These mice also demonstrate impaired limb perfusion recovery in hindlimb ischaemia experiments [101]. Interestingly, animal studies utilising type 1 and type 2 diabetes models as comparators demonstrate that although both had impaired indices of vascular renewal following hindlimb ischaemia, the defect was more marked in the type 2 diabetes model [151] - i.e. insulin resistant diabetes is associated with a more marked defect in vascular regeneration than insulin deficiency.

Recent work has highlighted the potential role of PGC-1α on angiogenic processes. PGC-1α is a transcription factor that regulates metabolism in numerous tissues and is up-regulated in both type 1 and type 2 diabetes, suggesting hyperglycaemia rather than insulin resistance is responsible for this. Sawada *et al* found that overexpressing PGC-1α in the endothelium of mice resulted in impaired endothelial cell migration, impaired vascular repair in a wire injury model, and impaired vascular regeneration in a hindlimb ischaemia model. They postulated that PGC-1α mediated up-regulation of Notch activity, with resultant attenuation of Akt/eNOS signalling, was the underlying mechanism for this dysfunction [152]. Another recent study has suggested that hyperglycaemia-related oxidative stress prevents VEGFR2 membrane localisation, thereby leading to VEGF resistance and impaired angiogenic activity [153].

1.4.7 Abnormal insulin signalling

Discussion thus far has highlighted the complexity of the insulin resistant phenotype and the difficulty in assessing the impact that each facet of this phenotype can have on vascular regeneration. When considering the effect of insulin resistance and abnormal insulin signalling *per se* on vascular regeneration, it is important to note that the insulin receptor is found in many tissues beyond liver, skeletal muscle and fat. The insulin receptor can also be found in vascular endothelium, cardiac myocytes, leukocytes and many other cell types. Although few studies specifically address vascular regeneration, there is much data from murine studies utilising gene-modified strains with global or tissue specific alteration of insulin signalling.

The insulin receptor knockout (IRKO) mouse has global haploinsufficiency of the insulin receptor. This results in mild metabolic insulin resistance with normoglycaemia and mild hypertension [154]. Work in our lab has demonstrated that IRKO mice exhibit delayed re-endothelialisation following femoral artery denudation with an angioplasty wire [155]. Circulating progenitor cells (CPCs) were less abundant, as were blood derived EEPCs, and there was impaired CPC mobilisation in response to VEGF. Interestingly, c-kit$^+$ progenitors infused from wildtype (WT), but not IRKO donors normalised re-endothelialisation in IRKO recipients following wire injury. C-kit$^+$ cells from IRKO donors improved vascular repair, but not to the extent seen with WT cells, implying a defect in both CPC mobilisation and function [155]. However, the effect of global insulin resistance on vascular regeneration has not yet been addressed.

Recent work from our lab has also evaluated LEPC derived from South Asian men, who were relatively insulin resistant, compared with white European controls. These LEPC had impaired *in vitro* function, as demonstrated in tube forming and migration assays. The South Asian LEPC also did not augment vascular repair to the extent as white European cells when transfused into mice following femoral artery denuding injury. We linked these defects with a reduction in Akt/eNOS signalling, and were able to restore South Asian LEPC functionality via expression of a constitutively active Akt mutant [156].

Murine models of cell specific insulin resistance exist, and although few address vascular repair and regeneration, clues can be found in the existing literature. The most pertinent study, by Kondo *et al,* utilised mice with complete loss of insulin receptor expression in the vascular endothelium (VENIRKO). These mice exhibited a reduction in retinal angiogenesis in response to relative hypoxia, which was associated with reduced tissue VEGF and eNOS expression [157]. This supports the notion that insulin resistance can negatively impact vascular regeneration. However, the effect of systemic insulin resistance on angiogenesis in other vascular beds, and in response to other angiogenic stimuli remains to be clarified.

Other models of tissue specific insulin resistance provide insight with regards vascular regeneration. A muscle specific insulin receptor knockout model, which lacks insulin receptor in cardiac myocytes, exhibited a reduction in cardiac vascular density, both before and after ischaemia. This was associated with a subsequent reduction in VEGF expression, and suggests that insulin signalling is key in regulating tissue VEGF expression [158].

1.4.8 Insulin resistance and altered vascular regeneration; Molecular mechanisms

The preceding discussion has highlighted how different facets of the insulin resistant phenotype can impact on vascular regeneration. There are recurring mechanistic themes throughout, and to identify potential therapeutic targets it is of course vital to understand these. Reduced PI3-kinase mediated signalling, reduced nitric oxide bioavailability, perturbations

in growth factor sensing/signalling, and oxidative stress have all been implicated; the following section will expand on these.

1.4.8.1 PI3 kinase/Akt/eNOS signalling

As already outlined, the PI3-kinase/Akt signalling pathway results in many of the metabolic actions of insulin, and in the vascular endothelium also induces NO production. It is widely accepted that signalling via this pathway is disturbed in insulin resistant states [13]. Examining models of altered PI3-kinase signalling may therefore provide insight into whether this is relevant to the diminished vascular repair and regeneration associated with insulin resistance. PI3-kinase has been shown to mediate VEGF expression and angiogenesis, with constitutively active PI3-kinase expression resulting in increased angiogenesis. Conversely, expression of a dominant negative PI3-kinase construct results in a reduction in angiogenesis [159]. Study of PI3-kinase-ɣ knockout mice has demonstrated a homing defect in their EEPCs; bone marrow derived progenitor cells from these mice are also less able to augment angiogenesis when transfused following hindlimb ischaemia induction, compared with cells derived from WT mice [160]. Akt1 knockout mice exhibit impaired ischaemia driven angiogenesis, as demonstrated using the hindlimb ischaemia model [161]. Seemingly eNOS is an important mediator of this pro-angiogenic function of Akt signalling, as studies in mice expressing mutant eNOS, resistant to phosphorylation at the site recognised by Akt1 also demonstrate impaired angiogenesis, which is overcome with the expression of constitutively active eNOS[162].

Nitric oxide (NO) bioavailablity (or eNOS signalling) has been shown to play a critical role in progenitor mobilisation. For instance, Dimmler *et al* demonstrated rescue of the defective ischaemia-associated neovascularisation seen in eNOS knockout mice by infusing wildtype progenitor cells. They suggested that a lack of eNOS expression in bone marrow stromal cells, and hence a reduction in NO bioavailabilty, resulted in a progenitor mobilisation defect that significantly impaired recovery from hindlimb ischaemia [82]. Our laboratory's work in insulin resistant South Asian men has also demonstrated that progenitor mobilisation depends significantly on NO, with the NOS inhibitor, L-NMMA, abrogating exercise stimulated CPC mobilisation [163]. These data highlight the important role of PI3-kinase/Akt/eNOS signalling in angiogenic processes, and given the defects seen in this signalling cascade with insulin resistance, it is reasonable to consider this as a likely mechanism underlying associated disturbances in vascular regeneration.

1.4.8.2 Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive oxygen based molecules, produced during normal cellular metabolism. The term ROS describes a group of molecules that can be divided into free-radicals (superoxide anion, peroxynitrite) and non-radicals (hydrogen peroxide, ozone). ROS are produced during oxygen metabolism by mitochondria, and by enzymes such as lipoxygenase and NADPH oxidases (NOX). NOX are the most pertinent group of enzymes with regards vascular regeneration as they are the predominant source of ROS in the vasculature [164]. ROS were

originally thought to represent the harmful by-product of processes required for normal cellular function, and indeed some aspects of this statement can be considered correct. ROS are indeed the by-product of many crucial cellular processes, most notably the production of energy via the metabolism of oxygen. It is also clear that ROS can be harmful, and this property is exploited by macrophages that kill bacteria after phagocytosis using a ‘respiratory burst’ of ROS. At high concentrations ROS can react with DNA, lipid, carbohydrate and cause impaired function or irreversible damage [164].However, it has become clear that ROS act in other ways that are crucial to normal cellular function, for instance as signalling molecules [164].

There are biological systems in place to limit the production and lifespan of ROS, in order to limit the damage they can cause in excess. The breakdown of ROS is mediated by enzymes including superoxide dismutase and catalase, which dismute superoxide anion into hydrogen peroxide and water and degrade hydrogen peroxide to water and oxygen respectively [164]. Oxidative stress occurs when ROS production outweighs the capacity of the cell to degrade ROS. Insulin resistant syndromes are associated with an increase in ROS [165].

As outlined before, it is established that ROS play an important role in normal cell physiology, but also have the potential to cause cell damage if present in excess. Intuitively therefore, it would seem that there is a window within which the concentration of ROS is optimal. There is evidence to suggest that increased ROS have a detrimental impact on progenitor biology. *In vitro*

studies have demonstrated that hydrogen peroxide negatively impacts upon LEPC proliferative capacity [166]. Another *in vitro* study, however, demonstrated an increase in angiogenic processes (migration, proliferation and tube forming) in bovine endothelial cells treated with lower concentrations of hydrogen peroxide [167].

Murine studies have also highlighted the necessity of ROS for angiogenesis. Mice lacking an isoform of NADPH oxidase, NOX1, exhibit reduced endothelial cell migration and tube formation [168]. Furthermore, mice with NOX2 knockout have impaired ischaemia and VEGF stimulated angiogenesis in a hindlimb ischaemia model[169].

NOX4, a source of hydrogen peroxide, has been implicated in vascular regeneration. NOX4 knockout mice have impaired neovascularisation in response to hindlimb ischaemia and lung endothelial cells isolated from these mice form fewer vascular tubules *in vitro*, possibly due to reduced expression of eNOS [170].

In addition to the data discussed that highlights the importance of ROS to physiological angiogenesis, there is data to suggest that excess ROS negatively impact upon vascular regeneration. Studies show that in conditions characterised by oxidative stress, such as hypertension, there are impaired indices of vascular regeneration [171]. Eplerenone, a mineralocorticoid antagonist and losartan, an angiotensin receptor antagonist, have been shown to improve recovery from hindlimb ischaemia

in rats, via an antioxidant effect [105, 172]. Superoxide dismutase inhibition, and so elevation in superoxide concentrations, results in impaired collateral artery formation in a rat model of myocardial ischaemia [173]. Oxidative stress has been implicated as a mechanistic factor central to the impaired angiogenic response in type 1 diabetic mice post ischaemia [149] as well as host of other pathologies associated with reduced angiogenesis including hypercholesterolaemia [116]. In both of these studies the defect in angiogenesis was reversed by treatment with antioxidants or genetic ablation of proteins capable of producing ROS.

The apparent profound effect of ROS on angiogenesis has in part been attributed to its effect on VEGF signalling. Data shows that it impacts on many facets of VEGF signalling and action, including VEGF receptor dimerisation and activation, and VEGF mediated endothelial cell migration and proliferation [174]. For example, signalling pathways involving ROS are crucial to the action of VEGFR2. In order for the VEGFR2 to function normally it must be auto phosphorylated and this is regulated by protein tyrosine phosphotases (PTPs), via dephosphorylation at important sites of the VEGFR2. PTP activity is inhibited by oxidation, which occurs via superoxide anion [175] and therefore ROS indirectly regulates VEGFR2 activity. Another study by Warren *et al* [153], discussed in more detail in the following section, provides a link between diabetes, oxidative stress and abnormal VEGF activity and thereby provides a potential mechanistic link between diabetes and impaired vascular regeneration.

Evidence suggests that ROS are clearly very important to physiological angiogenic processes. There is also ample data to demonstrate that oxidative stress is detrimental to vascular regeneration, and often mediates the negative impacts on angiogenesis of a number of pathologies that co-exist in insulin resistant syndromes. On balance current literature supports the notion that there is probably an 'optimal zone' for ROS, and if levels of ROS fall either above or below this zone it is detrimental to angiogenesis and vascular regeneration.

1.4.8.3 VEGF signalling

As has been already discussed, VEGF is the chief angiogenic mediator, and its expression is under the control of hypoxia inducible factors (HIFs). Interference with this hypoxia sensing mechanism, and subsequent VEGF signalling, leads to changes in angiogenesis. There is data to suggest that these systems are affected in insulin resistant syndromes. It has been shown that monocytes from diabetic patients do not migrate effectively to VEGF; this was associated with significantly higher plasma levels of VEGFA in these subjects. However, their monocyte migration to formylMetLeuPhe appeared intact [176], and mechanistic studies suggested hyperglycaemia induced ROS inhibited protein tyrosine phosphatases required to terminate signal transduction. This may lead to constitutive activation of VEGFR1, and so prevent directed migration toward exogenous VEGF ligands. The authors of this study suggested that because monocytes lack VEGFR2, the receptor by which VEGFA-directed migration is chiefly mediated in endothelial cells, extrapolating these findings to endothelial cells is difficult. The findings do,

however, raise the concept of VEGF resistance in tandem with insulin resistance. More recent work supports this notion, with the demonstration that hyperglycaemia induced ROS results in abnormal VEGFR2 phosphorylation dynamics. This results in reduced VEGFR2 trafficking to the cell surface and a muted response to VEGFA despite increased circulating levels [153]. These experiments were done using two murine models of diabetes; *ob/ob* mice and streptozotocin induced diabetic mice, in which results were similar. This therefore implicated hyperglycaemia, rather than insulin resistance, in the signalling abnormalities and apparent VEGF resistance. In contrast to this, it is known that insulin can induce VEGF expression in a PI3K/Akt dependant manner, and when this insulin signalling is reduced VEGF mRNA levels fall [159]. VEGF production in response to ischaemia is reduced in diabetic rats [177]. Therefore, both 'VEGF resistance' and impaired VEGF production in response to ischaemia offer reasonable mechanisms by which the impaired vascular regeneration seen in diabetes occurs. Although insulin resistance may be implicated by association, it is not possible to accurately define the link between abnormal VEGF signalling and insulin resistance.

In addition to the apparent abnormalities in VEGF expression and responsiveness in insulin resistant syndromes, there is data to suggest significant crosstalk between insulin and VEGF signalling pathways. It is therefore conceivable that defects at certain levels of the insulin signalling cascade could modulate VEGF signalling. Evidence for this is provided by Senthil *et al* who demonstrated that insulin receptor substrate 1 (IRS1) is

phosphorylated in response to VEGF exposure in mouse kidney epithelial cells and rat heart endothelial cells [178]. Al-Mahmood *et al* also demonstrated that IRS-1 is upregulated in angiogenic conditions, and that by reducing the expression of IRS-1 with an antisense oligonucleotide, *in vivo* and *in vitro,* angiogenesis is reduced [179]. Data is also available to show that important cross talk occurs at the PI3-K signalling node. PKC activation, as seen in diabetic states, has been shown to phosphorylate the p85 subunit of PI3-K at Thr86 [180]. This modification is inhibitory, and results in decreased PI3-K/Akt activation in response to not only insulin exposure, but also to VEGF exposure, and therefore highlights another important node of the insulin signalling cascade that is shared with the VEGF signalling pathway.

1.5 Cell therapy

As insulin resistant syndromes become increasingly prevalent, novel treatment strategies have been sought to address the associated excess cardiovascular mortality and morbidity. One such therapeutic modality that has received recent attention is cell-based cardiovascular repair. The following discussion will focus on cell therapy, rather than other novel strategies, as it informs some of the work that has been conducted as part of this project.

Therapeutic strategies utilising cells of various lineages have been, and continue to be, evaluated in human clinical trials and animal studies. One such human study injected EEPCs or bone marrow derived cells into the

coronary arteries of patients that had recently had a myocardial infarction. They found a modest improvement in cardiac dysfunction, which was most pronounced with the use of bone marrow derived cells [181], recreating the findings of earlier studies [182, 183]. Although these studies are generally relatively small, 5 year follow up in the TOPCARE-AMI trial suggests that transfusion of bone marrow derived cells is a relatively safe procedure, and improvements in ejection fraction are maintained [184]. The REPAIR-AMI study, also showed a reduction in death, recurrent myocardial infarction and revascularisation at 1 year [185]. One study has failed to show any benefit with the transfusion of autologous bone marrow derived cells [186], but the methods used to store cells prior to use may have had negative effects on SDF-1 signalling. Encouraging results have been described in the SCIPIO trial where c-kit^{+} myocardium derived cells where injected into the coronary arteries of patients with ischaemic cardiomyopathy post bypass surgery. Patients receiving the c-kit^{+} cells had an 8% absolute increase in ejection fraction (EF), compared with no change in EF in the control group [187]. Similar results have been observed with the intracoronary delivery of cardiosphere derived cells [188], although both studies were not powered to assess anything other than safety measures. The balance of evidence remains unclear, and further work, powered to look at ‘hard’ outcomes, is required to address this. Furthermore, a recent meta-analysis has added to this lack of clarity, with the authors questioning the quality of the evidence base and highlighting a correlation between the number of discrepancies in a study and the magnitude of effect on left ventricular ejection fraction [189]. It

should be noted that some success has also been observed with the use of adult stem cells in the setting of peripheral arterial disease [190].

In particular relevance to work that will be carried out as part of this project, human LEPC have been used in pre-clinical animal models. LEPCs have been used in a murine hindlimb ischaemia model and were found to enhance neovascularisation; this effect was enhanced if the cells were co-injected with EEPCs [191]. They have also been used in models of porcine myocardial infarction, with the intra coronary injection of LEPC being associated with an increase in neovascularisation, and improvements in left ventricular remodelling, versus mesenchymal stem cells or vehicle [192].

Human studies so far encourage cautious optimism with regards cell therapy, although none have specifically recruited patients with insulin resistance or diabetes. One important consideration from the preclinical data outlined earlier is that these disorders appear to negatively impact upon progenitor cell function. It is therefore likely that autologous cell-based therapies in patients with insulin resistance or diabetes will require *ex vivo* pharmaceutical or genetic manipulation of cells in an attempt to improve their function.

1.6 South Asian ethnicity and cardiovascular risk

1.6.1 South Asian ethnicity

Novel strategies, such as cell-based therapies, may offer a means by which cardiovascular events can be prevented in high risk patients, such as those with diabetes. Another group at high risk for cardiovascular disease, and associated insulin resistant disorders, are those of South Asian ethnicity; published data from our group suggest progenitor cell dysfunction is also present in this population. Before discussing these issues in more detail, it is important to first define South Asian ethnicity. Ethnicity encompasses a person's religion, diet, culture, language and geographical heritage [193]. Biomedical research often assigns subjects to a particular ethnic group based on their geographical heritage. This method has its limitations as it fails to address other aspects of ethnicity that could impact on biology, and result in significant heterogeneity within these groups. There is no overriding consensus in how to address this, but for the purposes of this discussion, South Asian ethnicity represents those with Indian, Pakistani, Bangladeshi, Sri Lankan or Napalese heritage, unless stated otherwise.

1.6.2 South Asian ethnicity and cardiovascular risk

1.6.2.1 Epidemiology

Epidemiological studies conducted in the United Kingdom (UK) have consistently shown that South Asian ethnicity is associated with an increased risk of cardiovascular disease (CVD) [194, 195]. Cross-sectional analysis of UK mortality data identified an excess mortality from ischaemic heart

disease, compared with the UK average, of approximately 70% in South Asians in 1970, and 50% in 1990. When looking at cerebrovascular disease, the difference in mortality was more marked, with South Asians having standardised mortality ratios of 240 in 1970, and 150 in 1990.

Table 1. Prevalence of CVD/IHD/IHD or stroke, by age within minority ethnic group and sex. From: Health Survey for England 2004: The Health of Minority Ethnic Groups

Age: 55+		**Indian (%)**	**Pakistani (%)**	**Bangladeshi (%)**	**Chinese (%)**	**Carribean (%)**	**Gen Pop (%)**
IHD	**Male**	23.9	35.1	18	7.2	12.8	17.9
	Female	14.7	13.7	12.7	8.2	6.3	4.1
IHD or Stroke	**Male**	26.9	41.1	24.2	8.7	20.1	21.6
	Female	18.9	17.6	18.2	9	10.8	5.8

Data collected during the Health Survey for England in 2004 [196] again demonstrated the increased burden of cardiovascular disease in those of South Asian ethnicity. Table 1 shows data for both men and women over the age of 55. The highest prevalence of ischaemic heart disease (IHD) and stroke were seen in men of Pakistani heritage, with the prevalence of both IHD and stroke being elevated in men and women originating from Pakistan, India and Bangladesh.

This high prevalence of cardiovascular disease in UK South Asians is mirrored in other settings. Epidemiological data from the USA demonstrates this, with studies from 1996 quoting a three-fold increase in prevalence of angina or myocardial infarction in Asian Indian men, compared with the

general population; this was despite lower levels of cigarette smoking and obesity [197]. Other data point toward higher rates of ischaemic heart disease in urban, compared with rural, India. A systematic review carried out in 2005 used ECG criteria to define ischaemic heart disease, and concluded that the prevalence of IHD in urban areas was approximately double that seen in rural areas [198]. These findings, along with data from migrant populations in Western countries, imply that migration and the adoption of an urban or ‘Western’ lifestyle is important when considering the increased IHD risk seen in South Asian populations. Along with increased prevalence, there is evidence to suggest South Asian patients have more severe disease, with a tendency to suffer earlier and larger myocardial infarctions (MI) than those from other ethnic groups [199]. Recent angiographic studies in men of South Asian ethnicity in the US demonstrate that this group has much higher rates of 3 vessel coronary artery disease (CAD) than their Caucasian counterparts. South Asian men in this cohort had significantly higher levels of co-existing diabetes, hypertension, obesity and dyslipidaemia, but the Caucasian cohort contained more smokers. There was a strong trend towards South Asian ethnicity being an independent risk factor for the presence of 3 vessel disease CAD [200]. South Asian ethnicity has itself been mooted to be an independent risk factor for cardiovascular disease, with the excess risk not simply being explained by differences in conventional CVD risk factors, compared with Caucasian populations[199].

1.6.2.2 Pathogenesis

Some published data suggest that the increased CVD risk in South Asian populations can be accounted for by conventional risk factors such as hypertension, smoking and hyperlipidaemia [201]. However, there is also data to counter this that demonstrates that, in fact, excess CVD cannot completely be explained by an increase in conventional CVD risk factors [202]. When considering the impact of migration and the adoption of a 'western' lifestyle on vascular disease patterns it is interesting to note that other migrant populations, for example, Chinese immigrants in the UK, do not experience an increase in vascular disease, and in fact, have lower rates of IHD than the general population [203]. This suggests that the increased CVD risk in UK South Asians is therefore unlikely to solely be accounted for by migration and the adoption of a 'Western' lifestyle and that other factors play a significant role in the development of the observed CVD.

1.6.2.2.1 *Socioeconomic factors and healthcare provision*

The impact of socioeconomic status on the health of ethnic minority populations may also be important. A recent population study in Finland provides a good example of the impact socioeconomic deprivation can have, with higher socioeconomic status correlating with a favourable cardiovascular risk profile, and a slower rate of progression of carotid atheroma [204]. UK census data from 1991 showed that although there were higher rates of CVD in South Asian populations, these were proportionally spread across socioeconomic classes comparably to the general population.

This suggests that the higher rates of CVD seen in UK South Asians are not related to socioeconomic factors [205].

Recent data suggest that access to healthcare, and appropriate investigation and treatment, is not affected by ethnic status or socioeconomic status [206]. This conflicts with earlier work that suggested that thrombolysis occurred less frequently in UK South Asians [207]. Differences have also been seen historically in less acute settings, with data from the early 1990s demonstrating a longer time from experience of first angina symptoms to referral for secondary care input. The authors of this work suggested that language barriers, or perhaps referral bias on the part of general practitioners, could have been potential factors [208]. Subsequent work with South Asian focus groups acknowledges that language barriers play a part when considering access to health services [209]. Another study in 2002 concluded that although South Asian patients were less likely than white patients to receive revascularisation, despite being deemed appropriate candidates, this was not due to physician bias [210]. Available data has identified that socioeconomic factors do indeed impact on cardiovascular risk, but the impact this has on cardiovascular risk on UK South Asians, at least, is not disproportionate in comparison to the general population. On balance, although socioeconomic factors and access to healthcare have been shown to affect UK South Asians, it is probably not a major determinant of the excess CVD seen in this population.

1.6.2.2.2 Classical CVD risk factors

When considering CV risk in UK South Asians, or any other population, it is important to consider the presence of classical CV risk factors, such as hypertension, smoking, hyperlipidaemia and diabetes, and comparisons of risk profiles between populations may help to define reasons for inter-ethnic differences. In keeping with the heterogeneity of South Asian ethnic groups, tobacco use rates vary widely within the population. Studies can be found quoting lower overall rates of smoking [197], and smoking in Indian women is not commonly encountered [211]. A cross-sectional study of South Asian immigrants in the UK found that smoking prevalence was comparable to white Europeans, except for the Punjabi Sikh group, whose smoking rates were much lower [212]. Available data suggests that total cholesterol levels are broadly similar in South Asian and Caucasian populations. It is noted that south Asians do generally have lower levels of HDL cholesterol and higher triglycerides than other ethnic groups [197]. Furthermore, there are often higher levels of small, dense LDL cholesterol [213]. Interestingly, this pattern is reflective of the typical lipid profile seen in insulin resistant syndromes and the metabolic syndrome. Although this hints at the presence of insulin resistance, which could certainly contribute to the elevation of CVD rates in this ethnic group, and will be discussed further, differences in circulating lipids are not stark and it suggests that hyperlipidaemia *per se* is probably not the only mechanism by which CVD risk is elevated in South Asians. The Health Survey for England in 2004 found that levels of systolic blood pressure in those of South Asian origin were lower than the general population, and therefore cannot explain the increase in CVD seen, at least

in UK South Asians. UK census data again provide valuable insight to the prevalence of diabetes in those of South Asian ethnicity, reporting marked increases in rates of DM in those of South Asian origin, compared to the general population.

Table 2 Prevalence of type 2 Diabetes Mellitus within minority ethnic group and sex. From: Health Survey for England 2004: The Health of Minority Ethnic Groups

Age: 55+		**Indian (%)**	**Pakistani (%)**	**Bangladeshi (%)**	**Chinese (%)**	**Carribean (%)**	**Gen Pop (%)**
Type 2 DM	**Male**	24.3	25.3	29.9	16.1	24.8	9.3
	Female	20.5	44.4	13.5	13.1	25.7	6.9

1.6.2.2.3 *Obesity and Insulin resistance*

It is clear that the high prevalence of diabetes in South Asian populations is likely to be a major contributor to their increased CVD risk. Interestingly, given the close links between obesity, insulin resistance, and diabetes, this hugely elevated diabetes risk in South Asians is seen with body mass indices (BMIs) comparable to the general population. Evidence suggests, however, that despite having 'normal' BMI, South Asian populations have different body compositions to Caucasian counterparts, with increased body fat, and in particular visceral adiposity. Moreover, data produced using the gold standard test of insulin resistance, hyperinsulinaemic euglycaemic clamping, suggest a majority of healthy South Asian men are insulin resistant [214]. Similar data has been reproduced in UK cohorts, where despite similar BMI, UK South Asians are insulin resistant relative to Caucasian counterparts.

Again, this work provided evidence of differing body composition, with a propensity towards central adiposity seen in the South Asian group [215]. As a result of this apparent increased diabetes risk at ‘normal’ BMI levels, the International Diabetes Federation now recommends the application of ethnicity-specific thresholds for waist circumference when diagnosing the metabolic syndrome [104].

The aetiology of this central adiposity, increased visceral fat distribution, and resultant insulin resistance seen in South Asians migrating to urban/Western areas has been linked to the so-called thrifty phenotype phenomenon. Originally described with respect to the metabolic syndrome by Hales and Barker, this theory suggests that exposure to environmental factors *in utero* can lead to ‘foetal reprogramming’, and permanent adaptive changes that can influence the risk of developing disease in later life [216]. In the case of obesity and diabetes that are seen in migrant South Asian populations, it is postulated that *in utero* under-nutrition (e.g. during periods of famine) leads to adaptations which conserve metabolic homeostasis via induction of insulin resistance. It is suggested that these initially adaptive metabolic changes can become maladaptive in later life, if challenged by caloric excess and physical inactivity, resulting in the manifestation of obesity and frank diabetes. This theory is supported by the observations that birth weight in South Asian populations is amongst the lowest in the world, and this remains the case in babies born in the UK to parents of South Asian origin [217].

The fact that insulin resistance (or diabetes) is one of the few consistent differences in the cardiovascular risk profiles of UK South Asian groups, versus the general population, suggests that it is an important pathogenic factor in the development of CVD in this group, and this suggestion is supported by the findings of the Southall study, where diabetes or insulin resistance accounted for 70% of Q wave ECG abnormalities in migrant South Asians [218]. There are established mechanisms that link insulin resistance and endothelial dysfunction, the antecedent of atherosclerosis [10]. Perturbed insulin signalling in the vascular endothelium leads to a reduction in nitric oxide (NO) bioavailability [154], a hallmark of endothelial dysfunction. Diabetic humans have been shown to exhibit endothelial dysfunction [219], and this has also been demonstrated in animal models of insulin resistance [154]. Furthermore, there is evidence to show that young South Asian men have endothelial dysfunction, manifest by impaired flow mediated dilatation [220]. This impairment was, however, found to persist despite adjustment for insulin resistance as well as other common CVD risk factors, and perhaps implicates genetic differences, altered risk factor sensitivity or novel risk factors. The reasons for the endothelial dysfunction seen in young South Asians are not fully understood. A possible explanation is oxidative stress mediated reduction in NO bioavailability [221]; as highlighted in previous sections, many facets of the metabolic syndrome are characterised by oxidative stress. Also, the actions of insulin on the endothelium include the production of NO via Akt/PI3-K/eNOS signalling [222] and this could have implications in South Asian populations with insulin resistance. Whatever the cause(s) of such endothelial dysfunction, a

decrease in NO results in the reduction of, or loss of, its beneficial anti-coagulant, anti-inflammatory and vasodilatory properties and is a significant step in the development of overt atherosclerotic disease.

A common theme throughout this discussion is that a number of factors often combine to result in an observed phenotype. This is also the case with insulin resistance, and insulin resistance *per se* is unlikely to exclusively account for inter-ethnic differences in CVD risk. This is highlighted by studies in people of African origin. Despite exhibiting high levels of diabetes and insulin resistance [223], this population experiences low rates of CVD, something that is demonstrated in UK population studies (see Table 1). Further observations of this study were a lack of central adiposity despite the presence of insulin resistance and the presence of a favourable lipid profile in comparison to European counterparts [223]. It may be extrapolated therefore, that South Asian groups cluster more features of the metabolic syndrome; an atherogenic lipid profile along with insulin resistance for example, and it is this coming together of factors that increases CVD risk. In addition, physical inactivity and other factors that are more difficult to define, such as diet, are likely to play an important environmental role in the development of insulin resistance, indeed a sedentary lifestyle, possibly related to migration and urbanisation, has been shown to be a potential contributor to insulin resistance in British Asians [224]. The markedly increased rates of type 2 diabetes and CVD seen in UK South Asians is undoubtedly due to a combination of factors, the impact of which cannot be assessed individually. Genetic, intra-uterine and environmental factors

combine to result in physiological and anatomical differences, and differing body fat distribution. The culmination of these factors is ultimately insulin resistance, and this, along with a cluster of other factors, results in endothelial dysfunction; the first pathological step to overt atherosclerotic disease.

1.7 South Asian ethnicity and progenitor cell dysfunction

Work carried out in our lab already has demonstrated impairment in progenitor cell mobilisation, abundance and function in healthy South Asian volunteers. CPC mobilisation in response to exercise is impaired in healthy South Asian subjects [163]; this correlated with impaired flow mediated dilatation (FMD), and hence endothelial dysfunction. In further experiments, L-NMMA, the nitric oxide synthase inhibitor, abrogated CPC mobilisation; this is suggestive of reduced NO bioavailibity in the South Asian group leading to the CPC mobilisation defect [163]. This echoed findings of previous work demonstrating a reduction in basal CPC number and function, along with endothelial dysfunction, in apparently healthy, but relatively insulin resistant, UK South Asian men [225].

A further study in our lab used 12 healthy South Asian and white European volunteers to study the biology of late outgrowth endothelial progenitor cells (LEPCs). All baseline characteristics were comparable, except that the South Asian cohort was relatively insulin resistant, as measured by the HOMA-IR index.

LEPCs derived from the South Asian cohort grew less readily, formed fewer vascular networks on Matrigel, had impaired migration to VEGF and were more senescent. When infused in a murine vascular injury model, they did not encourage re-endothelialisation beyond that seen by vehicle alone, yet the white European cells did. These *in vitro* and *in vivo* functional deficits were associated with a reduction in Akt, eNOS, pAKT and peNOS levels, key components of the insulin signalling cascade. The defect in Akt expression was then corrected by lentiviral expression of a constitutively active Akt1 mutant (E17KAkt) in the south Asian LEPCs. All experiments were then repeated and E17KAkt expressing South Asian LEPCs were found to augment re-endothelialisation to a level comparable with white European LEPC [156]. This work suggests a link between South Asian LEPC dysfunction and metabolic insulin resistance, although did not specifically address insulin resistance in LEPC *per se*. It also shows that by modulating a critical node of insulin (amongst many others) signalling cascade rescues the capacity of these cells to augment vascular repair. However, it is yet to be elucidated whether South Asian LEPCs can augment vascular regeneration in the context of tissue ischaemia.

Chapter 2. Aims and Hypotheses

A body of work, including work done in our lab, demonstrates that South Asian ethnicity is associated with defects in progenitor cell function and our lab has demonstrated that LEPC from healthy South Asian subjects do not augment vascular repair. It is not known however, how LEPCs from this group affect vascular regeneration, an important question as these cells are potentially important therapeutic tools in the management of ischaemic disorders. South Asian populations are a group that has been shown to exhibit insulin resistance and are also at relatively high risk of cardiovascular disease. But, as has been highlighted in the introduction, dissecting and defining the impact of ethnicity from insulin resistance on biological processes such as vascular repair is difficult. We will attempt to investigate this further by assessing the impact of critical insulin signalling node modulation in LEPCs on vascular regeneration and we will do this by manipulating late outgrowth endothelial progenitor cells (LEPCs) from South Asian men. This aspect of the project will therefore set out to answer these questions:

1. Does the ability of LEPCs from South Asian men to augment angiogenesis *in vivo* differ from the ability of LEPCs from white European men to do so?
2. If LEPC from South Asian men do not augment angiogenesis to the extent of those from white European men, can modulation of a critical insulin signalling node (Akt) improve their angiogenic function?

As mentioned earlier, separating the impact of ethnicity from insulin resistance in human studies is difficult. Therefore, another set of studies will be undertaken to determine the impact that insulin resistance *per se* has on vascular regeneration. As discussed during the introduction, diabetes, a major global health problem, is a leading cause of premature death and much of this is driven by cardiovascular disease and ischaemic disorders. A body of evidence demonstrates that diabetes is associated with defective angiogenesis, but what is less clear, however, is the impact that insulin resistance *per se* has on angiogenesis. Occult insulin resistance is present long before the onset of diabetes, and importantly it has been shown that these patients also experience increased cardiovascular morbidity and mortality. An interventional target with potential to reduce the CVD burden seen in patients with insulin resistant syndromes could therefore be revealed, if the impact that insulin resistance has on vascular regeneration could be clarified. In order to investigate this we have planned a series of experiments that will aim to answer the following questions:

3. Does isolated insulin resistance impair vascular regeneration in response to ischaemia *in vivo*?
4. Does insulin resistance impair VEGF driven angiogenesis *in vitro?*
5. If insulin resistance is associated with impaired angiogenesis, what are the mechanisms responsible and how does this modulate VEGF action?

These questions translate into four key hypotheses that will be addressed within two major sub-sections of the results chapter. Firstly aims 1 and 2 will

be addressed with studies utilising LEPC derived from South Asian and Caucasian men. The null hypotheses for this section are:

1. LEPCs from South Asian men augment angiogenesis in a similar fashion to LEPCs from Caucasian men.
2. Modulating Akt signalling in LEPCs from South Asian men does not improve their vascular regenerative function.

Further sub-sections of the results chapter will address questions 3, 4 and 5 with a series of *in vivo* and *in vitro* assays, using murine and cell culture based models of insulin resistance. The null hypotheses for this section are:

1. Insulin resistance does not impact upon vascular regeneration.
2. Insulin resistance does not modulate VEGF action.

Chapter 3. Materials

3.1 Demographic and biochemical assessment

- Human insulin ELISA (Mercodia #10-1113-10)
- Syphygmomanometer (Omron MX3 Plus)
- Blood analyses performed in the laboratories of Leeds General Infirmary:
    - Full blood count
    - Urea and Electrolytes
    - Liver function tests
    - Plasma glucose
    - Lipid profile

3.2 LEPC derivation and culture

- Phosphate buffered saline (Sigma)
- Ficoll Paque PLUS (GE Healthcare)
- Biocoat fibronectin coated plastic culture vessels in 6-well, 24-well, T25 flask and T75 flask format (Becton Dickinson)
- EGM-2 culture medium ± EBM2 Singlequots growth factor supplements (Lonza)
- Foetal calf serum (Biosera)
- Trypsin/EDTA 0.025% solution (Gibco BRL)
- Cryomedium (Cryo-SFM) (Promocell)

3.3 Lentiviral transduction

- QuickTiter™ Lentivirus Titer Kit #VPK – 107 (Cell Biolabs)
- Akt Kinase assay kit #9840 (Cell signalling)

3.4 Hindlimb ischaemia and Murine femoral artery injury

- Microscopes
    - Nikon SMZ1500 Stereo microscope for dissecting tissue
    - Olympus SZ61 with up to 45x magnification with QiCam Olympus digital camera
- Fluovac anaesthetising chamber with gas scavenging system, Harvard apparatus
- Compact anaesthesia system AN001 (Vet Tech Solutions Ltd, UK) Fluovac system, Harvard apparatus
- Isoflurane key fill applicator AN003B
- Heating plate (Vet Tech Solutions Ltd, UK)
- Thermal cage (HE-011(Vet Tech Solutions Ltd, UK))
- Light source (Schott KL 1500 LCD)
- Guide wire 0.014” – Hi-Torque Cross-IT 200XT (AbbotVascular, USA)
- Micro serrifine curved (Inter Focus #18055-05)
- Forceps type clip applicator (Inter Focus #18057-14)
- Needle holder (World Precision Instrument #14109)
- Forceps and scissors:
    - Vannas scissors for arteriotomy (World Precision Instrument #501778)

  - Vannas scissors for cleaning vessels (Inter Focus #91501-09)
  - Fine Iris scissors for skin incision (Inter Focus #14094-11)
  - Tweezers Dumont (World Precision Instrument #500339)
  - Tweezers Dumont (World Precision Instrument #500234)
- Syringes
  - Insulin syringe 0.3ml with needle 29G x 13mm (Terumo)
  - Insulin syringe 0.5ml with needle 27G x 13mm (Terumo)
  - Insulin syringe 1ml with needle 29G x 13mm (Terumo)
- Surgical sutures
  - VICRYL absorbable surgical suture, 6-0, ETHICON (Johnson & Johnson)
  - VICRYL absorbable surgical suture, 8-0, ETHICON (Johnson & Johnson)
- Microscope slide (Cell Path)
- Microscope Coverslip 22 x 22mm (SLS)
- Others
  - Cotton tips
  - Gauze
  - Hair removal cream (Veet)
  - Adhesive tape
- Reagents

  - Isoflurane-Vet (Merial Animal Health Ltd, Essex, UK)
  - 4% paraformaldehyde (Fisher)
  - Rodent No.1 maintainence diet (Specialist Diet Service, UK)
  - Evans Blue (Fluka)
  - Buprenorphine (Alsatoe Animal Health, UK)
- Serum free protein block (DAKO, UK)
- Tissue-Tek OCT compound (Sakura, Netherlands)
- rabbit polyclonal antibody to mouse/human CD31 - ab28364 (Abcam,UK)
- goat polyclonal anti-rabbit conjugated to Chromeo642 - ab60319 (Abcam, UK)
- DAPI-Fluoromount-G (Southern Biotech, AL, USA)
- Laser Doppler analysis (Moor LDI2-HR, Moor systems, UK)
- LSM 700 laser scanning confocal microscope (Zeiss)

3.5 Akt Kinase activity assessment and Western Immunoblotting

- Akt Kinase assay Kit (Nonradioactive) (Cell signalling)
- Tris-Buffered saline with Tween-20
- Dried skimmed milk (Marvel)
- Bovine serum albumin (Santa Cruz Biotechnology)
- NuPAGE® LDS sample buffer (Invitrogen)
- NuPAGE® Sample reducing agent (Invitrogen)
- MES SDS Running Buffer (Invitrogen)
- 4-12% Bis-Tris Gels 1.5mm 10 well (Invitrogen)

- Precision Plus Protein Western C Standards (Bio-Rad)
- Precison Strep Tactin HRP Conjugate (Bio-Rad)
- Immobilon Western Chemiluminescent HRP Substrate (Millipore)
- Syngene G box imaging system
- Akt Rabbit Antibody (New England biolabs)
- Phospho-Akt Ser 473 Rabbit Antibody (New England biolabs)
- eNOS Mouse Antibody (BD Biosciences)
- Phospho-eNOS Ser 1177 Mouse Antibody (BD Biosciences)
- ERK 2 Mouse Antibody (Santa Cruz Biotechnology)
- Phospho-ERK Tyr 204 Mouse Antibody (Santa Cruz Biotechnology)

3.6 In vitro Angiogenesis, Boyden chamber migration assays and Invasion studies

- Growth factor reduced BD Matrigel™ matrix (Becton Dickinson)
- Human Recombinant Vascular Endothelial Growth Factor 165 (Peprotech)
- Biocoat fibronectin coated plastic 24-well culture vessels (Becton Dickinson)
- Polycarbonate Boyden chamber inserts with 8µm pore size (Becton Dickinson)
- Boyden chamber inserts with 8µm pore size, pre-coated with Matrigel (Corning)

- L-NMMA (Calbiochem)
- Olympus CKX41 Flourescence microscope with ‘Cell F’ software
- Haemotoxylin and Eosin (Sigma)

3.7 Murine aortic ring assay

- Phosphate buffered saline (Sigma)
- Opti-MEM + GlutaMAX-I (Gibco)
- Foetal calf serum (Biosera)
- Dulbecco’s modified Eagle’s medium (Gibco)
- Penicillin-streptomycin (Gibco)
- Collagen type I, rat tail (Millipore)
- Human Recombinant Vascular Endothelial Growth Factor 165 (Peprotech)
- Triton X-100 (Sigma)
- Protein block, serum free (Dako)
- BS1 lectin-FITC (Sigma)
- LSM 700 laser scanning confocal microscope (Zeiss)

3.8 Senescence

- Image J software - National Institutes of Health, USA
- Senescence β-Galactosidase Staining Kit (Cell signalling)
- Olympus CKX41 Flourescence microscope with ‘Cell F’ software

3.9 Survival studies

- Recombinant human tumour necrosis factor (TNF) – alpha (R&D systems)
- Hydrogen peroxide (Sigma)
- Lactate dehydrogenase (LDH) activity kit (Sigma)
- MRX TC 2 microplate reader (Dynex technologies)

3.10 Akt silencing

- Lipofectamine RNAiMax (Invitrogen)
- Akt1 (GCGUGACCAUGAACGAGUUTT, siRNA ID: s659) and control scrambled siRNA (Invitrogen)
- Opti-MEM serum free media (GIBCO)

3.11 Quantitative PCR

- Trizol reagent (Sigma)
- Isopropanol (Sigma)
- PTC Pellier thermal cycler (MJ Research)
- ABI prism 7900 HT thermal cycler (Applied Biosystems)
- Nano drop machine
- Reverse transcriptase kit (Applied Biosystems)
- Taqman PCR probes (Applied Biosystems)

3.12 PEC isolation

- anti-mouse PECAM-1 antibody (CD-31) (BD Biosciences)
- anti-mouse ICAM-2 antibody (CD-102) (BD Biosciences)
- anti-rat IgG Dynabeads (Invitrogen)

- Magnetic Particle Concentrator (MPC) (BD Biosciences)
- DMEM (Gibco)
- Collagenase/Dispase (C/D) (Roche)
- Cell strainer 70 µm nylon (Becton Dickinson)
- 14G Cannula (BD Biosciences)
- 10ml Syringes (BD Biosciences)
- Petri dishes (Nuncon)
- MACSmix™ Tube Rotator (Miltenyi Biotec)
- Scalpel blades (Swann-Morton)
- Bovine serum albumin solution (Gibco)
- Gelatin from Bovine skin (Sigma)

3.13 Scratch wound assay

- Sterile, non-coated, cell culture treated 24 well plate (BD Falcon)
- Olympus CKX41 Flourescence microscope with ‘Cell F’ software
- Mitomycin C from Streptomyces caespitosus (Sigma)

3.14 Proliferation assay

- LSM 700 laser scanning confocal microscope (Zeiss)
- Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life technologies)
- Phosphate buffered saline (Sigma)
- 4% paraformaldehyde (Fisher)

Chapter 4. Methods

4.1 LEPC derivation

4.1.1 Subject selection

Posters seeking healthy volunteers were displayed within the university campus after seeking appropriate ethical approval. Subjects were given written information prior to providing written consent. Ethical approval was provided by the Harrogate and district, and Leeds Central, NHS research ethics committees (06/Q1107/32 and 11/YH/0030). Ethnicity was defined by subjects from a list used in UK healthcare monitoring [226]; those selecting 'Asian or British Asian' were allocated to the South Asian cohort and those selecting 'White' were allocated to the white European cohort.

Subjects were required to be male, aged 18-40, and other exclusion criteria included; smoking within the past year, obesity (BMI>30), diabetes, hypertension (>160/90mmHg), hypercholesterolaemia (Total cholesterol >7mmol/L) and not taking regular medications. These criteria were chosen to minimise variability not related to ethnicity. Subjects were screened for the above criteria with a questionnaire, clinical exam and blood testing. Twelve white European and twelve South Asian men were recruited.

Blood pressure, body mass index, waist-hip ratio, full blood count, urea and electrolytes, liver function tests, fasting lipid profile, fasting insulin and fasting glucose were all measured in each recruit. All blood tests were analysed by the laboratory at the Leeds General Infirmary, with the exception of fasting

plasma insulin levels. This was measured in house with the use of an ELISA as per manufacturer's instructions. HOMA-IR, the index of insulin resistance, was calculated as fasting insulin x fasting glucose/22.5 [227].

4.1.2 LEPC derivation

Blood samples were taken and stored in EDTA coated tubes. 30mls of blood was mixed with an equal volume of phosphate buffered saline (PBS) and layered on top of Ficoll Paque PLUS prior to density gradient centrifugation (30mins, 300G, 22ºC, brake off). Peripheral blood mononuclear cells (PBMCs) were aspirated from the buffy layer and washed in 50mls of PBS and re-centrifuged at 400G for 10 minutes. The cell pellet was washed once more in 50mls of PBS at 400G for 10 minutes. The resultant cell pellet was re-suspended in 6mls of supplemented EGM2 media before being split, in equal volumes, into 3 wells of a fibronectin coated 6-well plate. Non adherent cells were removed daily for the initial 7 days of culture with a full culture media change and PBS wash. Half media changes were then performed on alternate days. Colonies of late outgrowth endothelial progenitor cells, with typical cobblestone morphology, were identified at approximately days 21 to 28 of culture (see Figure 4-1). These populations were then expanded via serial passage. After 2 passages the cells were detached from the culture vessel using 0.025% Trypsin/EDTA and centrifuged at 400G for 10mins. The LEPCs were re-suspended in 1 ml of cryo-medium in order to be stored in liquid Nitrogen for use at a later date. Prior to placement in liquid Nitrogen, cells were placed in a -80ºC freezer inside a freezing container containing isopropyl alcohol for 24 hours. This results in slow cooling, lowering the

temperature by 1ºC per minute, which is necessary for cryopreservation of cells.

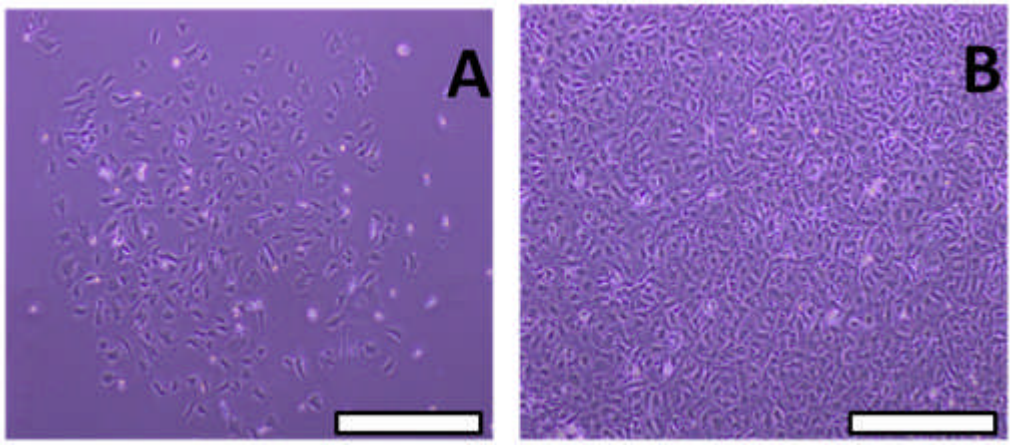

Figure 4-1: Example of LEPC colony and subsequent expansion. Scale bars denote 500µm.

4.1.3 Lentiviral transduction

As discussed previously, work in our lab has already identified dysfunction in the LEPC from healthy south Asian men and associated this with down regulation in the Akt/eNOS signaling axis. This dysfunction was reversed by over expressing Akt and this method was repeated for my studies to assess any effect on South Asian LEPC performance in a hindlimb ischaemia protocol.

Recent reports have identified a somatic mutation of Akt1 found in human breast, ovary and colorectal cancers. This mutation results in the substitution of lysine for glutamic acid at amino acid residue 17 (E17K) [228]. This mutation alters the pleckstrin homology domain (PHD) of the protein and results in constitutive localization to the plasma membrane. Once localized at the plasma membrane Akt is readily phosphorylated at serine 473 and

threonine 308. This mutation therefore renders Akt constitutively active. A lentivirus (pHVSLS2) was used to deliver this constitutively active form of Akt1 to the South Asian LEPCs. Lentiviral delivery (pSINCSGWdlNot1) of enhanced green fluorescent protein (EGFP) to matched LEPC samples was performed as a control to account for the effect of viral infection on the function of the cells.

pSINCSGWdlNot1 was a kind gift of Dr. Yashiro Idea (Mayo Clinic, Minnesota, USA). The EGFP cassette of pSINCSGWdlNot1 was replaced by E17KAkt to generate pHVSLS2. The SIN-lentiviral vectors were generated in accordance to established protocols [229]. Production of the lentivirus and subsequent cell transduction was performed by Dr Sam Stephen. Viral titration work was carried out by Dr Hema Viswambharan.

The lentiviral vectors were titred using QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24 ELISA kit [Cell Biolabs]) in accordance with manufacturer's protocol. The method is as follows. 100µl of inactivated lentiviral sample or p24 standard was added to a p24 antibody coated plate and incubated for 1 hour at 37ºC. After washing the plate with provided buffer 100µl of FITC-conjugated anti-p24 antibody was added to each well. This was agitated at room temperature for 1 hour and then washed 3 times with the provided wash buffer. These were repeated with a HRP-conjugated anti-FITC antibody in place of the FITC-conjugated anti-p24 antibody. Provided substrate solution (100µl) was added to each well and the plate was left for a further 30 minutes at room temperature. 100µl of stop solution

was then added to each well and absorbance at 450nm was measured immediately with a plate reader. According to the manufacturer's instructions, the results were used to calculate the concentration of lentiviral particles.

LEPCs were then infected with differing amounts of virus to infer the appropriate ratio of virus particles to LEPCs (multiplicity of infection, or moi) required to increase Akt activity by the desired amount; one moi of virus was used to increase Akt activity by a factor of 3 (as defined in pilot studies for a previously conducted project).

Prior to use in experiments semi-confluent second passage LEPC were transduced at one multiplicity of infection (moi) of appropriate lentiviral vector for use 4 days later.

4.2 LEPC transfusion studies

4.2.1 Preparation of cells for transfusion

On the day of hindlimb ischaemia induction, LEPCs were prepared in the following manner. Cells were labelled with CMDil cell tracker to enable subsequent in vivo tracing studies; this was done by reconstituting one vial of CMDil cell tracker (containing 50µg of CMDil) with 50µl of DMSO. This solution was then added to 50mls of complete EBM2 (supplemented with bullet kit and 10% FCS). Media was aspirated from the LEPCs and replaced with 2mls per well (6well plate) of the CMDil/EBM2 mixture and cells were placed in the incubator for 5 minutes. Cells were then detached from the

plate using Trypsin/EDTA solution and enumerated with a haemocytometer. $3x10^5$ LEPCs were used for each injection, and these cells were resuspended in 300µl of complete EBM2 media. The cells were aspirated into a 0.5ml syringe with a 27G needle attached and transported on ice to the animal facility to be transfused following surgery.

4.2.2 Hindlimb ischaemia

CD1 Immunodeficient mice were used to assess the capacity of LEPC to augment angiogenesis *in vivo*; this was done with a hindlimb ischaemia protocol. 24-72 hours prior to femoral artery ligation hair removal was undertaken. This was done after anaesthetic induction with 5% inhaled isoflurane in an anaesthetic chamber. Anaesthesia was maintained with 1-2% inhaled isoflurane delivered via face mask. Appropriate anaesthesia was confirmed by loss of withdrawal reflexes in the paw. Hair was carefully removed with clippers to the naval level, front and back. To remove residual hair, depilation cream was applied and gently removed with water and tissue paper. The mice were then recovered in a controlled manner and left until the following day, when cutaneous inflammation had resolved, and they were ready for the surgical procedure.

Anaesthesia was carried out as described above for the femoral artery ligation, along with an intraperitoneal injection of buprenorphine (0.25mg/Kg). An incision was made over the right inguinal ligament and the femoral vessels were identified and dissected. The femoral artery was ligated proximally at the inguinal ligament and distally at the bifurcation giving rise to

the popliteal and saphenous vessels. The ligated section of femoral artery was then removed and the wound was closed. Laser Doppler was performed 2 hours post-operation to confirm induction of hindlimb ischaemia.

Six hours following the induction of hindlimb ischaemia $3x10^5$ pre-prepared CMDil tracked LEPCs in EBM2, or vehicle alone as negative control, were injected into the contralateral femoral vein at the time of sham surgery. Following the sham procedure assessment of limb perfusion was performed via laser Doppler analysis.

4.2.3 Laser Doppler

Laser Doppler was undertaken to confirm ischaemia and to monitor recovery of perfusion 14 days later. Laser Doppler measurement was performed under anaesthesia. Before Doppler analysis, the machine was calibrated with the kit provided by the manufacturer. Mice were anaesthetised using 5% inhaled isoflurane (in Oxygen) for induction and maintenance was achieved with 1-2% inhaled isoflurane. Mice were gently immobilised with tape by the front paws in a supine position. The hindlimbs were gently extended and externally rotated and held in place by a 3-5mmx2mm section of double sided tape to allow visualisation of the appropriate section of hindlimb. The limbs were then imaged by the laser Doppler and limb perfusion was calculated by analysing signal flux below the ankle with the software provided. The flux arising from the injured limb was divided by the flux from the non-injured limb, after correction for the area that was measured, to give an ischaemic/non-ischaemic flux ratio. The mouse was then woken and

placed back with its litter mates, typically in cages of 2-4 mice. Mice were not housed alone unless fighting between siblings occurred. Mice were euthanised on day 14 after contucting repeat laser Doppler analysis. Adductor and gastrocnemius muscle was harvested from both limbs at this point for use for histological analysis. See Figure 4-2 for representative image following laser Doppler.

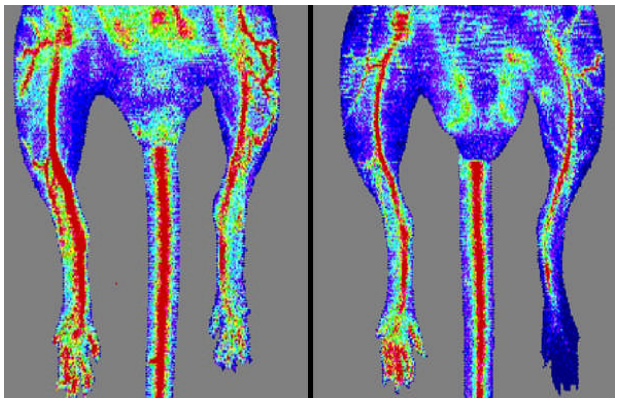

Figure 4-2: Laser doppler imaging; pre and post hindlimb ischaemia induction. Red indicates areas of high flux and therefore areas with increased perfusion. Conversely, blue is indicative of low flux and low or absent perfusion. Green represents areas with intermediate flux/perfusion.

4.2.4 Histological analysis

On day 14 post-surgery, perfusion fixation with 4% paraformaldehyde was carried out under isoflurane anaesthesia. Ischemic and contralateral-limb gastrocnemius muscle was then harvested and embedded in OCT, prior to snap freezing in liquid nitrogen and cryosectioning at 10µm thickness on to glass slides pre-coated with polylysine. Specimens were then blocked with serum free protein block (Dako) and incubated for 2 hours at room temperature with a rabbit polyclonal antibody to mouse/human CD31 (1:500

dilution) and washed with PBS. This was followed by a 1 hour incubation with a goat polyclonal anti-rabbit conjugated to Chromeo642 (1:100 dilution) then mounted on slides with DAPI to define nuclei. Confocal microscopy (Zeiss LSM 510 META Axioplan 2) was used to count engrafted LEPC, defined by nuclei with peripheral DiI and Chromeo642 fluorescence, per mm^2.

4.3 Transduced LEPC functional studies

4.3.1 Tubule formation

The required number of wells of a non-coated 24-well plastic cell culture plate were coated with 200µl of standard concentration, growth factor reduced, Matrigel and placed in a 37ºC incubator for 30 minutes. To render the cells quiescent, they were incubated in EGM-2 with 1% foetal calf serum for 4 hours. After this, cells were detached from the culture vessel with trypsin and enumerated using a haemocytometer. LEPCs (5×10^4 cells in 1ml of EGM-2 with 1% foetal calf serum with or without 50ng/ml $VEGFA_{165}$) were seeded in triplicate then cultured for 24 hours; in a subset of experiments 0.1mmol/L L-N^G-nitro-l-arginine methyl ester (L-NMMA), a nitric oxide synthase antagonist, was also added to culture medium. After 24 hours the plates were imaged with a phase contrast microscope (4 images per well at pre-defined positions, to avoid bias) and analysed. Tubules were defined as contiguous structures (see Figure 4-3: **Representative image of tubules on Matrigel. Scale bar denotes 2500µm. x40 magnification.**Figure 4-3 for example of contiguous structure) and a mean number of tubules was calculated per sample by calculating the mean number of tubules per high

power field from the 3 wells, i.e. 12 images (HPF: 7150x5300µm (x40 magnification)).

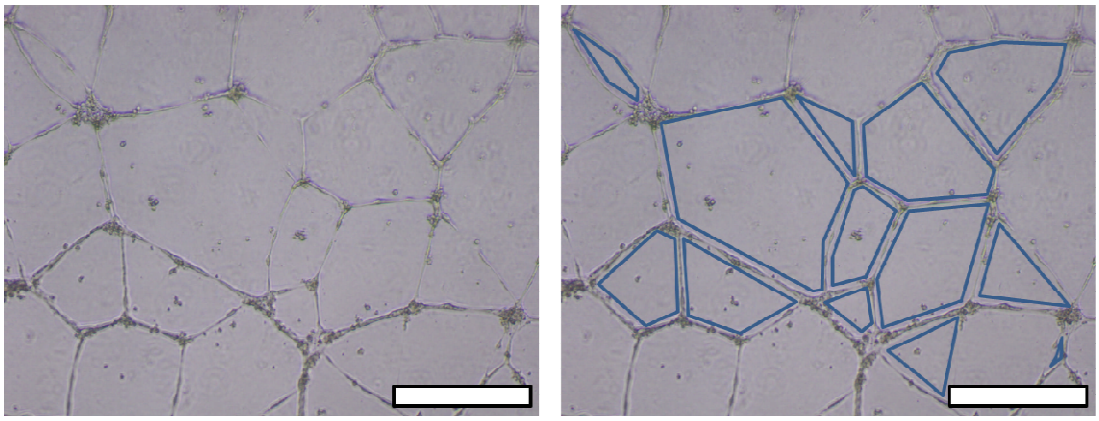

Figure 4-3: Representative image of tubules on Matrigel. Scale bar denotes 2500µm. x40 magnification. The panel on the right demonstrates the definition of all contiguous structures within that field of view.

4.3.2 Migration studies

LEPC were serum starved for 4 hours in EGM2 supplemented with 1% FCS prior to being treated with Trypsin to aid detachment from the culture plate and enumeration. 4×10^4 LEPC, suspended in basal EGM2 medium (1% FCS, without other additives), were placed in the upper compartment of modified Boyden chamber apparatus. The lower compartment contained basal EGM2 medium with 1% FCS, with or without (control) 50ng/ml Vascular Endothelial Growth Factor ($VEGFA_{165}$). After 24 hours, membranes were fixed in 70% ethanol before mechanical removal of cells on the upper surface, and Haematoxylin/Eosin staining; migrant LEPC were enumerated in 10 high power fields (HPF: 1430x1060µm). Experiments were conducted in triplicate. See Figure 4-4 for a representative image.

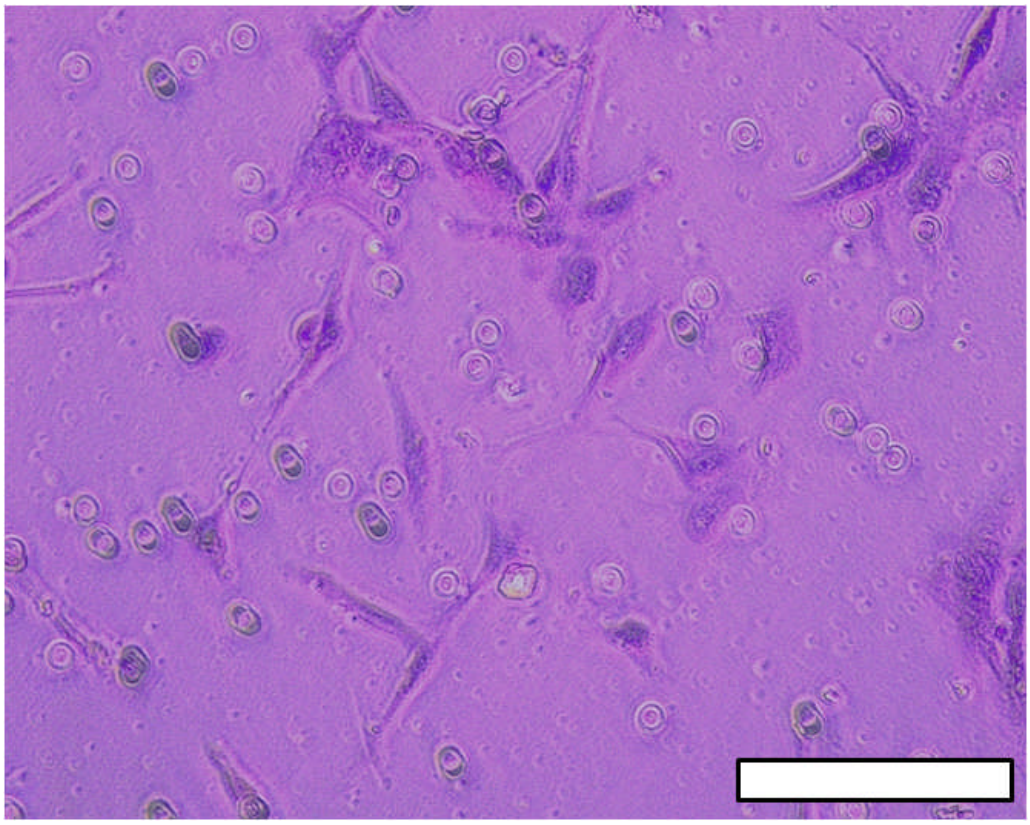

Figure 4-4. Representative image of Boyden chamber experiment x200 magnification. Scale bar denotes 500µm.

4.3.3 Senescence

Sub-confluent 3rd passage LEPCs were studied using a senescence-associated β-galactosidase staining kit. Cells were washed with PBS before treatment with the provided fixative solution. After 15 minutes the cells were washed two further times with PBS before adding the provided β-galactosidase staining solution and incubating overnight at 37ºC in ambient air. Senescent cells were identified by observing those cells that had developed a deep blue colour. Senescent EPCs were counted using Image J software and expressed as % of total cells. See Figure 4-5 for a representative image.

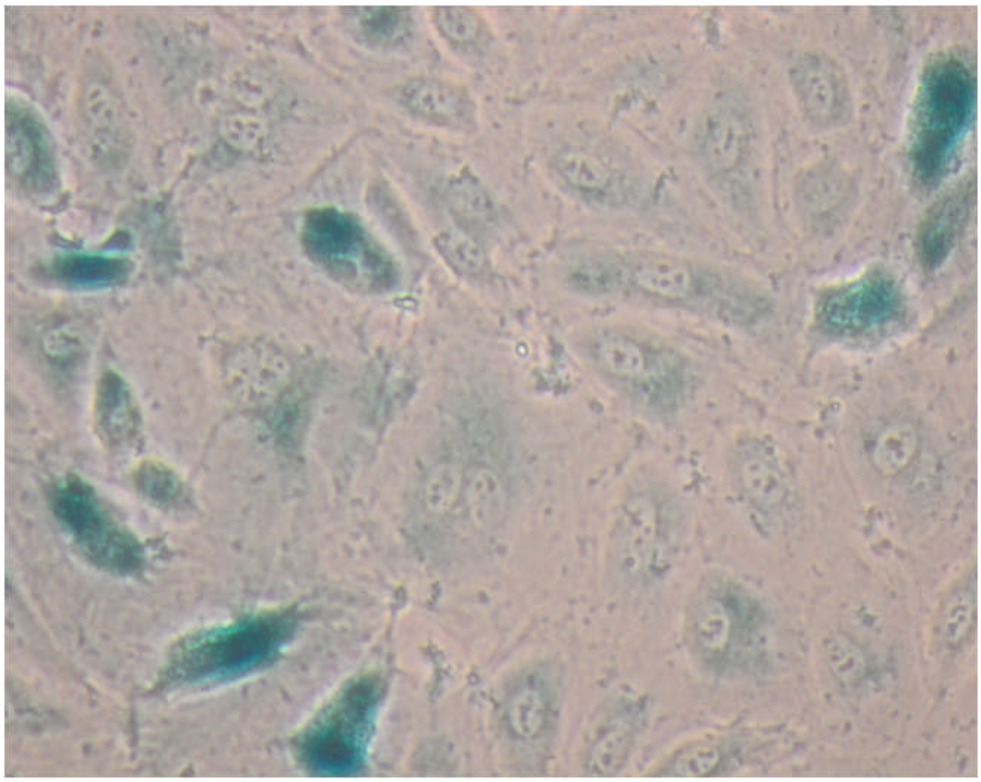

Figure 4-5. Representative image of senescence experiment x200 magnification. Cells with a deep blue colour were identified as being senescent.

4.3.4 Survival studies

An assessment of cell survival was carried out in LEPCs from South Asian donors transduced with E17KAkt or EGFP. This was done using a toxicology assay kit purchased from Sigma. This previously published method [98], measures LDH release from cells, a surrogate marker for cell membrane damage and cytotoxicity. $1x10^5$ LEPCs (transduced with EGFP or E17KAkt) were plated per well in 24w plate and allowed to adhere for 4 hours. Confluence was confirmed, and media was prepared to simulate an 'infarct environment', in order to 'stress' the cells and induce cytotoxicity. This was done by adding 0.5% heat inactivated (to denature LDH) FCS, 20ng/ml TNF-

alpha, and 500µM hydrogen peroxide, to basal EBM2. The cells incubated in the infarct environment media were then placed in a 1% O_2, 5% CO_2 incubator at 37°C for 24 hours. Half of the conditioned media (450µl) was collected from each well from the plate and transferred into Eppendorfs, and then centrifuged to remove cellular debris. 50µl of the supernatant was taken and added to a separate 96 well plate in duplicate; as a control, we also added infarct environment media in 2 wells to confirm low background LDH activity. 50µl of the provided LDH assay lysis solution was added to each well of the 24 well plate, which was then placed in a standard cell culture incubator for 45 minutes, agitating every 15 minutes. During these 45 minutes a reaction mixture was prepared according to manufacturer's instructions by combining equal volumes of assay substrate solution, assay cofactor preparation, and assay dye solution.

After 45 minutes, all of the lysed cell conditioned media was aspirated and into Eppendorfs and centrifuged to pellet the debris. 50µl of the supernatant was then added to a 96 well plate in duplicate. 100µl of the prepared reaction mixture added to each well of a 96 well plate. This plate was then stored in the dark for 25 minutes prior to stopping the reaction by adding 15µl 1M HCl to each well of the 96 well plate. A plate reader was used to measure 490nm and 690nm absorbance (the latter to normalise plate-related absorbance differences). LDH activity was then inferred using the normalised absorbance (490nm-690nm). We measured LDH activity in the conditioned medium from the cells exposed to the infarct environment and normalised to total cellular LDH.

4.3.5 Invasion studies

Invasion assays were carried out in an identical manner to the migration studies described in section 4.3.22, other than using Boyden chamber inserts pre-coated with Matrigel (Corning), to define VEGF-directed invasion, versus vehicle control.

4.4 LEPC Akt knockdown studies

4.4.1 Akt knockdown

confluent OEC were transfected with 20nM Akt1 siRNA (GCGUGACCAUGAACGAGUUTT, ID: s659) or control, scrambled Sub-siRNA (Invitrogen) using Lipofectamine RNAiMax (Invitrogen), according to the manufacturer's instructions. Confirmation of Akt1 knockdown, and functional studies, were conducted 48 hours later. In brief the method used was as follows; the cells were ready to use when 60-80% confluency was reached. The siRNA and Lipofectamine were both diluted in Opti-MEM media and mixed in a 1:1 ratio. This mixture was incubated for 5 minutes at room temperature and then added to the cells. The cells were incubated for 24 hours at 37^{o}C, 5% CO_2, for 24 hours and then washed before adding standard growth media. Knockdown was confirmed at 48 hours by western blotting for total Akt1 in cell lysates. All functional studies were conducted at this point.

4.4.2 Femoral artery injury studies using transfected LEPCs

CD1 Immunodeficient mice were used to assess the reparative capacity of the LEPCs from white European volunteers following Akt1 silencing. Mice were anaesthetised using isoflurane before three 1.5cm passes of a 0.014-inch-diameter angioplasty guidewire was used to denude the endothelium from the femoral artery. The contralateral artery underwent sham operation, without arteriotomy or wire injury. Animals received buprenorphine 0.25mg/Kg analgesia. Six hours following this wire-induced injury, equal numbers ($3x10^5$) of cell tracked LEPC in EBM2 were injected into the external iliac vein. After 4 days, 50µl of 0.5% Evans blue dye was injected into the inferior vena cava and the mice were perfusion fixed with 4% paraformaldehyde. Areas staining blue represented persistent denuded endothelium. See Figure 4-6 for representative images. Conversely, re-endothelialisation was defined as the region of absent Evan's blue staining in relation to a total area of a 5mm long section of vessel, commencing 5mm distal to the aortic bifurcation. Vessels were then blocked (Serum free protein block) and incubated for 2 hours at room temperature with a rabbit polyclonal antibody to mouse/human CD31 (1:500 dilution), this was followed by a PBS wash step. Samples were then incubated for 1 hour at room temperature with a goat polyclonal anti-rabbit antibody conjugated to Chromeo642 (1:100 dilution), washed with PBS and then mounted *en face* on slides with DAPI to define nuclei. Confocal microscopy (Zeiss LSM 510 META Axioplan 2) was performed on the intima of femoral artery specimens and adherent, fluorescently labelled cells that co-stained for CD31 and CMDiI cell-tracker were enumerated to define the abundance of LEPCs

incorporated into the endothelium. All murine studies and analysis were performed blinded to ‘treatment’ allocation.

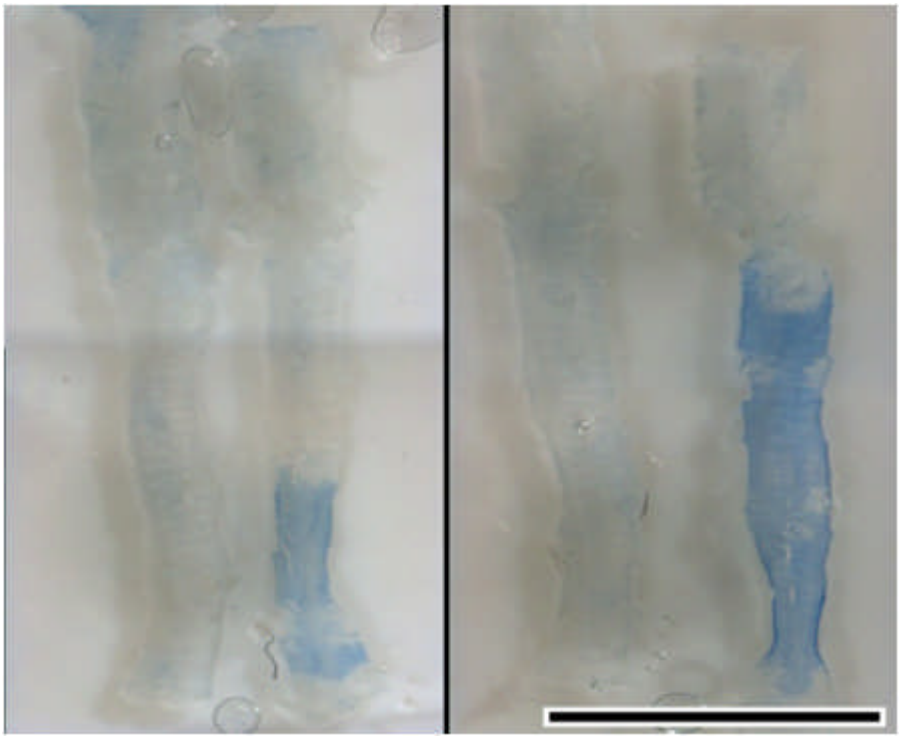

Figure 4-6. Representative image of explanted femoral arteries stained with Evan’s blue. Blue areas represent areas that have yet to re-endothelialise. In each panel there are two vessels from the same mouse; on the left is the uninjured vessel and on the right is the injured vessel. The vessel in the panel on the right demonstrates increased Evan’s blue staining, and therefore delayed endothelial regeneration, in comparison with the vessel in the left panel. Scale bar denotes 5mm.

4.5 Animal Husbandry

4.5.1 Insulin receptor knockout mice

Insulin receptor knockout mice (IRKO) were originally developed at the National Institutes of Health, Bethesda, USA [230]. IRKO mice have global

hemizygous null mutations of the insulin receptor. These mice were created by targeting the insulin receptor gene in a manner that results in a null allele. Gene inactivation is achieved by first constructing a targeting vector that will introduce a sequence including a neomycin resistance cassette and a premature termination chain. Genes were transferred via electroporation into embryonic stem cells. Stem cells were allowed to grow and colonies containing the mutant gene sequence were selected on the basis of neomycin resistance and expanded before being injected into day 3.5 murine embryos and implanted into female mice resulting in chimaeric offspring. Chimaeric males, determined by coat colour, were mated with C57Bl6 females, and the presence of germline mutation in the insulin receptor was confirmed via southern blotting. Offspring were used to establish colonies. Mice homozygous for the null allele ($IR^{-/-}$) develop normally but quickly develop hyperglycaemia and die after 48-72 hours of ketoacidosis. Mice heterozygous for the null allele ($IR^{-/+}$ or IRKO) exhibit apparently normal *in utero* development, and are insulin resistant without demonstrating overt hyperglycaemia [231]. The IRKO model has been chosen for this project so that the effects of insulin resistance, rather than hyperglycaemia, on vascular regeneration can be observed. Genetic manipulation was not undertaken as part of this project, as breeding colonies were already established at the University of Leeds at the outset. Mice were originally bought from the Jackson laboratory stock and bred with C57Bl6 females.

4.5.2 Breeding conditions

Breeding trios were set up to continue the colony. This comprised of 1 male and 2 females. Only the male ever carried the null allele and all mice were on a C57Bl6 background. Mice used for breeding were at least 6 weeks of age and preferably under 6 months of age. Access to food and water was not restricted.

4.5.3 Nude Mice

Nude mice were first discovered by Dr. N. R. Grist in 1962 at the virus laboratory at Ruchill hospital, Glasgow. They are hairless, hence the 'nude' nickname, but also have a genetic mutation resulting in the deletion of the FOXN1 gene, which consequently results in the absence of a thymus gland. Due to the lack of a thymus gland these mice are unable to produce mature T lymphocytes and the result of this is impairment in an array of immune responses. A particularly useful characteristic, in terms of research, is the inability to reject donor material and this has led to widespread use in tumour research. We chose this mouse as it will not reject human donor cells, namely LEPCs.

9-13 week old (weight 25-33g) male immuno-deficient CD1 nude mice (Charles River Labs) were utilized for the hindlimb ischaemia experiments required for *in vivo* assessment of human LEPC function.

4.5.4 Housing and experimental conditions

All experiments were conducted in full accordance with Home Office UK regulations, as mandated by the Experimental Animals (Scientific Procedures) Act 1988. Mice were housed in the animal facility at the University of Leeds under standard laboratory conditions in humidity (55%) and temperature (21ºC) controlled conditions with a 12-hour light-dark cycle. Animals were fed a standard chow diet and normal drinking water. Access to food and water was not restricted. Offspring were weaned at approximately 3-4 weeks onto a standard chow diet and at this point the mice were separated from their parents into male and female cages. Ear notching was performed in conscious mice at age 3-4 weeks, to allow identification, and to provide tissue for genotyping. Mice were housed in cages, irrespective of genotype, with a maximum of 5 mice per cage. Housing of single animals was avoided wherever possible. Only male animals were used to exclude the potential effects of cyclical variation in oestrogens. Mice were aged 8-12 weeks when taken for experiments. When mice were euthanised this was done via exposure to rising concentrations of CO_2 in a commercially sourced cabinet. This was done over a 10 minute cycle after which death was confirmed via cervical dislocation. The exception to this was when experiments required euthanasia with terminal anaesthesia.

4.5.5 Genotyping

4.5.5.1 DNA extraction

DNA extraction was performed using ear notches and incubating them in 100µl of 25mM NaOH/0.2mM EDTA solution for 20 minutes at 95ºC. After 20 minutes 100µl of 40mM Tris-HCL was added and the sample was vortexed.

4.5.5.2 PCR

PCR was performed in 25µl reaction volumes consisting of 2.5µl PCR buffer (containing 750mM Tris-HCl, 200mM (NH4)2SO4, 0.1% Tween 20), 2µl of MgCl (25mM), 0.5µl of dNTP, 0.5µl of primers, 0.2µl of Taq enzyme,17.3µl of water and 1µl of sample. Three primers were obtained commercially (Invitrogen) and sequences were as follows: TTA AGG GCC AGC TCA TTC CT CC (forward), AGC TGT GCA CTT CCC TGC TC AC (forward) and TCT TTG CCT GTG CTC CAC TCT CA (reverse). After mixing DNA samples with the reaction mix, tubes were placed in a thermal cycler. After completion of 31 amplification cycles (94ºC for 4 mins; 94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min and 72ºC for 4 mins) the products were identified by electrophoresis on a 1% agarose gel containing 3 µl of ethidium bromide (10mg/ml) (Sigma). Imaging of bands was performed by fluorescent imaging using a 100 base pair reference ladder and compatible software (ChemiLmager 5.5). The WT allele product is 232 base pairs, the null allele gives a product that is 255 base pairs in length. See Figure 4-7 for representative image of imaged gel.

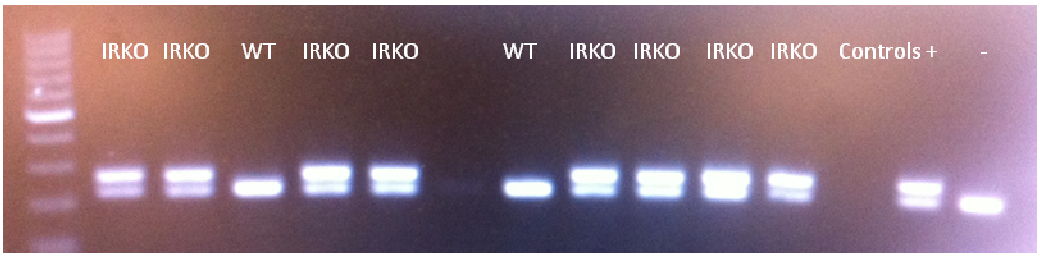

Figure 4-7 Representative image of gel used to genotype IRKO and WT mice. IRKO; Insulin receptor knockout, WT; Wildtype.

4.6 Western blotting

Cell lysates were generally produced when cells were approximately 95% confluent in 6 well plates. Following quiescence of cells, by incubation in serum free, unsupplemented media for 4 hours, cells were lysed in their basal state or following stimulation with insulin or VEGF. Lysis of cells was done as follows: they were first washed twice with ice cold PBS in attempt to arrest any cellular processes. 70µl of ice cold RIPA buffer, including protease and phosphotase inhibitors, was added to the cells and a cell scraper was used to detach and disrupt cells before lysates were aspirated and decanted into 1.5ml Eppendorf tubes. Cell lysates were then left on ice for 30 minutes whilst being mixed intermittently to facilitate complete cell lysis. Samples were then centrifuged at 13000rpm for 15 minutes at 4ºC. The resultant supernatant was then aspirated and decanted into clean 1.5ml Eppendorf tubes. The remaining pellet was discarded.

Before beginning western blotting, protein quantification was undertaken to allow for the same amount of protein to be loaded for each sample in subsequent electrophoresis and to ultimately allow accurate comparison.

Protein quantification was done using a commercially acquired bicinchoninic acid (BCA) assay. The method used was as follows: samples were diluted 1:8 with distilled water and were mixed before being pipette into a 96 well plate (25µl per well). 9 different, known concentrations of bovine serum albumin (BSA) were also placed (25µl per well) into the 96 well plate. All samples and protein standards were set up in duplicate. 200µl of BCA reaction mix, containing BCA reagent A and BCA reagent B (50:1 ratio) was then added to each well and the plate was covered with sealing tape and incubated for 30 minutes at 37ºC. Absorption spectra were measured at 562nm and Protein quantification was done in reference to the known concentrations of BSA and correction for the initial 1:8 dilution.

When the protein concentration was known, 30µg of sample was taken and mixed with loading dye to achieve a (1:4) dilution and reducing buffer (1:10) dilution. RIPA buffer was added to make the total volume 50µl. Each sample was then heated to 95ºC for 5 minutes. Samples were loaded into the wells of a pre-cast 4-12% polyacrylamide gel. One well was loaded with a protein reference ladder. The gel was placed in a tank and 500ml of running buffer was added (25ml MES SDS running buffer, 475mls distilled water). Samples were then electrophoresed at 160V for 1 hour.

Following electrophoresis, the gel was then left to soak in transfer buffer along with filter paper, sponges and a PVDF membrane. Before soaking the PVDF membrane was first washed with methanol and rinsed with distilled water. A sandwich was then assembled before protein transfer that consisted of: sponge, filter paper, gel, PVDF membrane, filter paper, sponge and held

firmly in a plastic cassette. The cassette was then placed in a transfer tank in the correct orientation (gel closest to centre of tank) to allow protein transfer. A mixer was then placed in the tank and it was filled with transfer buffer. An ice pack was also placed in the tank before the lid was connected to a power pack (100V for 45minutes).

Following protein transfer the PVDF was air dried prior to immunostaining for the protein of interest and then washed with methanol and rinsed with distilled water. The membranes were 'blocked' using milk buffer (containing 1.25g milk powder in 25ml of TBS tween) for 1 hour. The antibody of interest was then added to a pot containing the membrane and left to roll overnight at 4ºC. The antibodies were prepared in milk buffer and diluted according to the manufacturer's guidance, which differed depending on the antibody. The antibodies used and the dilution are indicated in Table 3.

Table 3 Antibodies used for Western blotting

Antibody target	**Antibody type**	**Manufacturer**	**Dilution**
eNOS	Monoclonal mouse	BD	1:1000
Phospho-eNOS (Ser 1177)	Monoclonal mouse	BD	1:1000
Akt	Monoclonal rabbit	Cell Signalling	1:1000
Phospho-Akt (Ser 473)	Monoclonal rabbit	Cell Signalling	1:1000

ERK 1/2	Monoclonal mouse	Santa cruz	1:500
Phospho-ERK 1/2	Monocloal mouse	Santa cruz	1:500
Beta actin	Monoclonal mouse	AbCam	1:3000
HRP conjugated 2º antibody	Mouse/Rabbit	Dako	1:1000

After being left overnight the membrane was then washed 3 times with TBS tween for 10 minutes. A secondary, HRP conjugated antibody directed at either mouse or rabbit, was then prepared in milk buffer and the membrane was incubated with this for 1 hour at room temperature. The membrane was then washed once with TBS tween for 10 minutes, once with TBS tween + 1:25000 Precison Strep Tactin HRP Conjugate for 10 minutes and finally once more in TBS tween for 10 minutes. The membrane was then incubated for 5 minutes with Immobilon Western Chemiluminescent HRP Substrate and sandwiched between 2 acetate films and imaged using the Syngene G box imaging system.

Once imaged the resultant bands were quantified into arbitrary units via densitometry using Image J software.

4.7 Akt activity

Akt activity was determined via a nonradioactive immunoprecipitation-kinase assay protocol as per the manufacturer's instruction. Cell lysates were made as per the western blotting protocol outlined above. Cell lysis buffer provided in the kit was used in place of RIPA buffer. Prior to centrifugation to remove cellular debris the samples were sonicated on ice. Beads, conjugated with Akt primary antibody were provided. Prior to use, they were slowly defrosted on ice to lower the viscosity of the buffer in which they were suspended. After protein quantification, 30µg of protein was made up to 200µl by adding lysis buffer. 20µl of the antibody/bead slurry was then added to the sample and this mixture was left agitating on a roller for 2 hours at room temperature. The lysates/antibody/bead mixture was then centrifuged at 14000G for 30 seconds and the pellet was washed twice with ice cold lysis buffer. The samples were kept on ice during the wash steps. The pellet was then washed twice more with kinase buffer, provided in the kit, before the pellet was suspended in 40µl of kinase buffer, and ATP and GSK-3 fusion protein were added. This reaction mixture was then incubated for 30minutes at 30ºC. After 30 minutes the reaction was terminated by the addition of SDS sample buffer. The sample was then used in a western blot protocol exactly as described above and immunostaining for phospho-GSK-3 was done using an antibody provided in the kit. The phosphorylated product that was measured was produced in a reaction catalysed by Akt present in the original cell lysates and is therefore indicative of the Akt activity of the sample.

4.8 In vivo assessment of angiogenesis in the IRKO mouse

4.8.1 Hindlimb ischaemia protocol

Hindlimb ischaemia was undertaken in IRKO and WT mice in the same manner that has been described in section 4.2.2. Conditions did not differ but no cells were administered in this subset of experiments.

4.8.2 Laser Doppler

Laser Doppler was undertaken as described in section 4.2.3, although was conducted every 7 days for 3 weeks after the initial assessment on day 0 to confirm appropriate induction of hind-limb ischaemia.

4.8.3 Histological analysis

On day 21 post-surgery, perfusion fixation with 4% paraformaldehyde was carried out under isoflurane anaesthesia. Ischemic and contralateral-limb gastrocnemius muscle was then harvested and snap frozen in liquid nitrogen and embedded in paraffin prior to sectioning at 5µm thickness on to glass slides pre-coated with polylysine. Specimens were then blocked with serum free protein block (Dako) and incubated for 2 hours at room temperature with a rabbit polyclonal antibody to mouse/human CD31(1:500 dilution) then washed with PBS. A further 1 hour incubation at room temperature with a goat polyclonal anti-rabbit conjugated to Horse Radish peroxidase (1:100 dilution) followed. The specimens were then incubated with DAB substrate and mounting on slides was completed with the addition of a coverslip following staining with Haemotoxylin and Eosin. Brightfield microscopy was

used to count capillaries. These were identified as brown structures running between myocytes. See Figure 4-8 for a representative image.

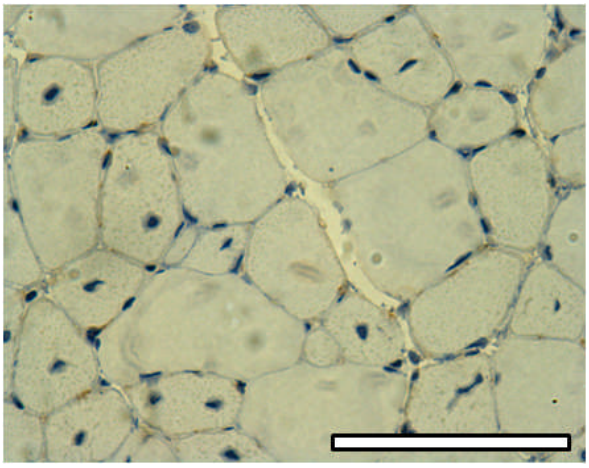

Figure 4-8. Representative image taken following histological analysis of gastrocnemius muscle harvested following hindlimb ischaemia. X400 magnification. Scale bar denotes 250µm.

4.8.4 Quantitative PCR

Gastrocnemius and adductor muscle were harvested following hindlimb ischaemia for analysis of VEGF expression, which was done via quantitative PCR. The methods used are described below.

4.8.4.1 RNA Extraction

Prior to RNA extraction tissue samples were placed in a 1.5ml Eppendorf tube containing 1ml of Trizol reagent (Sigma) and lysed using a mechanical tissue lyser. The resultant mixture was centrifuged at 10,000g for 10 minutes. The supernatant was transferred to a fresh tube and 200ul of phenol chloroform was added. The tubes were shaken vigorously for 15 seconds and left to settle for 3 minutes before another centrifugation step (12,000g for

15 minutes). The resulting clear layer was carefully removed with a pipette and decanted into a clean tube. 500µl isopropanol was added before being gently mixed and left at room temperature for 10 minutes. The samples were placed in the centrifuge and span at 12,000g for 10 minutes, the resulting supernatant was discarded and the pellet was mixed 1ml of 75% ethanol and span at 7,500g for 5 minutes. The supernatant was carefully removed and the samples were left to air dry for 10 minutes. Samples were mixed with 20µl of RNase free water and placed on ice for 20 minutes. RNA concentration was then measured using a nanodrop system and relevant software. Samples were stored at -80ºC until needed.

4.8.4.2 Reverse transcriptase PCR

Before performing quantitative PCR, RNA obtained from the extraction process was converted to complimentary DNA (cDNA). 1µg of RNA was added to a mastermix, containing water, random primers, dNTPs, multiscribe enzyme (reverse transcriptase) and buffer. The RNA was diluted in water (10 µl total volume) and this was added to the mastermix to give a final reaction volume of 20µl. This was then placed in a thermal cycler (25ºC for 10 minutes, 37ºC for 2 hours 85ºC for 5 minutes) and then either used for quantitative PCR or stored at 4ºC.

4.8.4.3 Quantitative PCR

Quantification of RNA expression was done using cDNA obtained as outlined above. The PCR reaction was performed in a thermal cycler (ABI Prism 7900HT, Applied Biosystems) using Taqman as the fluorescent probe to

detect PCR products. Samples were analysed in triplicate and an average of the 3 readings was used to calculate the relative expression using the comparative Ct method. Reaction volumes were 19 µl and made up as follows; water 8µl, Taqman 10µl, primer 1µl. Beta-actin was used as the house keeping gene for normalisation and the sequence was as follows: forward TTCTACAATGAGCTGCGTGTG and reverse GGGGTGTTGAAGGTCTCAAA. The insulin receptor primers used had the following sequence: forward TGCCACCAACCCCTCTGT and reverse CGGAGGGTGGTTTCCACTT. Other primers used included an 18s Taqman probe; product code Mm02601777-g1, and a VEGFA Taqman probe; product code Mn01281449-m1.

4.9 PEC Isolation

This previously described method [232] was used to isolate pulmonary endothelial cells (PECs) from murine lungs. The endothelial cells cultured were then use in an array of *in vitro* functional assessments and signalling studies.

4.9.1 PECAM bead preparation

Prior to obtaining tissue for pulmonary endothelial cell (PEC) isolation antibody/bead solutions were prepared. 200µl of magnetic bead solution was aliquoted into a 1.5ml Eppendorf tube. This was then loaded onto a magnetic column and left for 1 minute. The supernatant was then removed and the magnetic beads were washed four times with a 0.5% BSA/PBS mixture. The

beads were then resuspended in 500µl of 0.5% BSA/PBS and mixed with 10µl of anti cd-31 (anti PECAM). This mixture was agitated overnight at 4ºC. The antibody/Bead solution was then loaded onto the magnetic columns and washed with 0.5% BSA/PBS four more times in the manner described above. The antibody/bead solution was finally re-suspended in 200µl of 0.5% BSA/PBS and stored at 4ºC for up to two weeks. The above steps were repeated in the same manner to prepare the anti CD102 (anti ICAM)/bead mixture. This mixture was stored at 4ºC for up to two weeks also.

4.9.2 PEC Isolation

Mice were euthanized at ages 8-12 weeks by exposure to a rising concentration of CO_2. Death was confirmed via neck dislocation. Subsequently, the lungs were carefully dissected and transferred into Hank's buffered salt solution (HBSS) and kept on ice until ready to process further. In a cell culture hood, the lungs were placed into 10cm Petri dishes and minced with two scalpel blades until the lungs were cut into approximately $1mm^3$ cubes. Minced lungs were then mixed with 10mls of a collagenase/dispase solution (1mg/ml prepared in Dubellcos modified eagle medium (DMEM)) and agitated for 45 minutes at 37ºC. The mixture was then passed through a 70µm cell strainer and the collagenase/dispase was neutralised with complete endothelial cell media containing 10% FCS. The solution was then triturated with a 14G cannula and 10ml syringe in an attempt to achieve a single cell suspension. The solution was then centrifuged for 8 minutes at 400g. The cell pellet was re-suspended in 3mls of a 0.5% BSA/PBS mixture before the addition of 20µl of PECAM/Magnetic

bead slurry. The solution was agitated for 15 minutes at room temperature before proceeding with the magnetic separation of endothelial cells. The cell/bead mixture was split into three 1.5ml sterile Eppendorf tubes and loaded onto the magnetic columns. After 1 minute the supernatant was removed and the cells were washed with 1ml of 0.5% BSA/PBS. The cells were then loaded onto the magnetic column again. This process was repeated five times and then the cells were suspended in 10mls of endothelial cell growth media and plated onto T75 cell culture flasks that were pre-coated with 2% Gelatin. After twenty-four hours the media was completely replaced and thereafter half the media was replaced on alternate days. Clusters of cells then began to grow and when 70% confluency was achieved, typically after 10-14 days, a second bead separation was performed. The cells were detached from the flasks using a 0.5% Trypsin/EDTA solution. When detached from the flask the Trypsin was neutralised with endothelial cell growth media and the solution was centrifuged for 8 minutes at 400g. The cell pellet was then re-suspended in 2mls of a 0.5% BSA/PBS mixture and 10µl of the ICAM/Bead slurry was added. This mixture was agitated at room temperature for 15 minutes. The solution was then split into two 1.5ml Eppendorf tubes and loaded onto the magnetic columns. The cells were washed as describe with the first bead isolation and then re-suspended in 10mls of media once more. These cells were plated onto a T75 flask pre coated with 2% Gelatin and allowed to grow to confluency, typically taking approximately 5-7 days. The cells were then passaged without a bead separation into 3 T75 flasks and allowed to grow to confluency. At this point they were use for an array of *in vitro* studies.

4.9.3 Characterisation of murine PECs

Methods used to confirm the endothelial nature of the cells isolated from murine lungs included immuno-fluorescence based imaging techniques and western blotting to confirm the presence of eNOS.

4.9.3.1 Immunocytochemistry

Initially PECs were seeded onto fibronectin coated coverslips and were allowed to recover and incubated as normal in standard growth media overnight. The cells were fixed using ice cold acetone for 10 minutes at room temperature. The use of acetone also permeabilised the cell whilst preserving membrane bound antigens. The cells were washed twice with ice cold PBS prior to the blocking stage. Cells were incubated in 1% BSA in PBSTween for 30 minutes to block subsequent non specific binding of the antibodies. Cells were incubated with antibody overnight. The antibody used was an anti-VE Cadherin rabbit polyclonal antibody or a Rabbit IgG Isotype Control. The cells were washed 3 times with PBS, at each wash step the PBS was left for 5 minutes. The secondary antibody, again diluted in 1% BSA in PBSTween, was then added and the cells were incubated for 1 hour at room temperature in the dark. The secondary antibody was an Alexa Fluor® 647 Goat Anti-Rabbit IgG (H+L) Antibody. The cells were then washed a further 3 times (for 5 minutes) in PBS before they were counter-stained with DAPI for 1 minute. The cells were rinsed again with PBS and imaged using a confocal microscope. Negative control samples were processed identically, other than omitting the addition of the primary VE-Cadherin antibody (See Figure 4-99 for a representative image). This

confirms the presence of VE Cadherin in the anticipated pattern, supporting their endothelial phenotype. Western blotting confirmed the enrichment of eNOS in bead purified samples (See Figure 4-1010).

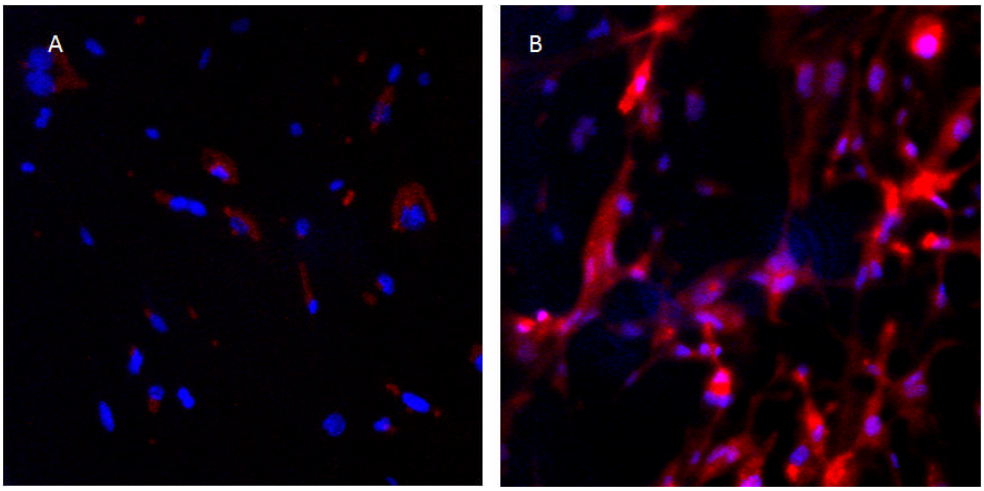

Figure 4-9 Panel A: Representative confocal microscopy image of PECs following immune-staining with nuclear stain, DAPI in blue, and Alexa Flour 647 isotype control antibody in Red (Negative control). Panel B: Representative confocal microscopy image of PECs following immune-staining with nuclear stain, DAPI in blue, and VE Cadherin antibody with Alexa Flour 647 secondary anti rabbit IgG antibody, in Red.

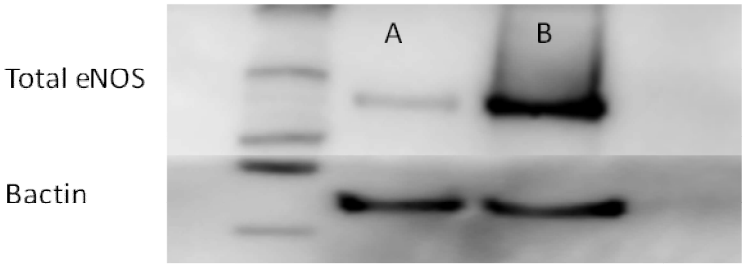

Figure 4-10. Western blot of PEC lysates. Lane A from a lysates made using PECs derived without two step bead purification. Lane B is from a lysates made from the PECs derived by the method used for this thesis.

4.10 In vitro assessment of angiogenesis in the IRKO mouse

4.10.1 Aortic ring sprouting assay

Mice were culled at age 8-12 weeks by exposure to a rising concentration of carbon dioxide; subsequently death was confirmed via dislocation of the neck. Aortae were harvested into ice cold Opti-MEM media. A dissecting microscope was used to facilitate the removal of peri-vascular fat and trimming of side branches. After cleaning, the aortae were gently flushed with 1 ml of Opti-MEM media using a 1ml syringe and 27G needle. The aortae were cut into approximately 20 rings of approximately 1mm thickness. These rings were then incubated overnight in serum free Opti-MEM at 37°C in 5% CO_2. The following day (day 0), each ring was placed in individual wells of a 96-well plate and embedded in a plug consisting of DMEM and 1mg/ml rat tail collagen. The 1mg/ml collagen DMEM mixture was prepared on ice and 75µl volumes were aliquoted quickly into each well of a 96 well plate. Only 5 wells were aliquoted at a time to prevent premature polymerisation prior to embedding of the aortae. The rings were placed so the lumen of the aorta was in a vertical orientation. Aortic rings from IRKO and WT littermates were exposed to either Opti-MEM media containing 2.5% FCS alone, or 2.5% FCS plus 50ng/ml of $VEGFA_{165}$. Penicillin-streptomycin was added to the media to prevent the development of infection. Media was replaced on day 3 and the rings were fixed and stained on day 5 in preparation for analysis on day 6. On day 5 all wells were washed with PBS and then incubated for 30 minutes at room temperature with 4% paraformaldehyde. The fixative was removed prior to 2 further 15 minute incubations with PBS plus 0.25% Triton-X 100.

Each well was then incubated with 1 drop of DAKO protein blocker for 30mins at 37°C. The rings were then incubated overnight with PBS plus 0.1% Tween-20 and 0.1mg/ml of BS-1 lectin-FITC. The following day each well was imaged using a laser scanning confocal microscope (Zeiss LSM700 laser scanning confocal microscope). Tiled images were taken (3x3) to allow complete visualisation of the ring and endothelial sprouts. See Figure 4-111 for representative images. The data was analysed by measuring the 3 longest sprouts and counting the number of sprouts per ring. Data from the rings of one mouse was collated and a mean value (mean number of sprouts, mean sprout length) was calculated for each mouse. Each sprout was counted as it emerged from the base of the ring before it had branched. Data was compared between groups using an unpaired students t test and statistical significance defined as $p<0.05$.

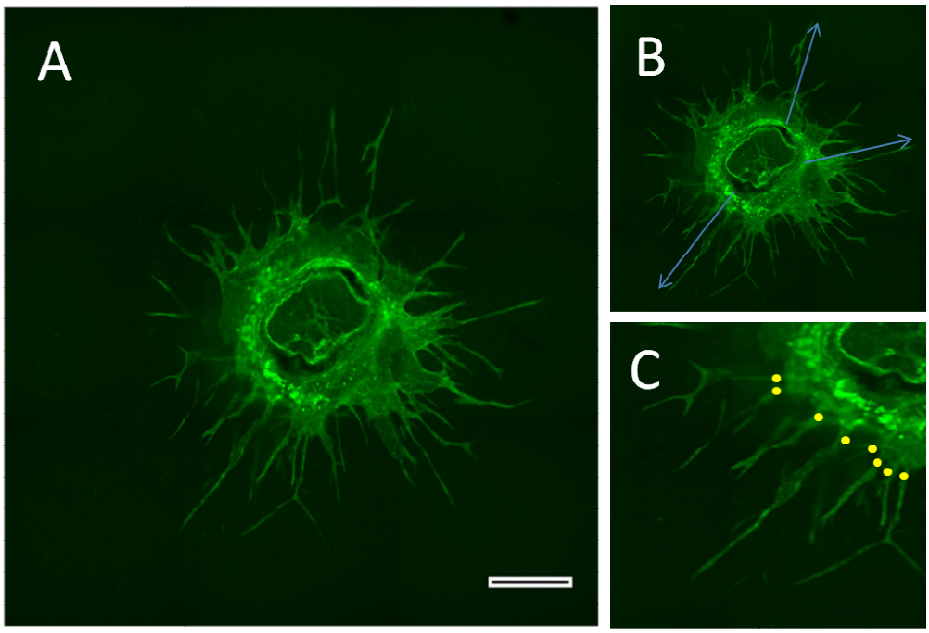

Figure 4-11. Panel A: Representative confocal microscopy imaging of aortic rings following lectin staining. Scale bar denotes 500µm. X200 Magnification.

Panel B: Method used to determine sprout length; 3 longest sprouts and measures taken are shown. Panel C: Identification of sprouts for enumeration; sprouts were counted prior to branching.

4.10.2 Tubule formation

Murine pulmonary endothelial cells (PECs) were isolated as described and used at passage 3 to assess tubule formation capacity. Prior to cell seeding, Matrigel coated plates were prepared. Tube forming experiments were performed as described as described in section 4.3.1, except that $2x10^5$ cells PECs were used per well. Prior to seeding the cells were suspended in basal media containing 1% FCS +/- 50ng/ml of VEGFA. Wells were set up in triplicate for each condition and imaging was performed

4.10.3 Migration assay (Boyden chamber)

Murine PECs were isolated as described and identified for use in a Boyden chamber assay at passage 3. Boyden chamber experiments were done as described in section 4.3.2, except that $5x10^4$ PECs were used per well.

4.10.4 Migration assay (scratch wound)

The capacity for WT and IRKO PECs to migrate was assessed with a scratch wound assay. Cells were plated in triplicate for each condition on to 24 well plates that were pre-coated with 2% Gelatin and allowed to grow until confluent. Prior to plating, a line perpendicular to the intended scratch wound was drawn underneath the plate to aid localisation during imaging. When

confluent, a proportion of cells were incubated for 12 hours in complete endothelial cell growth media containing 1 µg/ml Mitomycin C in order to prevent cell division, hence allowing study of migration without proliferation. A horizontal scratch was then made using a p-200 Gilson pipette tip across the centre of the plate. Each well was then imaged using phase contrast microscopy (x40 magnification). Cells that had been incubated with Mitomycin C previously, remained in this media during scratch closure. Pilot studies indicated that after 16 hours the wound had closed substantially, but not completely, so this duration was used in all presented data. At this stage, each well was imaged again and the plate was discarded. Images were analysed using Image J software and the percentage of wound area reduction calculated (See Figure 4-122 for representative images at 0 and 16 hours).

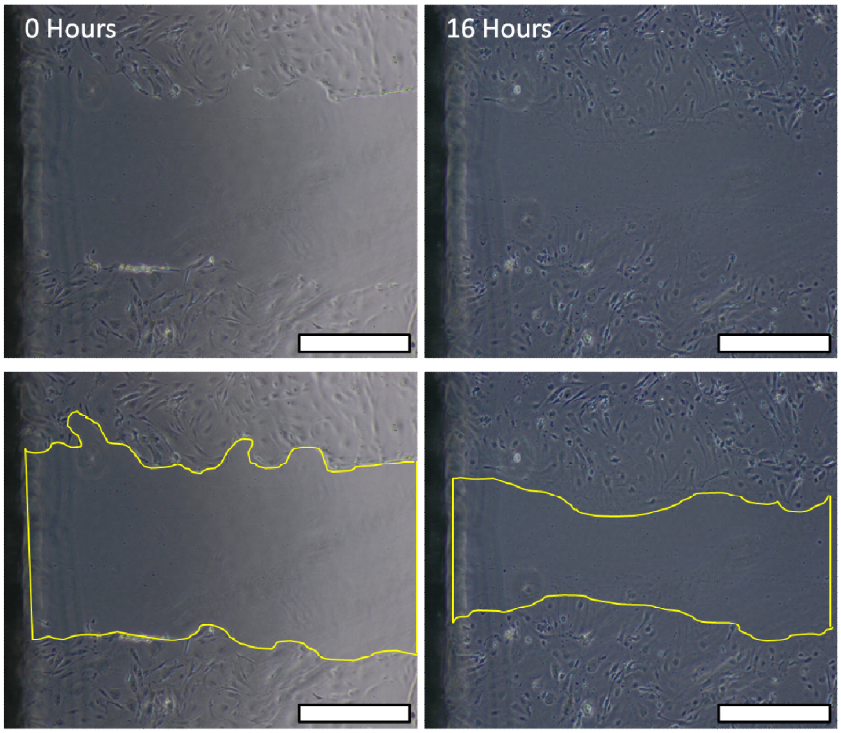

Figure 4-12. Representative image of scratch wound assay at 0 hours (left pictures) and 16 hours (right pictures). Scale bar denotes 500µm. X40

magnification. Bottom panels demonstrate the quantification of wound area at 0 and 16 hours.

4.10.5 Proliferation assay

Assessment of the proliferative capacity of murine PECs was performed via a commercially available kit. The Click-iT® EdU cell proliferation assay (Life technologies) utilises EdU (5-ethynyl-2´-deoxyuridine), a modified nucleoside which is incorporated into the DNA of actively dividing cells. This EdU is then detected with Click-iT™ EdU Alexa Fluor® azide reagent, and imaging identifies proliferating cells. Reagents required for the assay were prepared as per the manufacturer's instructions. The detailed method is as follows. PECs were plated onto a twenty-four well micro clear imaging plate and allowed to grow until approximately 75% confluent. Half of the media was then replaced with media containing EdU at a concentration of 20µM to achieve a final concentration of 10µM. The cells were then incubated for two hours in standard conditions (37ºC, 5%CO^2) before proceeding to fixation and staining. The media/EdU solution was removed from the cells and 1ml of 4% Formaldehyde was added to each well and left at room temperature for 15 minutes. The fixative was removed and the cells were washed twice with 1ml of a 3% BSA/PBS mixture. Cells were then permeabilised for 20 minutes at room temperature with 1ml of 0.5% Triton X-100 in PBS in each well. The permeabilisation buffer was removed and each well was washed twice with 3% BSA/PBS. A reaction cocktail was prepared as per manufacturer's instructions and 0.5ml of this Click-iT® reaction cocktail was added to each well. The plate was left in the dark for 30 minutes at room temperature. All

wells were then washed with 1ml of 3% BSA/PBS before proceeding with nuclear counterstaining. Nuclear staining was undertaken with a Hoechst stain provided with the kit (30 minutes in the dark at room temperature with a 5µg/ml Hoechst in PBS solution). The cells were then washed again with 3% BSA/PBS mixture and stored in the dark at 4ºC until ready to image. Imaging was performed using laser scanning confocal microscopy. Total cell number was identified via the nuclear staining; proliferating cells were expressed as the percentage of total in each field of view (See Figure 4-133 for a representative image).

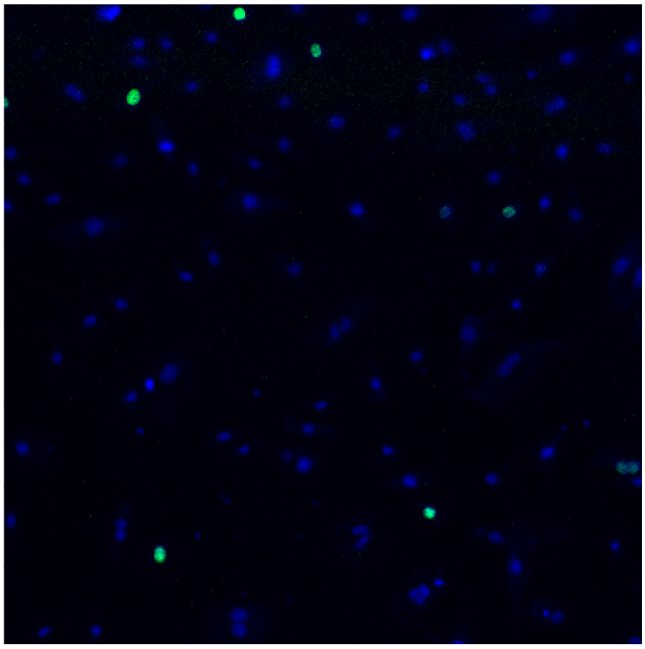

Figure 4-13. Representative confocal microscopy image of PECs following EdU proliferation assay. Nuclei are stained with Hoechst stain and appear blue. Proliferating cells have incorporated the EdU and therefore appear green.

4.10.6 Apoptosis assay

Apoptosis was inferred in PECs from IRKO and WT mice using Western blotting. A kit containing antibodies against total Caspase 3 and cleaved Caspase 3, along with positive and negative controls were used. Caspases are central regulators of apoptosis; once activated, initiation Caspases cleave and activate effector Caspases, one of which is Caspase 3, and in turn these proteins cleave other targeted cellular proteins, initiating apoptosis [233]. Hence, higher levels of cleaved Caspase 3 suggest a higher degree of apoptosis. Western blotting was carried out as described in section 4.6.

4.11 PEC signalling studies

PECs were isolated as has been described and were plated into gelatin coated 6 well plates at passage 2. Cells were serum straved to render them quiescent by incubating them in MV2 only + 1% FCS for 4 hours. Cells were then stimulated with VEGF (50ng/ml) and lysed at 0, 10, 15 and 30 minutes. Western blotting of lysates was performed as has been described. Proteins that were quantified included: enos, phospho enos S1177, Akt, phospho Akt S473, ERK1/2, phospho ERK1/2, and beta actin was measured to use as the house keeping protein by which protein levels were normalised.

4.12 Statistics

All data are displayed as mean ± SEM; $p<0.05$ is denoted by *. Comparative analysis between groups was performed using the unpaired student t test.

Statistical significance was taken as $P<0.05$. Statistical analysis was performed using Microsoft Excel 2007 software.

Chapter 5. Results: Part 1

Experiments were carried out to evaluate the ability of LEPCs from WE and SA donors to augment vascular regeneration. A group of 12 WE and 12 SA men were recruited to provide blood samples to allow the derivation of LEPCs. As highlighted in Table 4, these volunteers were well matched for all classical CVD risk factors. However, the SA group had a significantly higher fasting plasma insulin level and relative insulin resistance, as demonstrated by a higher HOMA-IR score.

Table 4: Demographic and cardiovascular risk profile (work carried out in conjunction with Dr Richard Cubbon and Dr Vivek Baliga)

	South Asian	**White European**	**p value**
Age (years)	30.9 (1.2)	29.7 (1.1)	NS
Body Mass Index (Kg/m2)	24.1 (0.7)	22.4 (0.5)	NS
Waist-Hip ratio	0.85 (0.01)	0.84 (0.01)	NS
Systolic Blood Pressure (mmHg)	116 (2.3)	116 (2.5)	NS
Diastolic Blood Pressure (mmHg)	70 (1.6)	69 (1.6)	NS
Total Cholesterol (mmol/L)	4.6 (0.1)	4.7 (0.1)	NS
Triglycerides (mmol/L)	1.1 (0.1)	1.2 (0.1)	NS
Glucose (mmol/L)	4.8 (0.1)	4.6 (0.1)	NS
Insulin (mu/L)	5.6 (0.8)	2.9 (0.3)	0.009
HOMA-IR	1.2 (0.2)	0.6 (0.1)	0.008

5.1 LEPC transfusion studies

Previous work in our lab has demonstrated that SA LEPCs have an impaired capacity to augment endothelial regeneration after vascular injury. This was associated with impaired indices of *in vitro* function also; SA LEPCs formed fewer vascular networks on Matrigel and displayed impaired migration towards VEGF and IGF-1 [156]. In an attempt to build on these data, we first assessed the capacity of SA LEPCs to augment vascular regeneration in comparison to LEPCs from WE donors. We began with an *in vivo* model, hindlimb ischaemia, and the results are presented below. As discussed in the introduction to this thesis, the impaired function, previously observed in our lab, was associated with reduced abundance of Akt and eNOS protein [156]. Restoration of Akt activity in SA LEPCs rescued their endothelial regenerative capacity [156], and this finding forms the basis for many of our experiments, the results of which, are presented in this chapter.

5.1.1 Hindlimb ischaemia laser Doppler

Transfusion studies were carried out following the induction of hind limb ischaemia in mice as described in section 4.2.1. WE LEPCs significantly enhanced recovery of limb perfusion (WE 66% [3] vs. SA 49% [2] vs. vehicle 50% [2] Ischaemic/Non-ischaemic Flux ratio, p=0.002 for WE vs. SA). The SA LEPCs, however, did not enhance reperfusion, at day 14 post operation, above the level observed with vehicle control (basal medium): Figure 5-1. Representative laser Doppler imaging is presented in Figure 5-2. Hindlimb ischaemia surgery was performed by Dr N Yuldasheva.

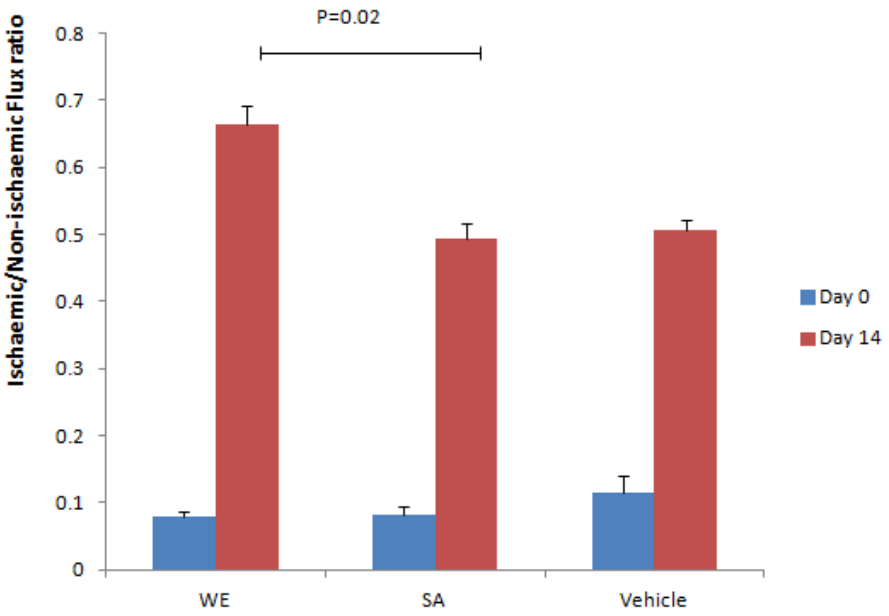

Figure 5-1. Limb blood flow, whilst comparable post-operatively, is greater on day 14 in mice receiving WE LEPC than those receiving vehicle or SA LEPC (n=5 per group)

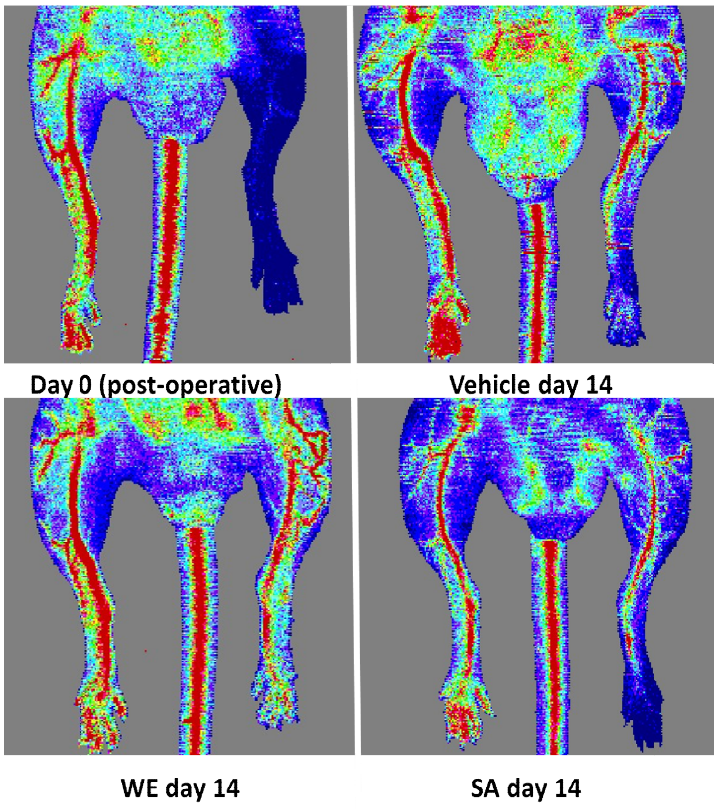

Figure 5-2. Representative laser Doppler imaging 14 days following hindlimb ischaemia induction and transfusion of vehicle control, WE LEPC and SA

LEPC. Red areas represent areas with increased blood flow; blue represents areas with lesser blood flow.

5.1.2 Hindlimb ischaemia histological analysis

Histological analysis was performed on muscle blocks taken at the end of the experiment as described in section 4.2.4. Engraftment of LEPCs was assessed by confocal microscopic imaging of muscle sections. The findings of reduced Doppler measures of tissue perfusion were supported by this histological data, and the number of engrafted fluorescently tracked LEPC, co-expressing CD-31, was significantly higher in mice that had received WE LEPCs (WE 80.9 [16.9] vs. SA 44.6 [5.2] LEPCs per mm^2, p=0.048, n=5). See Figure 5-3. Histological preparation was performed with Dr N Yuldasheva. Confocal microscopy was performed with Dr R Cubbon.

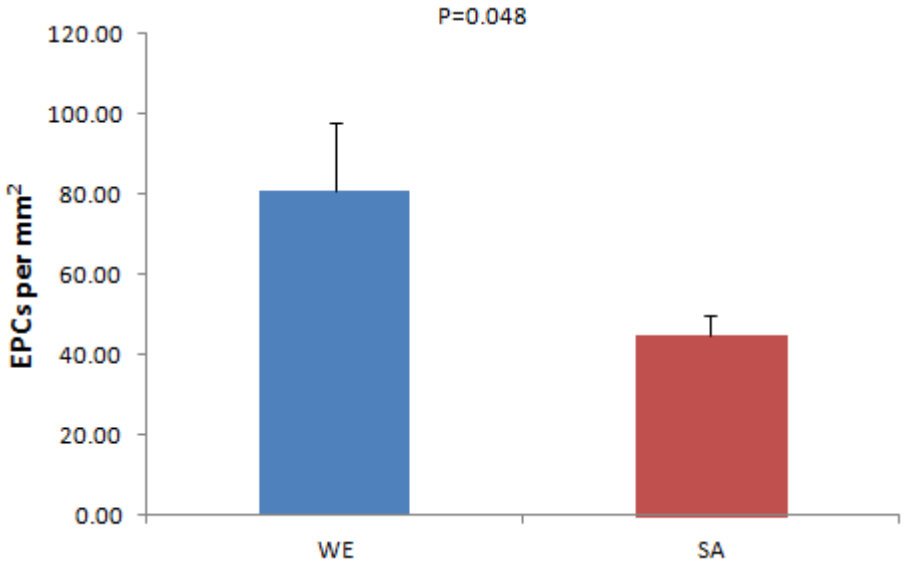

Figure 5-3. LEPC engraftment following hindlimb ischaemia. LEPCs were more abundant in the muscle of mice receiving WE LEPC.

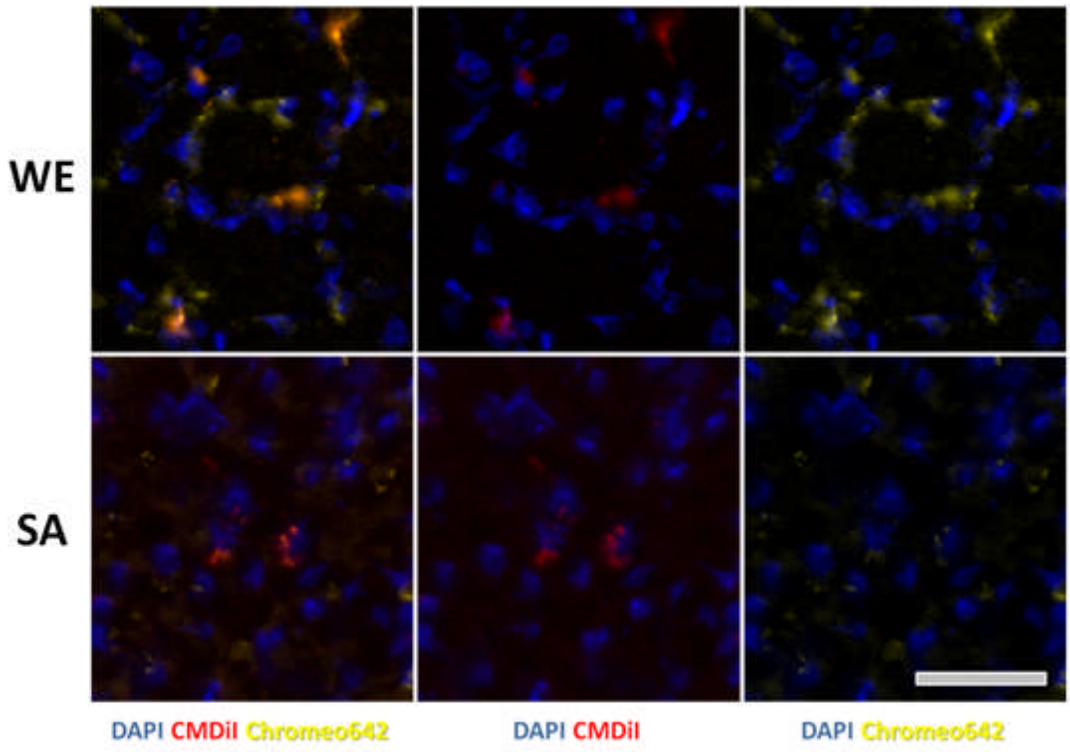

Figure 5-4. Representative confocal microscopy images of ischaemic gastrocnemius muscle. Increased engraftment of LEPC (red – CMDil cell tracker) from WE donors is evident. These cells also co-express CD31 (yellow – Chromeo642). Scale bar denotes 50µm.

5.1.3 Lentiviral transduction of South Asian LEPC

Previous findings from our lab of reduced protein levels of Akt and eNOS in SA LEPC led to the targeting of Akt as a means of restoring reparative capacity. The rationale for this was both the upstream location of Akt to eNOS, and published literature identifying these molecules as key angiogenic mediators [82, 161, 234]. We chose to augment SA LEPC Akt activity using a lentiviral vector as described in section 4.1.3. Viral titres were chosen to increase SA LEPC phospho-Akt S473 content by a factor of three, aiming to achieve a level comparable to that seen in the WE LEPCs.

E17KAkt expressing SA LEPC exhibited increased phospho-Akt S473 content and Akt activity as measured using a cell free GSK phosphorylation assay; Akt activity in EGFP expressing and native SA LEPC was comparable. E17KAkt expressing SA LEPC also demonstrated increased total and phospho-eNOS S1177. See Figure 5-5. Handling of virus and transduction of LEPCs was performed by Dr S Stephen and Dr H Viswambharan.

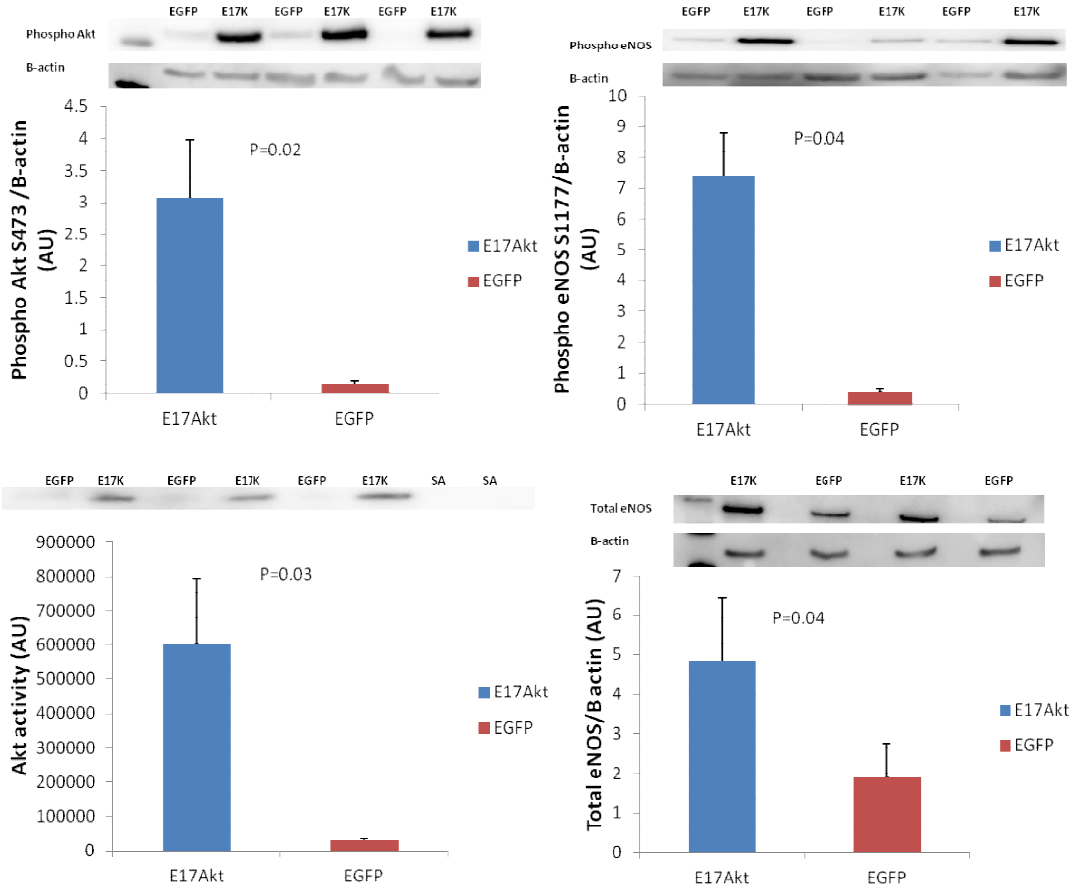

Figure 5-5. : E17KAkt expressing SA LEPC had higher levels of pAkt (S473), eNOS, peNOS (S1177) and Akt activity than EGFP expressing LEPC.

5.1.4 Hindlimb ischaemia following transfusion of E17KAkt expressing LEPC: laser Doppler

Hindlimb ischaemia experiments were repeated with SA LEPC expressing either E17KAkt or EGFP. Augmenting Akt activity restored the reperfusion promoting capacity of SA LEPCs to an extent comparable to that seen in WE LEPCs. EGFP expression had no effect on limb perfusion recovery as there was no difference seen between the EGFP expressing cells and vehicle control (basal medium) (E17KAkt 63 [1.2] vs. EGFP 48 [3.3] Ischaemic/Non-ischaemic Flux ratio, p=0.02, n=5). See Figure 5-7. See Figure 5-6 for representative laser Doppler images. Hindlimb ischaemia surgery was performed by Dr N Yuldasheva.

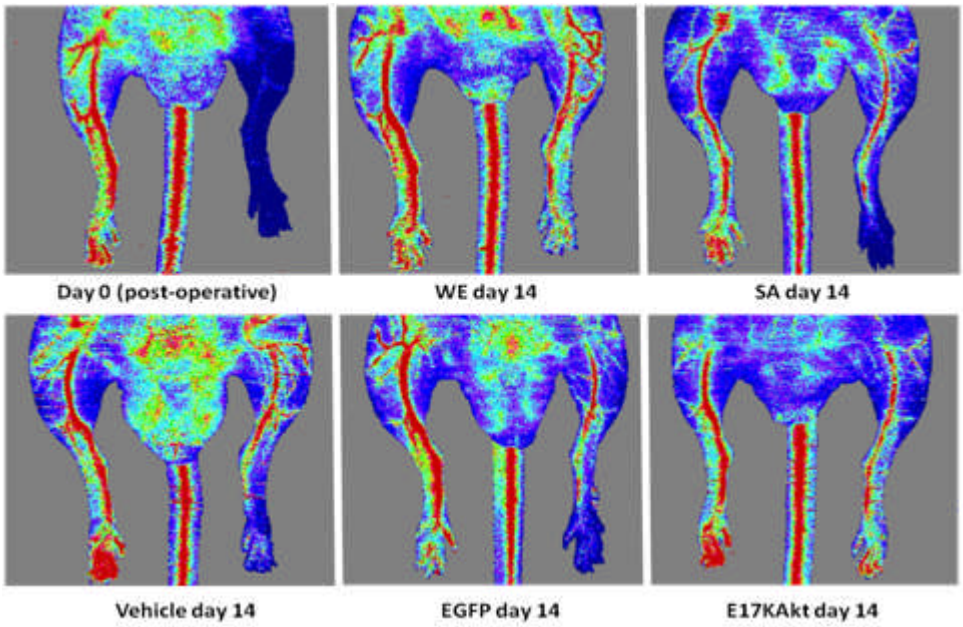

Figure 5-6: Representative laser Doppler images taken 14 days post hind-limb ischaemia induction and transfusion of WE LEPC, SA LEPC, E17KAkt-expressing SA LEPC or EGFP-expressing SA LEPC. It is evident that mice

receiving E17KAkt transduced SA LEPC had limb reperfusion comparable to the level seen in mice receiving WE LEPCs.

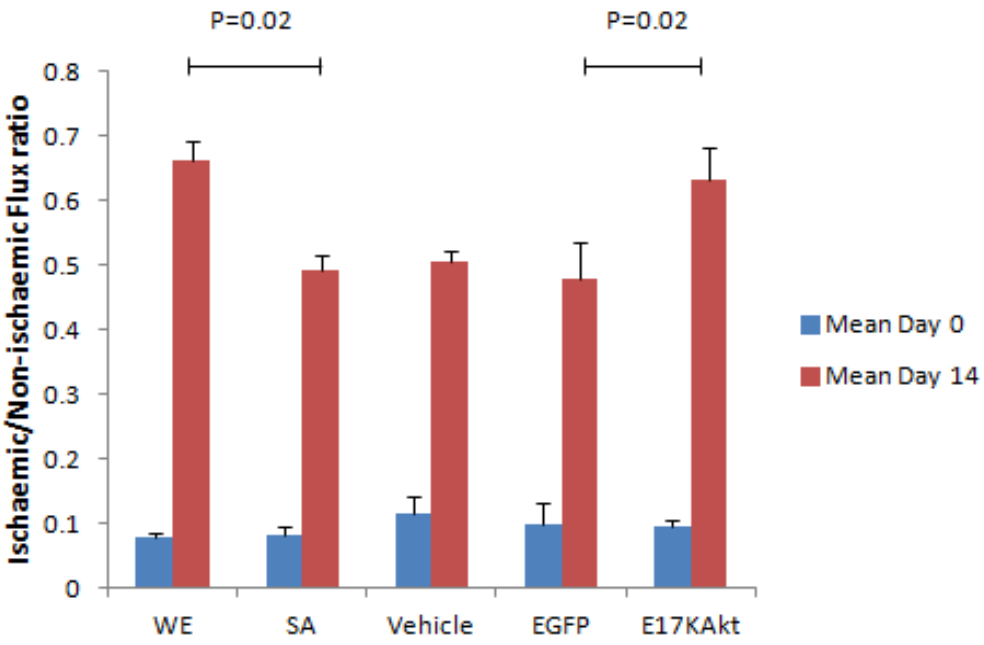

Figure 5-7: Mice receiving E17KAkt expressing LEPCs displayed improved limb perfusion recovery at day 14 in comparison to those receiving EGFP expressing LEPCs.

5.1.5 Hindlimb ischaemia following transfusion of E17KAkt expressing LEPC: histological analysis

The findings of increased Doppler measures of tissue perfusion with the transfusion of E17KAkt expressing SA LEPCs were supported by histological data. The number of engrafted fluorescently tracked LEPC, co-expressing CD31, was significantly higher in mice that had received E17KAkt expressing SA LEPCs, compared with EGFP expressing SA LEPCs (E17KAkt 111.4

[15.3] vs. EGFP 36.7 [4.1] EPCs per mm^2, p=0.001, n=5). See Figure 5-8. Histological preparation with performed by Dr N Yuldasheva. Confocal microscopy was performed with Dr R Cubbon.

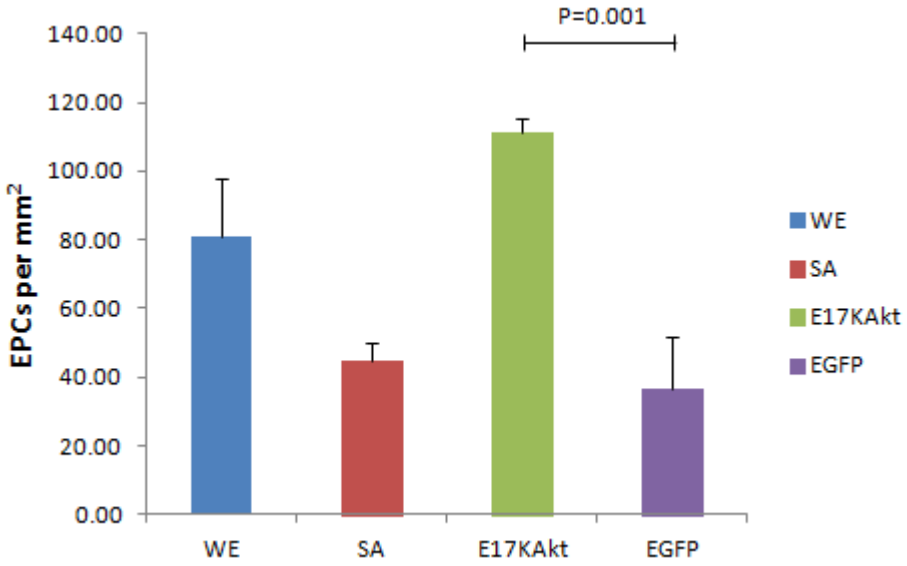

Figure 5-8: Engraftment of LEPC into gastrocnemius muscle following hindlimb ischaemia is significantly increased in SA LEPC expressing E17KAkt, versus EGFP.

5.2 *E17KAkt expressing LEPC: in vitro* studies

In order to gain further insight into the impact of modulating Akt activity in SA LEPCs on vascular reparative capacity, *in vitro* studies were performed. Previous work from our lab, as outlined, had identified deficits in the *in vitro* function of SA LEPCs in comparison to WE LEPCs. Specifically, defects in growth factor directed migration, angiogenic tubule formation on Matrigel, and premature senescence, were noted.

5.2.1 Tubule formation

SA LEPCs were transduced to express either E17KAkt or EGFP. Constitutively activating Akt in SA LEPCs, resulted in increased vascular tubule formation on Matrigel (E17KAkt 9.6 [1.4] vs. EGFP 5.8 [0.7] Tubules per field (x40), p=0.03, n=4). See Figure 5-9. The formation of tubules was abrogated by the addition of L-NMMA, a nitric oxide synthase (NOS) inhibitor, thereby causally implicating Akt/eNOS signalling, and increased NO production, in the improved angiogenic function seen with E17KAkt expression.

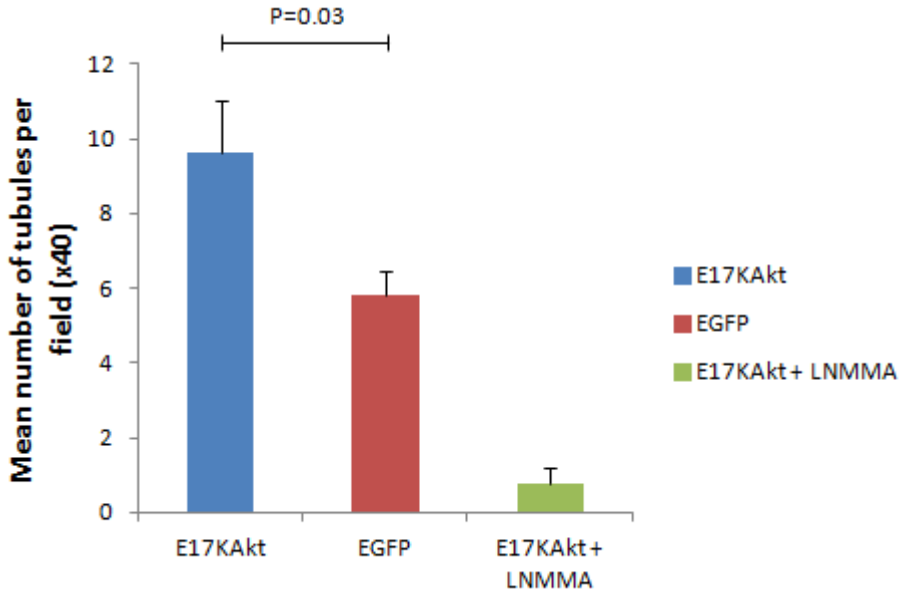

Figure 5-9. Mean number of vascular tubules formed by E17KAkt and EGFP expressing SA LEPCs.

5.2.2 Migration

Migration towards VEGF was assessed as described in section 1034.3.2 using SA LEPCs transduced with E17KAkt or EGFP. Increasing Akt activity

did not alter the chemotactic response to VEGF since the number of cells that migrated to the lower aspect of the Boyden chamber was similar when using both E17KAkt and EGFP expressing SA LEPCs (E17KAkt 25.3 [6.2] vs. EGFP 34.8 [3.5], p=0.2, n=4). See Figure 5-10.

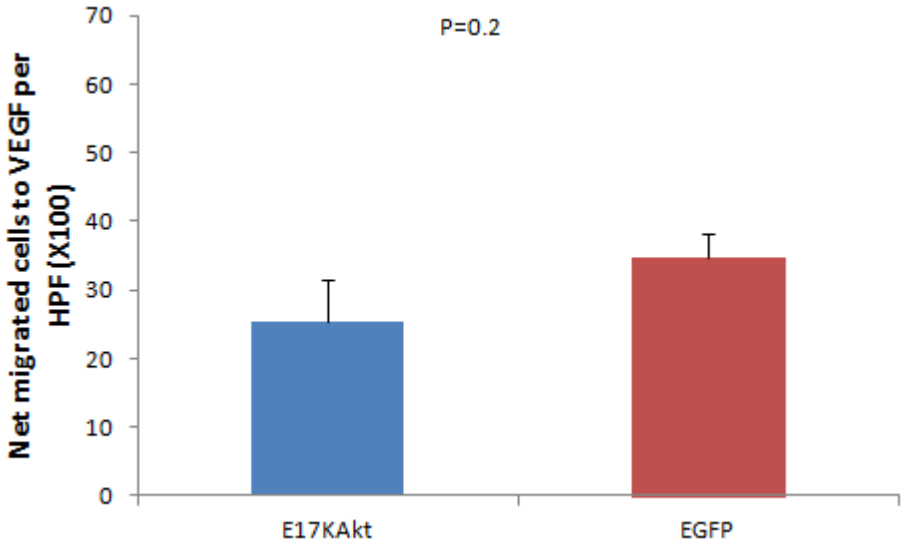

Figure 5-10. Mean number of migrated LEPCs per high powered field (x100).

5.2.3 Senescence

Previous work has highlighted increased levels of senescence in SA LEPCs in comparison to those derived from WE subjects, and therefore assessment of levels of senescence was undertaken in SA LEPCs expressing both E17KAkt and GFP. There was no demonstrable difference in senescence between E17KAkt and EGFP expressing cells at passage number 3 (E17KAkt 73.2% [5.8] vs EGFP 73.8% [3.7] % senescent cells, p=0.95, n=4). See Figure 5-11.

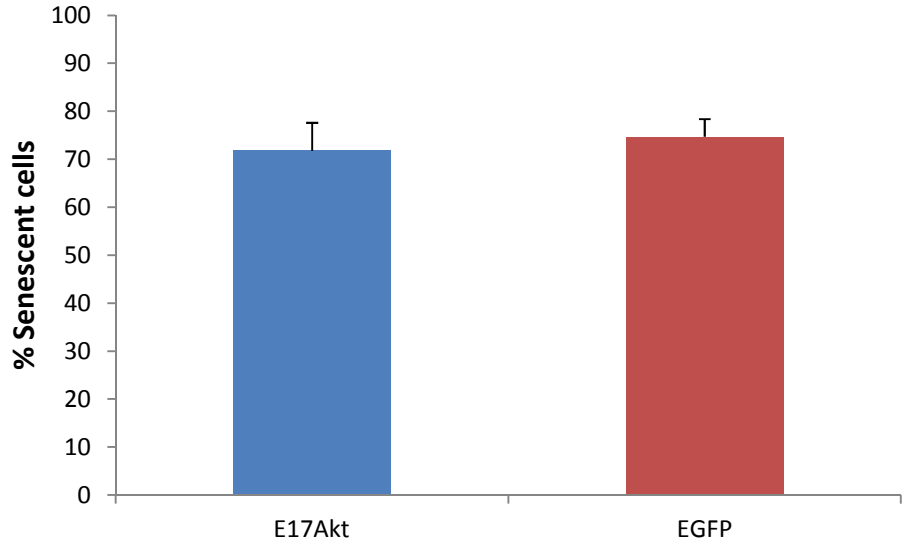

Figure 5-11. Percentage of senescent LEPCs at passage 5.

5.2.4 Survival studies

The effect of increased Akt activity on cell survival was assessed by simulating an 'infarct environment'. Lysates from E17KAkt expressing SA LEPCs had higher levels of total LDH (E17KAkt 1.51 [0.21] vs EGFP 0.78 [0.11] Total cell LDH (AU), p=0.045, n=4), a marker of cell survival, where as analysis of conditioned media demonstrated increased levels of released LDH (E17KAkt 0.13 [0.02] vs EGFP 0.35 [0.04] LDH release (AU), p=0.003, n=4), a marker of cytotoxicity, in the EGFP expressing SA LEPCs. See Figure 5-12 and Figure 5-13.

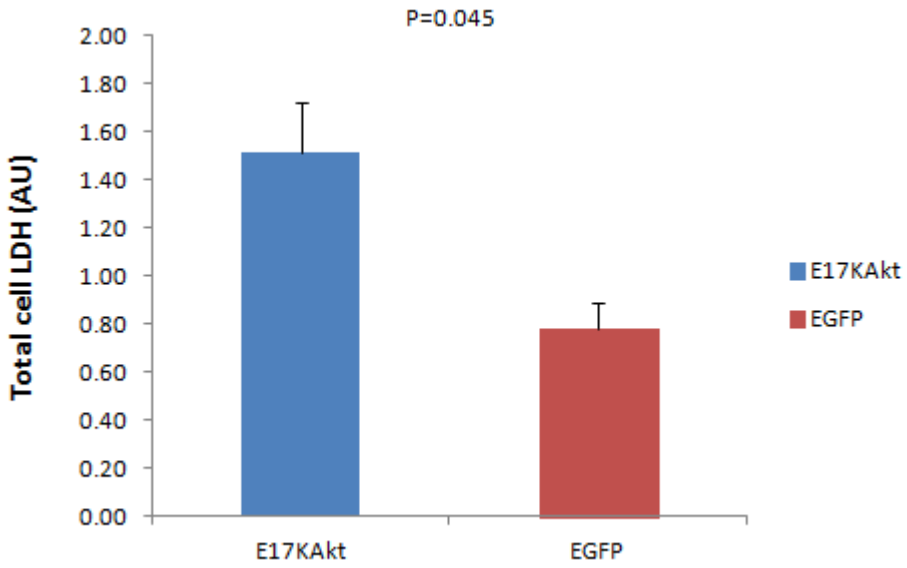

Figure 5-12. Levels of total LDH were elevated in lysates made from E17KAkt expressing cells compared with the EGFP expressing cells.

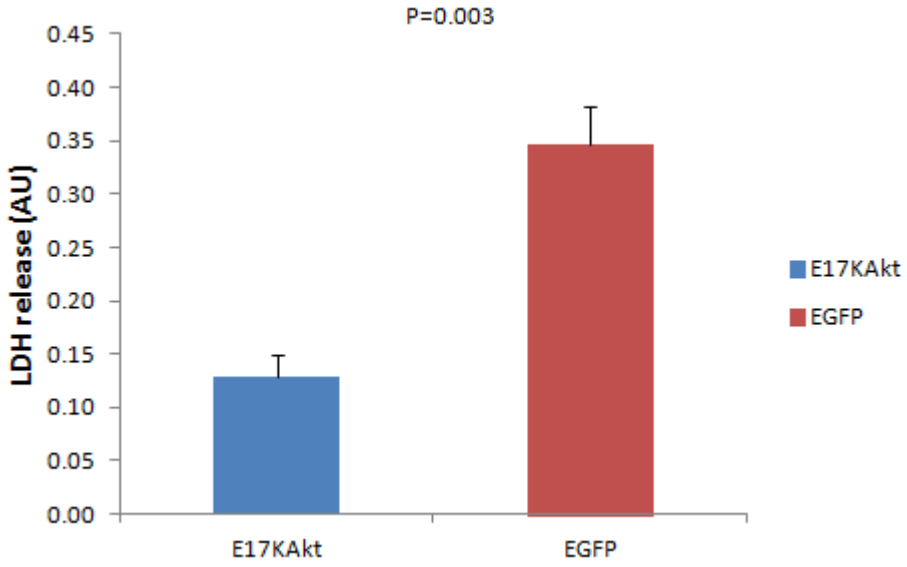

Figure 5-13. LDH release was higher from the EGFP expressing SA LEPCs than from the E17KAkt expressing SA LEPCs.

5.2.5 Invasion studies

Assessment of invasion was performed on LEPCs from SA and WE donors. There was no difference observed between the groups (SA 25.2 [4.9] vs WE 22.7 [2.8] Invaded cells, p=0.67, n=5). See Figure 5-14.

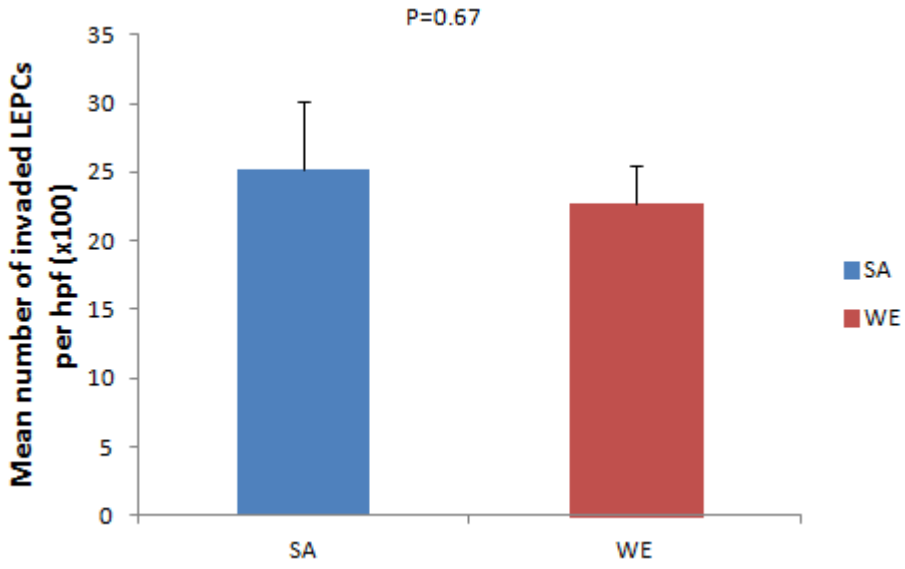

Figure 5-14. WE and SA LEPCs invade through a layer of Matrigel similarly.

5.3 LEPC Akt knockdown studies

5.3.1 Akt knockdown

In order to strengthen our proposed causal role for reduced Akt1 expression in SA LEPC dysfunction, we went on to silence Akt1 in WE LEPCs. WE LEPCs were transfected with scrambled siRNA as a control, or Akt1 siRNA to decrease Akt expression. Akt knockdown was confirmed using western blotting, with approximately 50% Akt knockdown being achieved (Scrambled 0.93 [0.12] vs. Akt siRNA 0.48 [0.08] Total Akt (AU), p=0.002, n=4). See Figure 5-15. Transfection of LEPCs was performed with Dr A Bruns.

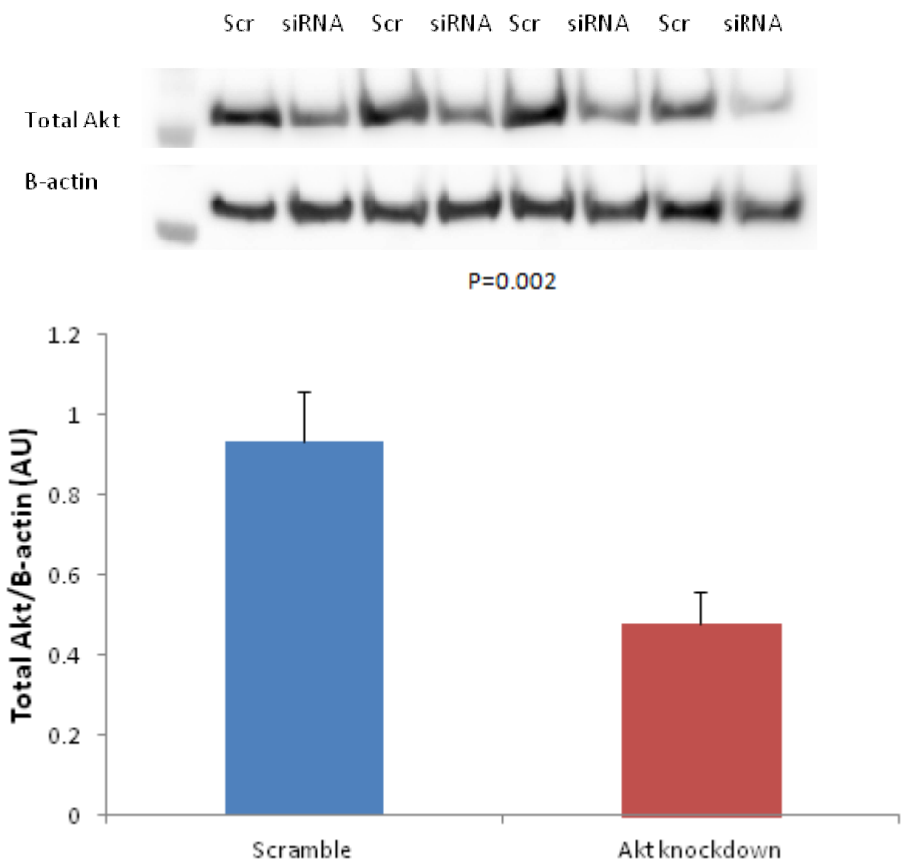

Figure 5-15. Demonstration of effective Akt knockdown in WE LEPCs using western blotting (including representative blots).

5.3.2 Femoral injury studies

WE LEPCs transfected with scrambled or Akt1 siRNA were transfused into mice following femoral arterial injury to assess endothelial regenerative capacity. WE LEPCs transfected with Akt1 siRNA augmented endothelial regeneration to a lesser degree than those transfected with scrambled siRNA (Scrambled siRNA 40.2% [2.0] vs. Akt1 siRNA 24.0% [1.6] endothelial regeneration, p=0.002, n=6). See Figure 5-16. These findings are in keeping with previous work from our lab demonstrating decreased augmentation of re-endothelialisation with SA LEPC compared with WE LEPCs, and also the

augmented re-endothelialisation seen with SA LEPC when expressing E17KAkt. Moreover, these findings further strengthen the causal role of reduced Akt1 activity in SA LEPC dysfunction. Femoral injury surgery was performed by N Yuldasheva.

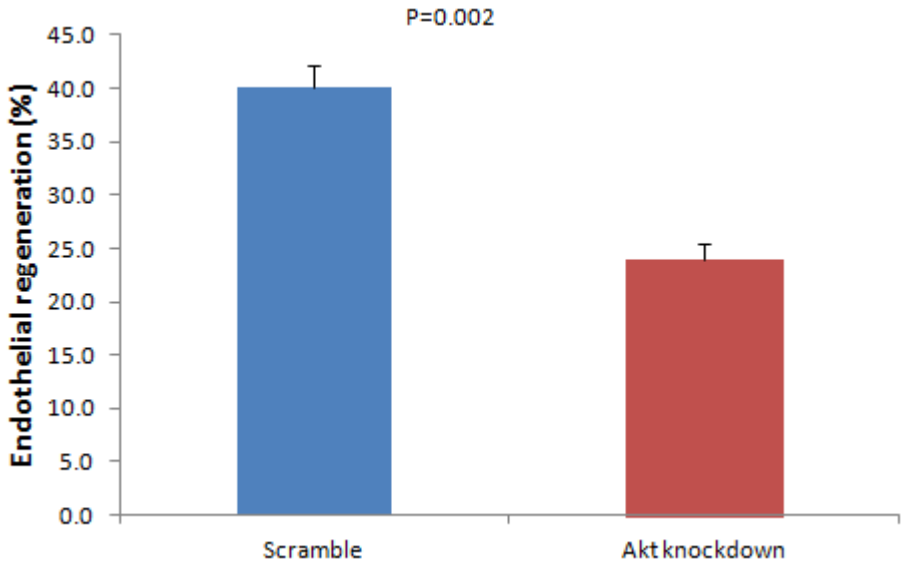

Figure 5-16. Augmentation of endothelial regeneration was reduced in WE LEPCs transfected with Akt1 siRNA compared with those transfected with scrambled siRNA.

This difference in endothelial regeneration was recapitulated when examining the incorporation of LEPCs into the intima of explanted femoral artery samples. This was done using confocal microscopy, and demonstrated a reduction in the number of engrafted cells in mice that had received Akt1 silenced WE LEPCs, compared with WE LEPCs transfected with scrambled siRNA (Scrambled 2214.1 [168.3] vs Akt knockdown 924.8

[122.0] engrafted LEPCs per mm^2, p=0.002, n=6). See Figure 5-17. Confocal microscopy was performed with Dr R Cubbon.

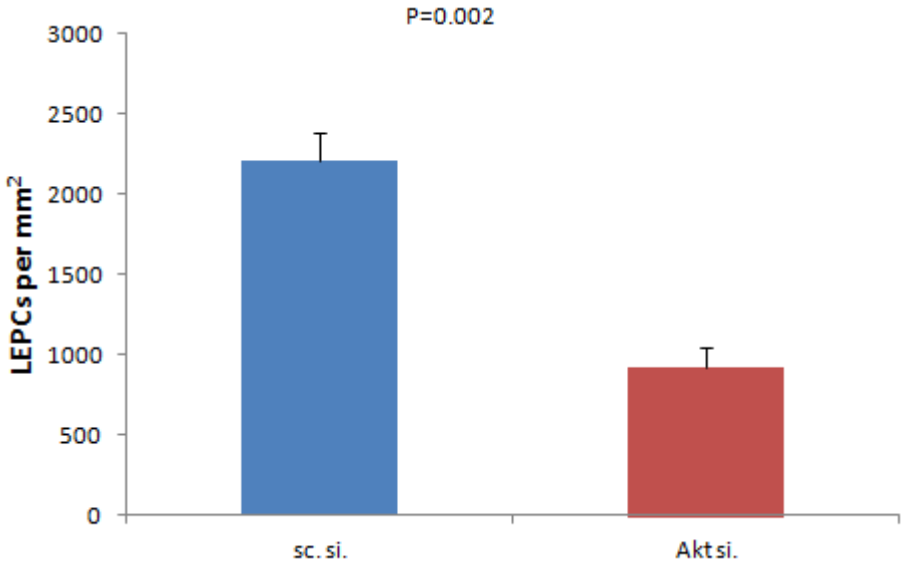

Figure 5-17. Number of LEPCs engrafting in murine femoral artery intima following femoral arterial denudation and LEPC transfusion. There were fewer engrafted WE LEPCs in mice that had received Akt silenced cells, compared with cells transfected with scrambled siRNA.

5.3.3 Migration

WE LEPCs transfected with scrambled or Akt1 siRNA migrated to the lower aspect of a Boyden chamber membrane when exposed to a VEGF concentration gradient. Akt silencing reduced the number of migrating cells (Scrambled siRNA 28.4 [5.8] vs. Akt siRNA 10.8 [3.3] migrated cells per high powered field (x100), p= 0.01, n=4). See Figure 5-18. These findings are in keeping with previous work from our lab demonstrating an impaired chemotactic response to VEGF in SA LEPCs compared with WE LEPCs.

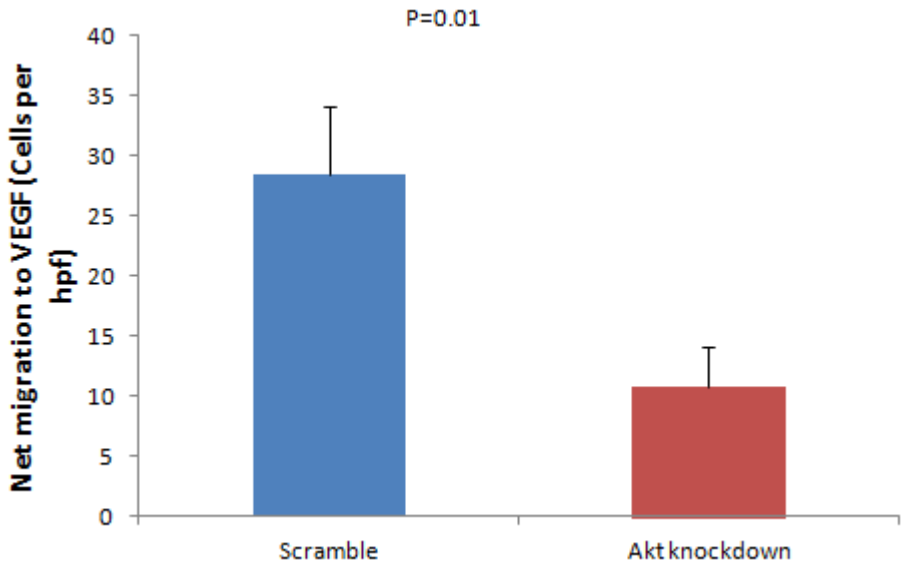

Figure 5-18. Migration of cells to VEGF in WE LEPCs transfected with scrambled siRNA or Akt1 siRNA.

Chapter 6. Results Part 2

6.1 Vascular regeneration is impaired in the IRKO mouse: In vivo data

6.1.1 Hindlimb ischaemia; laser Doppler

To assess vascular regeneration in response to ischaemia, a model of critical limb ischaemia was used. Hindlimb ischaemia was induced in both WT and IRKO mice as described in section 4.2.3. Laser Doppler analysis was carried out on day 0, day 7, day 14 and day 21. Representative laser Doppler images are presented below. Red areas indicate higher flux and represents increased blood flow, whereas blue indicates the opposite. Hindlimb ischaemia surgery was performed by Dr N Yuldasheva.

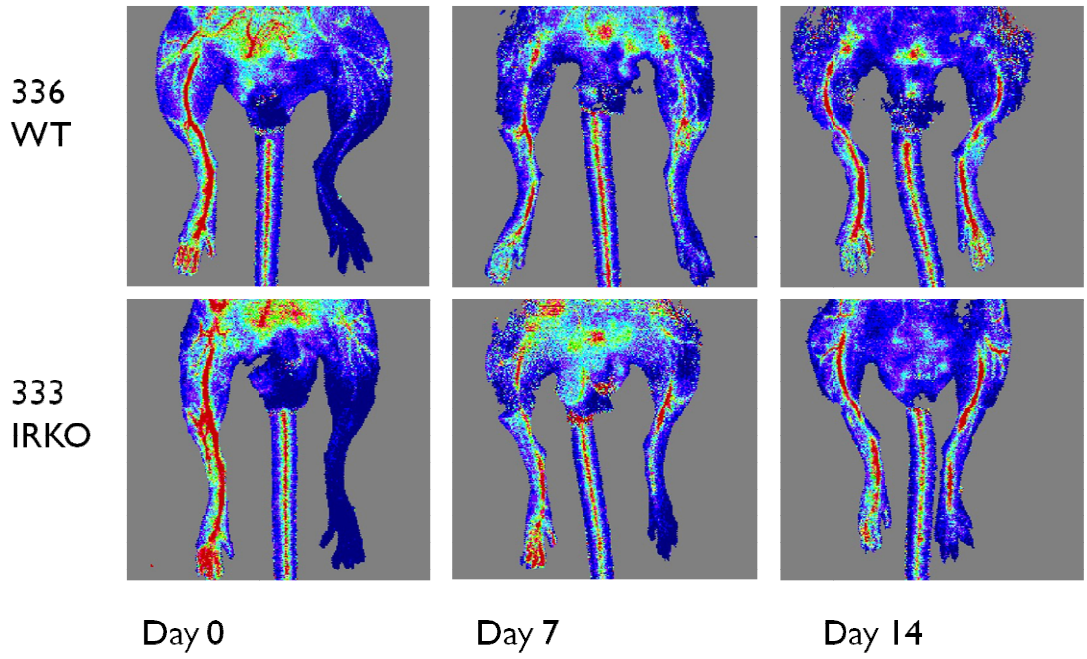

Figure 6-1. Representative laser Doppler images taken from WT and IRKO mice in a supine position immediately post operatively, 7 days post operatively and 14 days post operatively. The IRKO mouse has delayed recovery of blood flow in the ischaemic (mouse's left) limb.

Experiments were carried out in 14 IRKO mice and 13 WT. Laser Doppler analyses confirm a delay in the return of limb perfusion in the IRKO mice in response to critical limb ischaemia in comparison to WT mice. Ischaemia was confirmed post operatively in both genotypes with a similar ischaemic: non-ischaemic ratio being observed (WT 9.8%[1.3] vs. IRKO 7.7%[1.2], p=0.25). At days 7 (WT 47%[3.5] vs IRKO 36.4%[4.7}, p=0.04), 14 (WT 76% [5.4] vs. IRKO 57.4% [5.1], p=0.02) and 21(WT 82.2% [5.6] vs. IRKO 62.2% [7.1], p=0.04) there is increased ischaemic: non-ischaemic limb flux ratio in the WT mice in comparison to the IRKO mice. See Figure 6-2.

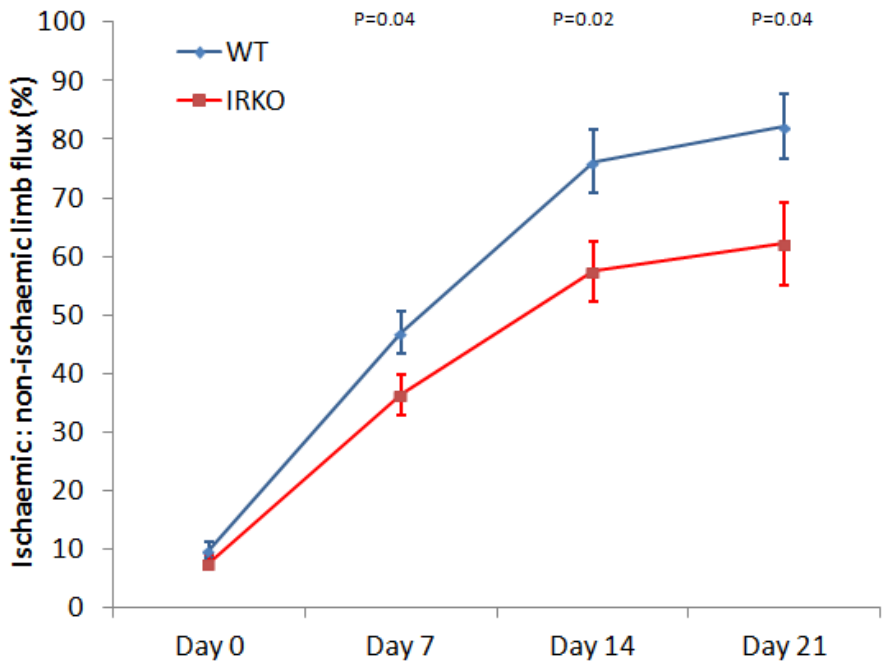

Figure 6-2. Limb perfusion recovery is blunted in the IRKO mouse following hindlimb ischaemia induction with a significant difference evident at day 7, day 14, and day 21.

6.1.2 Hindlimb ischaemia histological analysis

Muscles were analysed 21 days following hindlimb ischaemia induction. This demonstrates a strong trend towards a reduction in capillary density in the ischaemic gastrocnemius muscle from IRKO mice (WT 57.9 [3.3] vs IRKO 46.7 [6.3] Capillaries per HPF, n=9). This finding correlates with the perfusion defect seen at day 21 in the IRKO mice (r^2 0.254, p=0.037, see Figure 6-3 for scatter plot) and suggests that this is, at least partly, due to a defect in capillary formation and therefore angiogenesis.

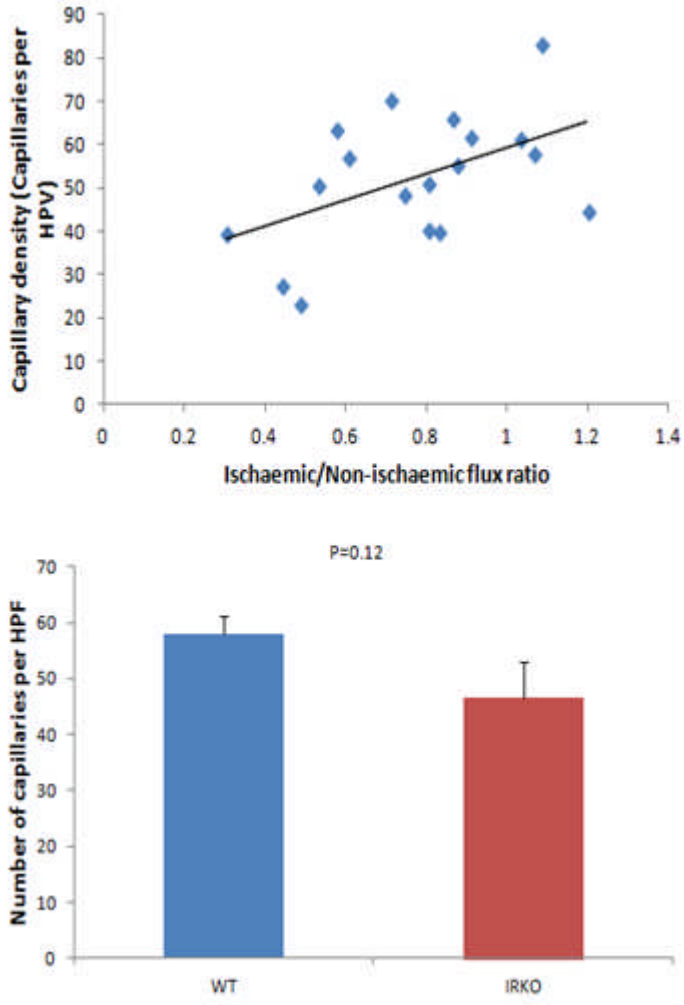

Figure 6-3. There was a trend towards a reduction in capillary density within the ischaemic gastrocnemius of IRKO mice.

6.1.3 Hindlimb ischaemia quantitative PCR data

Muscles from both the ischaemic and non-ischaemic legs were explanted from mice 3 days post hindlimb ischaemia induction. These tissue specimens were used for quantitative PCR analysis, which was conducted by Mrs Stacey Galloway. Levels of VEGF mRNA in non-ischaemic adductor muscle were similar in IRKO and WT mice (WT 1.67% [0.34] vs. IRKO 1.35% [0.26] of18S expression, p=0.47, n=4). See Figure 6-4. Levels of VEGF mRNA were significantly higher in the ischaemic adductor muscles of IRKO mice compared with WT mice however (WT 9.8% [2.5] vs. IRKO 31.4% [6.7] of 18S expression, p=0.04, n=4). See Figure 6-5. This therefore indicates that there is higher expression of VEGF in response to ischaemia in the musculature of IRKO mice despite perfusion recovery being delayed in this group when compared with WT littermates.

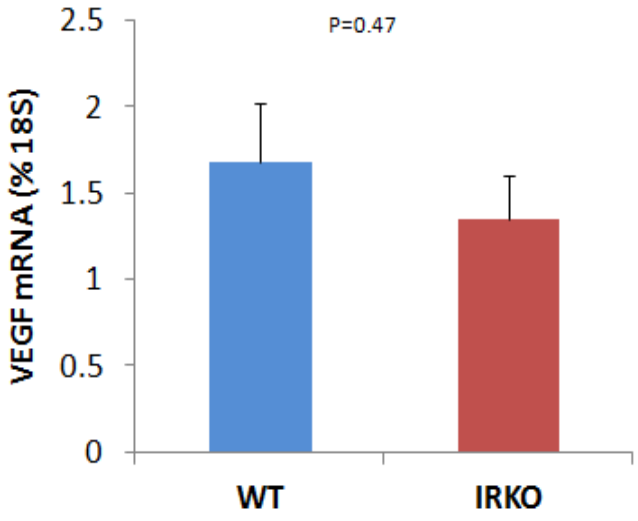

Figure 6-4. Basal levels of VEGF expression in adductor muscle is similar in IRKO and WT mice.

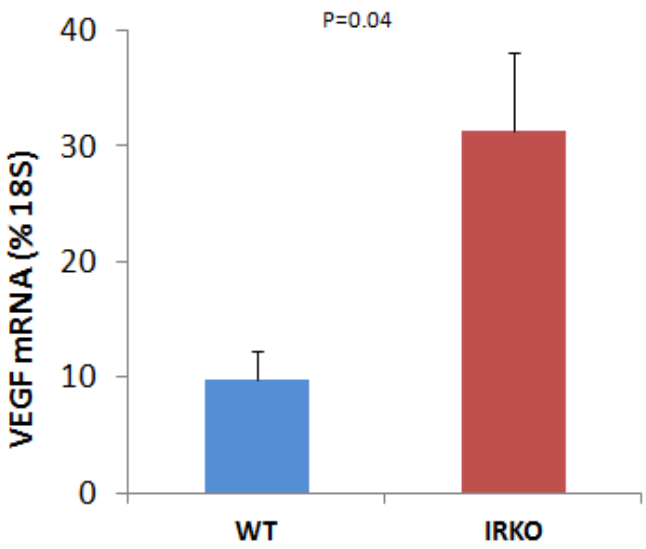

Figure 6-5. VEGF expression in ischaemic adductor muscle is increased in the IRKO mouse.

6.2 Angiogenesis is impaired in the IRKO mouse: In vitro data

6.2.1 Aortic ring sprouting angiogenesis

As described in section 4.10.1, this assay looks at *ex vivo* endothelial sprouting from segments of aorta, in response to VEGF. Aortic rings explanted from WT and IRKO mice formed vascular endothelial sprouts, identified by BS-1 lectin staining. Representative images obtained from confocal microscopy are presented below.

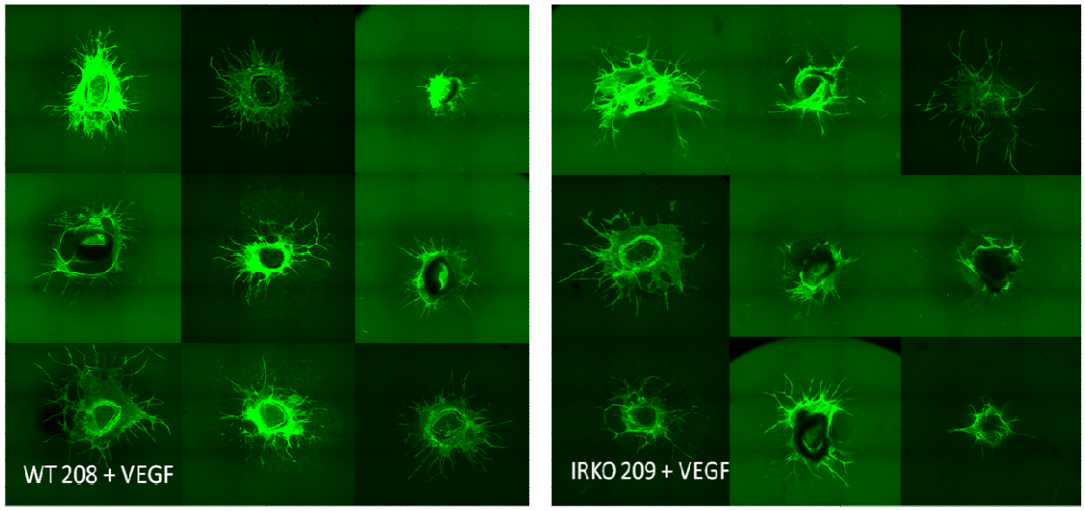

Figure 6-6: Representative confocal microscopy images of WT and IRKO aortic rings following immunostaining. Endothelial sprouts from the aortae of IRKO mice are smaller and less abundant.

Experiments were conducted using aortae form 8 WT mice and 8 IRKO mice. Mean sprout length in the rings cultured with VEGF from WT mice were significantly longer than those from IRKO mice (WT 1037.46µm [38.58] vs. IRKO 846.51µm [37.98], p=0.003; n = 8 IRKO, 8WT). See Figure 6-7. Sprout length in rings from WT and IRKO mice not cultured with VEGF was not significantly different (WT 553.8µm [62.13] vs. IRKO 419.63µm [30.23], p=0.10). Although there is a trend to diminished sprout length in the IRKO

compared with WT in conditions without significant growth factor addition, the difference is only statistically different during exposure to VEGF.

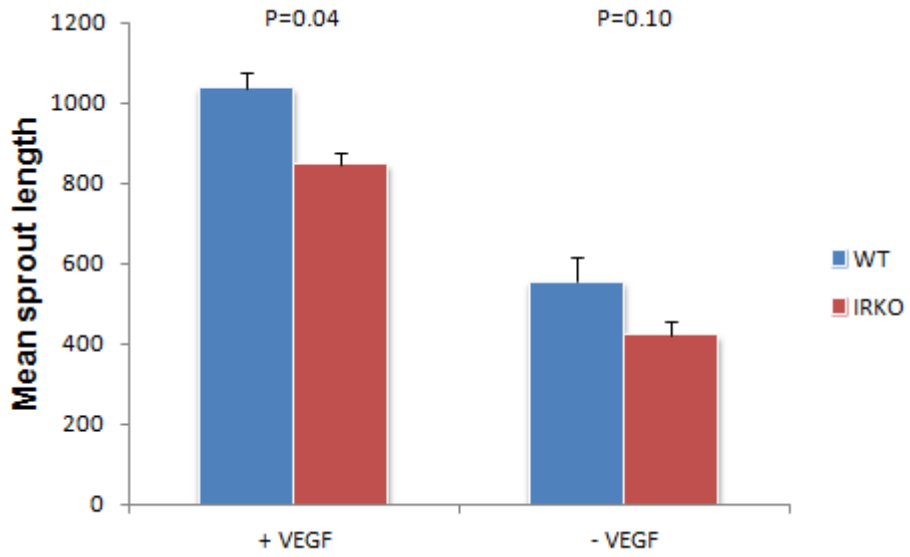

Figure 6-7. Mean length of endothelial sprouts, from aortic rings cultured in VEGF, is greater in WT compared with IRKO mice.

This difference in response to VEGF exposure is repeated when observing the number of endothelial sprouts present in aortic rings from WT compared with IRKO mice (WT 29.93 [2.19] vs. IRKO 21.82 [2.42] endothelial sprouts per ring, p=0.03). (n = 8IRKO, 8WT). See Figure 6-8. The number of sprouts emerging from rings cultured in media without addition of VEGF was comparable between WT and IRKO mice (WT 12.53 [1.38] vs. IRKO 12.18 [0.92] endothelial sprouts per ring, p=0.84).

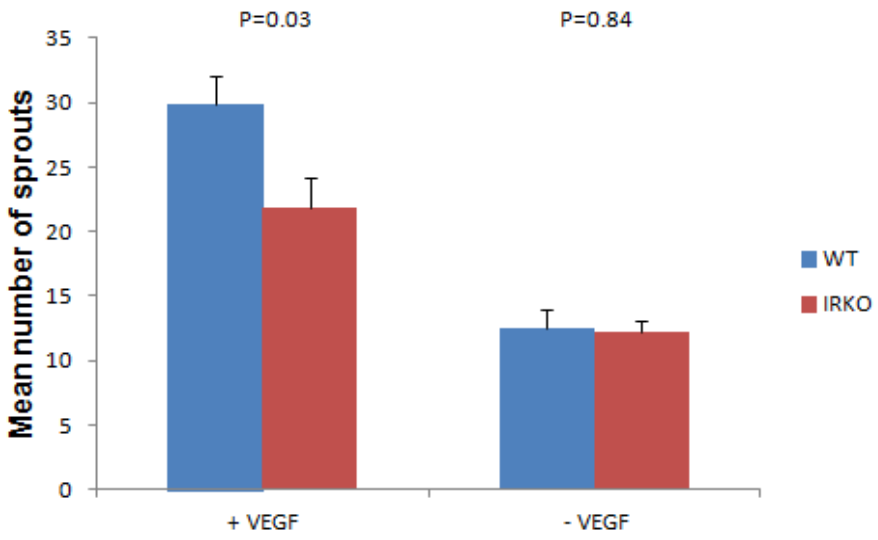

Figure 6-8. Mean number of endothelial sprouts, from aortic rings cultured in VEGF, is higher from WT compared with IRKO mice.

6.2.2 Tubule formation

PECs isolated from WT and IRKO formed tubular structures when seeded onto Matrigel and cultured with VEGF, by extending cellular processes to form cell-cell contacts. PECs from IRKO mice formed fewer tubular structures in comparison to WT PECs (WT 27.6 [2.1] vs IRKO 14.5 [2.4] structures per 40x microscopic field, p=0.004). (n= 7IRKO, 4WT). See Figure 6-9.

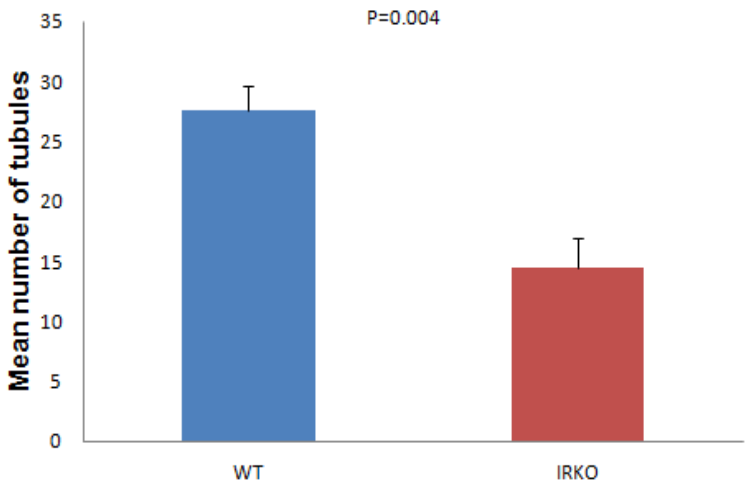

Figure 6-9. Tubule formation is reduced in PECs from IRKO compared with WT mice.

6.2.3 Migration assay (Boyden chamber)

Further studies were done to identify functional deficits that may contribute to the reduction in angiogenesis seen in the IRKO PEC during *in vitro* studies. PECs isolated from both WT and IRKO mice exhibited migration in response to vehicle (EBM + FCS 1%) solution and VEGF. In order to assess specific migratory response to VEGF, data was corrected on an individual sample basis for the migratory activity of cells exposed to vehicle solution alone. Migration towards VEGF was reduced in PECs isolated from IRKO mice (WT 7.5 [1.7] vs. IRKO 2.2 [1.1] cells per 100x high power field, p= 0.048; n= 5IRKO vs 4WT). See Figure 6-10.

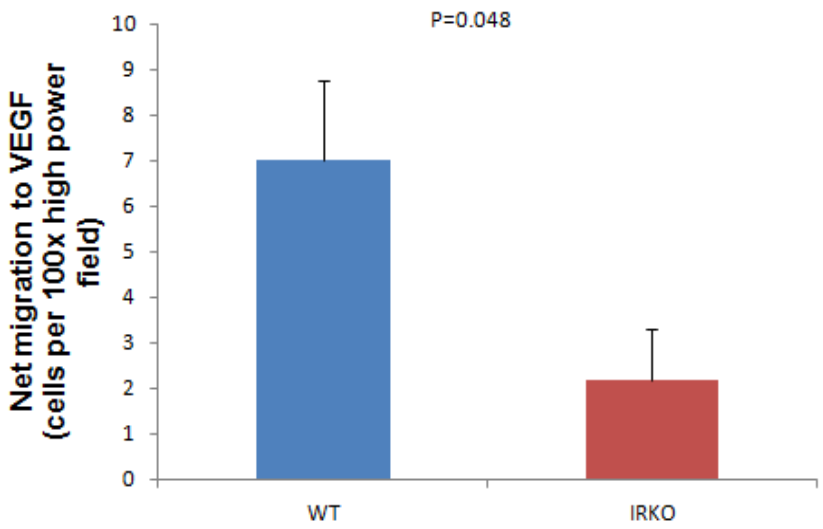

Figure 6-10. Migration to VEGF is reduced in PECs from IRKO mice compared to WT.

6.2.4 Migration assay (scratch wound)

PECs isolated from both WT and IRKO mice exhibited migration in response to the formation of a scratch wound. Cells were cultured in standard media supplemented with Mitomycin C (1µg/ml) to negate the impact of proliferation. There was no difference in the degree of wound closure achieved by WT cells compared with IRKO cells (WT 69.6% [14.8] vs. IRKO 67.4% [9.1] % wound closure, p=0.9: Figure 6-11; n= 4IRKO vs 3WT). Similarly, there was no difference in the absence of Mitomycin C (WT 65.2 [7.5] vs. IRKO 60.5 [1.9] % wound closure, p=0.6; n=3WT, 4IRKO). See Figure 6-12.

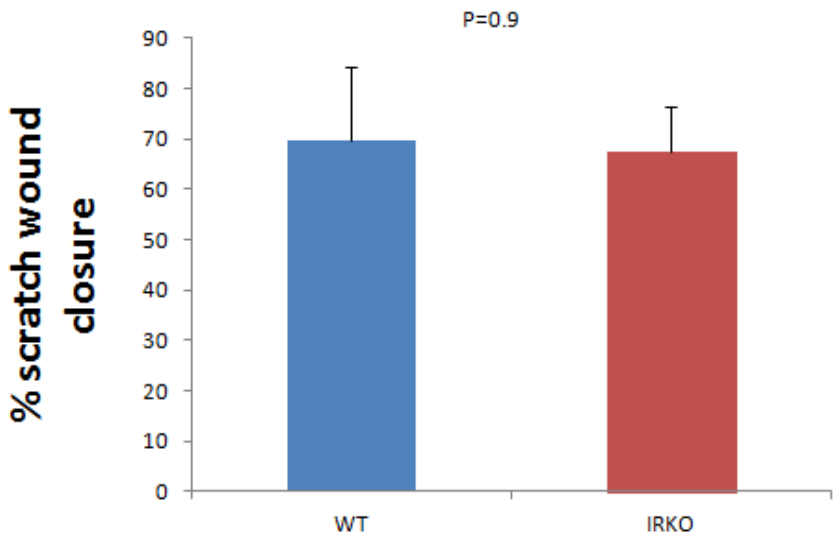

Figure 6-11. There was no difference in the degree of wound closure achieved by WT cells compared with IRKO cells in the presence of Mitomycin C.

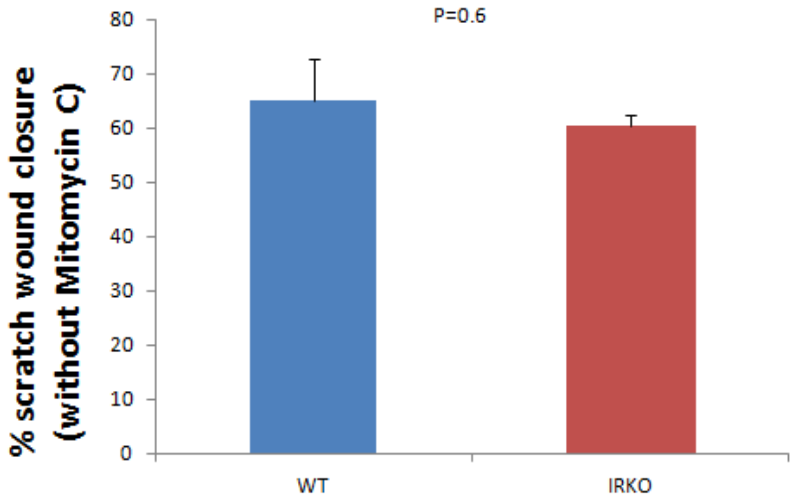

Figure 6-12. There was no difference in the degree of wound closure achieved by WT cells compared with IRKO cells in the absence of Mitomycin C.

6.2.5 Proliferation assay

The proliferative activity of PECs isolated from WT and IRKO mice was assessed by observing EdU uptake, as described in section 4.10.5 after passage number 3. The proliferative state of the PECs was comparable between genotypes (WT 0.1 [0.03] vs IRKO 0.09 [0.03] fraction of proliferating cells, p=0.77: Figure 6-13) (n=11IRKO vs 7WT). See Figure 6-13.

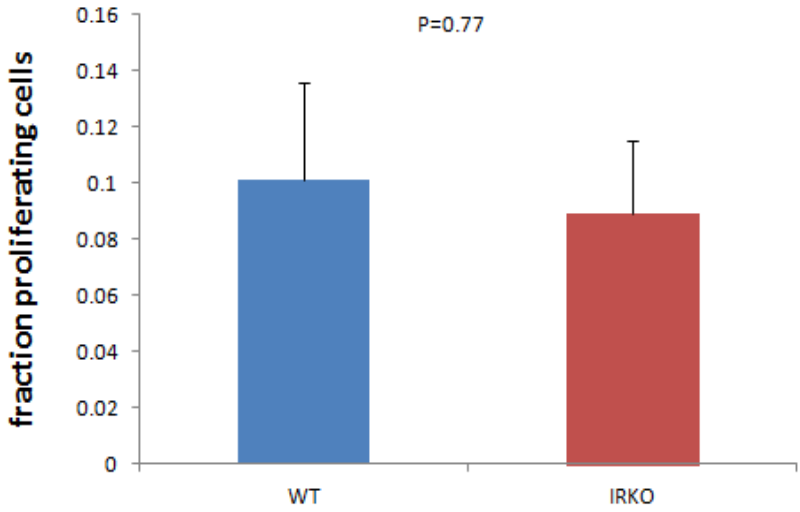

Figure 6-13. The proliferative state of the PECs was comparable between genotypes.

6.2.6 Apoptosis assay

The degree of apoptosis present amongst WT compared with IRKO PECs was assessed by measuring the levels of caspase 3 and cleaved caspase 3 present in cell lysates using western blotting, using a commercially available kit. The kit also provided control samples. The abundance of caspase 3 and cleaved caspase 3 in PEC lysates was similar in cells from WT and IRKO

mice (WT 0.51 [0.05] vs. IRKO 0.50 [0.04] Caspase 3/B actin (AU), p=0.87; n=3IRKO vs 3WT). See Figure 6-15. In fact, there was no detectable cleaved caspase 3 in any of the PEC lysates, an important finding, as it suggests that any differences in the functional performance of the cells is not likely to be due to cell death. Representative western blots are presented in Figure 6-14.

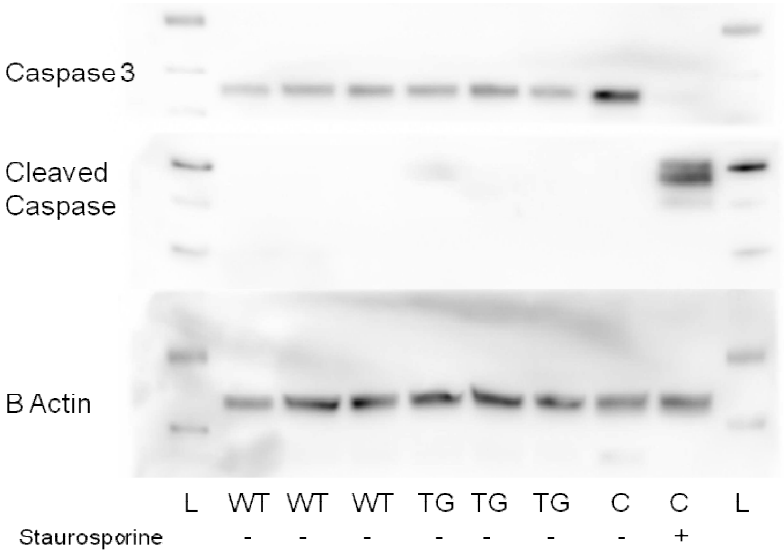

Figure 6-14. Representative western blot of PEC lysates and negative and positive (staurosporine treated) controls provided with the purchased kit. Abbreviations: L; Ladder, WT; Wildtype, TG; Transgenic, C; Control.

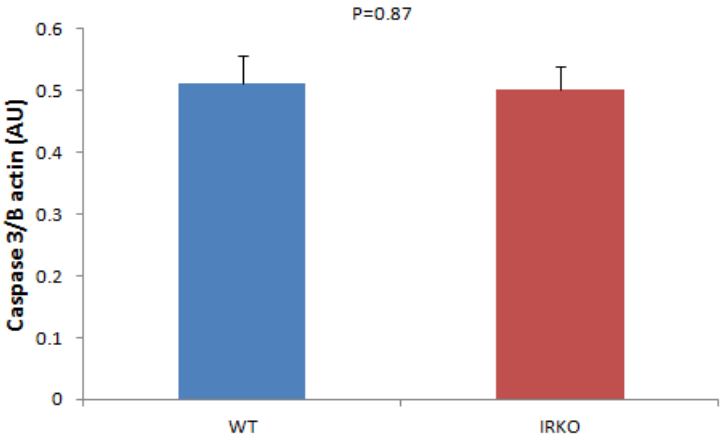

Figure 6-15. The abundance of caspase 3 and cleaved caspase 3 in PEC lysates was similar in cells from WT and IRKO mice.

6.3 PEC signalling studies

Stimulation of PECs was carried out as described in 4.11. Preliminary data suggests subtle differences in signalling responses to VEGF between PECs from WT and IRKO mice. With experiments being carried out in 4 WT and 4IRKO samples a trend is emerging with higher basal levels of Akt phosphorylation (S473) being evident in the PECs from IRKO mice. This difference is reversed after 15 minutes of exposure to VEGF, with Akt phosphorylation being increased in WT PECs. See Figure 6-17. Basal levels of eNOS phosphorylation (S1177) were similar between genotypes, but there was increased eNOS phosphorylation seen in the WT PECs compared with those from IRKO mice after 15 and 30 minutes of exposure to VEGF. See figure Figure 6-16. None of the differences observed are statistically significant. Further experiments will be conducted, and although interesting, the results should be interpreted as preliminary.

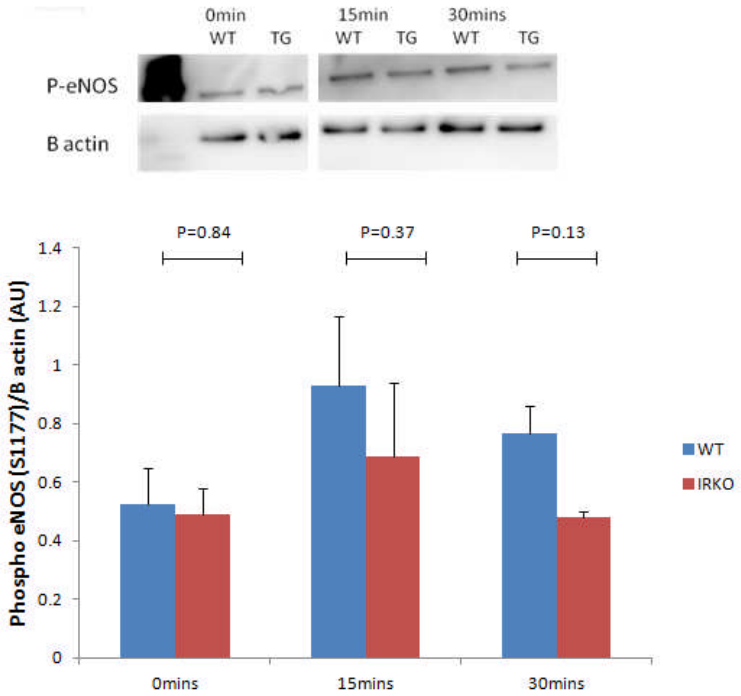

Figure 6-16. A trend towards reduced eNOS phosphorylation in response to VEGF exposure in IRKO PECs when compared with WT cells. (n=4 WT, 4IRKO).

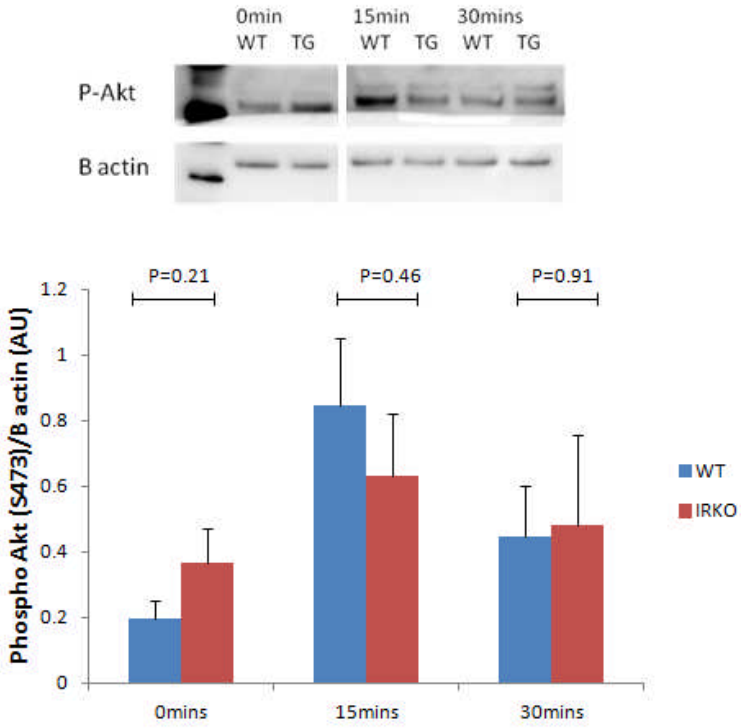

Figure 6-17. There is a trend towards increased Akt phosphorylation in IRKO PECs when compared with WT cells in basal, quiescent, conditions. There is

a trend towards reduced eNOS phosphorylation in response to VEGF exposure in IRKO PECs when compared with WT cells. (n=4 WT, 4IRKO).

Chapter 7. Discussion

7.1 Summary of key findings

This thesis presents a broad assessment of the impaired capacity of LEPCs derived from apparently healthy, but insulin resistant, SA men to augment vascular regeneration. Moreover we present a strategy to rescue their function, and in doing so causally implicate reduced Akt1 activity. As has been extensively discussed in the introduction, separating ethnicity from insulin resistance, as well as other potentially contributory factors, is difficult. In an attempt to address this issue, went on to characterise the adverse impact of insulin resistance in an animal model. The overall observations of the thesis will now be summarised.

Our cohorts of SA and WE men were well matched for classical CVD risk factors, including blood pressure, smoking status, lipid profile and BMI. The only apparent difference between the two groups was an elevated fasting plasma insulin level, and as a result an increased HOMA-IR score, in the SA group. We have demonstrated that, when transfused following hindlimb ischaemia induction, SA LEPCs are not able to augment vascular regeneration in the manner that WE LEPCs do. This data builds on an existing body of work, already carried out in our lab, demonstrating the impaired ability of these cells to facilitate re-endothelialisation of injured arteries. On the basis of previous findings from our lab showing reduced Akt phosphorylation in SA LEPC, we elected to assess whether increasing Akt1 activity in SA LEPCs, using lentiviral transduction of a constitutively active mutant, could rescue their angiogenic function. Indeed, this was the case in a

murine limb ischaemia model, with the level of regenerative function being comparable to non-manipulated WE LEPCs. *In vitro*, vascular network formation on Matrigel was significantly improved in SA LEPC expressing E17KAkt, and could be entirely abrogated by eNOS antagonism. Previous work from our lab highlighted an important reduction in total Akt, total eNOS, and their respective phosphorylated forms, in SA LEPC. By restoring Akt activity, and rescuing vascular regenerative function, we have causally implicated reduced Akt1 activity in SA LEPC dysfunction. The finding of relative insulin resistance in the South Asian group is also an important finding to bear in mind, since this is associated with reduced insulin mediated vascular Akt/eNOS phosphorylation in murine models. The subset of experiments demonstrating abrogated tubule formation in the presence of L-NMMA further implicates abnormal Akt/eNOS signalling and NO bioavailability in this observed SA LEPC dysfunction. Furthermore, by silencing Akt in WE LEPCs, we were able to recreate the dysfunction seen in SA LEPCs; Akt silenced WE LEPCs did not augment re-endothelialisation in a murine model of femoral arterial injury. *In vitro* dysfunction was also demonstrated with an impaired migratory response to VEGF evident in Akt silenced WE LEPCs when compared with control WE LEPCs. These data, again, strengthen the implication that impaired Akt1 signalling is important in the dysfunction seen in SA LEPCs.

Although we have identified that defective Akt1 signalling has important consequences for the vascular regenerative capacity of LEPCs derived from SA men, it is difficult to define the contribution of ethnicity or insulin

resistance to this dysfunction. We did, however, go on to perform further experiments to investigate the fundamental impact insulin resistance can have on vascular regeneration. Our *in vivo* studies in the IRKO mice, haploinsufficient for the insulin receptor, suggest that insulin resistance does indeed negatively impact vascular regeneration. Laser Doppler data gathered following the induction of hindlimb ischaemia suggest that limb perfusion recovery is delayed in the setting of insulin resistance. This defect in limb perfusion is associated with a reduction in capillary density when assessed at 21 days post ischaemia induction. Mechanistic insight is gained from quantitative PCR data obtained from the analysis of ischaemic musculature from both IRKO and WT mice. It is apparent that despite increased VEGF-A expression in the ischaemic adductor muscle of the IRKO mouse compared with the WT mice, perfusion recovery is impaired. This raises the possibility that functional resistance to the actions of VEGF-A are playing a role in the observed phenotype. These findings are also supported by preliminary data from western blots of lysates made from PECs following VEGF stimulation. These data suggests that the phosphorylation of key angiogenic mediators, Akt and eNOS, is blunted in endothelial cells isolated from IRKO mice when exposed to VEGF.

Findings from an array of *in vitro* studies complement our *in vivo* data; reduced VEGF mediated angiogenesis was evident in an aortic ring sprouting angiogenesis model, and IRKO PECs formed fewer vascular networks on Matrigel. Functional assays suggest an impaired migratory

response to VEGF in endothelial cells, a potential contributing mechanism by which angiogenesis could be impaired.

As outlined in the introduction, many facets of insulin resistant syndromes are associated with a reduction in angiogenesis and vascular regeneration. Our data demonstrates impaired function in a population of cells capable of aiding vascular regeneration, derived from a cohort of individuals with relative insulin resistance, and at higher risk of CVD. We have causally implicated decreased Akt1 signalling, a critical node in insulin signalling, in this dysfunction. We have gone on to demonstrate that insulin resistance *per se* is associated with reduced vascular regeneration. These data suggest that insulin resistance does impair vascular regeneration and that modifying insulin signalling in certain settings can improve vascular regenerative function.

7.2 Wider research context

7.2.1 SA LEPC transfusion studies

7.2.1.1 South Asian LEPCs do not augment vascular regeneration

Although a link between SA ethnicity and impaired vascular regenerative LEPC function has not been made before, there is data that demonstrates a link between SA ethnicity and dysfunction of other progenitor fractions. Murphy *et al* demonstrated a link between endothelial dysfunction in healthy SA men and a reduction in circulating CD133+/CD34+/KDR+ CPCs [225]. Further work demonstrated a reduced abundance of the same fraction of

CPCs in healthy SA men, both basally and when assessed following exercise, along with reductions in CD34+/CD45- CPCs [163]. Importantly, it is from within this rare latter fraction of cells that LEPC are derived [235], and newly published data from our lab demonstrates that LEPC colony formation is indeed reduced in healthy SA men. Furthermore, dysfunction is evident in LEPC from SA men [236]. *In vitro* studies demonstrate reduced proliferative capacity, increased senescence, impaired migration to VEGF and impaired vascular network formation on Matrigel with SA LEPCs compared with WE LEPCs. SA LEPCs did not augment re-endothelialisation in *in vivo* studies. Although *in vivo* vascular regenerative dysfunction has not been demonstrated previously, processes tested by the described *in vitro* studies, such as migration and proliferation, are important to angiogenesis and vascular regeneration, and our finding that SA LEPCs do not augment vascular regeneration is in keeping with the small existing body of evidence. The fact that LEPCs are capable of augmenting vascular regeneration is something that has previously been documented in the setting of murine hind limb ischaemia [191] and porcine myocardial infarction [192].

7.2.2 Akt signalling and vascular regeneration

The finding of reduced levels of total Akt and eNOS in SA LEPCs from previous work done in our lab [156] led to us restore Akt activity in SA LEPCs in an effort to reverse the observed inability to augment vascular regeneration. As outlined in the results section, modulating Akt1 activity via lentiviral delivery of a constitutively active Akt1 mutant, E17KAkt, did restore vascular regenerative function, as measured by an array *in vivo* and *in vitro*

assessments. Previous work by Ackah *et al* has highlighted the importance of Akt1 to ischaemia driven angiogenesis. This work identified that mice homozygous for Akt1 gene deletion had significantly impaired limb perfusion recovery following hind limb ischaemia, and impaired mobilisation of EEPCs. They also found that PECs isolated from these mice exhibited impaired migration to chemotactic stimuli in Boyden chamber experiments. The authors of this study concluded that, mechanistically, genetic loss of Akt1 resulted in the observed phenotype, due to impaired fibroblast and endothelial cell migration, reduced EPC mobilisation and reduced NO release [161]. Although different experimental models are used in this study, compared with ours, our findings build on the body of literature with regards the role Akt1 plays in vascular regeneration.

Interestingly, although our lab has previously found impaired SA LEPC migration to chemotactic stimuli, and impaired migration was noted in Akt1 silenced WE LEPC, we did not observe increased migration in E17KAkt expressing SA LEPCs. In fact a trend towards reduced cell migration was seen, compared with EGFP expressing SA LEPC, possibly because constitutive activation of Akt1 rendered the cells insensitive to further stimulation by VEGF.

Our finding of increased survival of E17KAkt expressing SA LEPCs offers potential mechanistic insight. If progenitor cells are to be used in the manner that has often been proposed, i.e. as a therapy to revascularise ischaemic tissue, they will have to function in an ischaemic environment that is hypoxic,

oxidative, and pro-inflammatory. All these factors have the potential to be cytotoxic, and interventions that infer resistance to these insults may enhance function. Another study that manipulated human LEPC by increasing Akt and heme-oxygenase-1 found that survival was enhanced; this was assessed using the same assay employed during our studies. They also found that vascular regenerative function of LEPCs was enhanced in a murine myocardial infarction model [98].

Our *in vitro* findings of abrogated angiogenesis in the presence of the NOS antagonist LNMMA, suggest that NO is critical to the beneficial angiogenic effects of enhanced Akt activity. The finding of increased eNOS expression in E17KAkt treated cells adds further support to this conclusion. These findings complement other data, published from our lab previously, that has demonstrated a reduction in flow mediated dilatation (FMD) [225] and a reduction in exercise induced CPC mobilisation in healthy SA men [163]. Both of these findings were found to relate to a reduction in NO bioavailability. Our suggestion, that NO mediates the improvement in LEPC function that accompanies increased Akt activity, is also supported by other work. Murohora *et al* demonstrated that eNOS is a critical mediator of ischaemic angiogenesis [234], and Sleicher *et al* demonstrated that the angiogenic effects of Akt1 are mediated via eNOS signalling by crossing Akt1 deficient mice with mice with a knock in mutation in a critical phosphorylation site of eNOS, which mimics phosphorylation (by Akt, for example). This led to rescue of the defective post-natal angiogenesis exhibited by Akt1 deficient mice [162].

7.2.3 *Ex vivo* modification of cells improves therapeutic function

Our data support the notion that LEPCs can augment vascular regeneration and be used in a therapeutic manner. It has previously been demonstrated that progenitor cells can be manipulated to improve their function; one example is the use of statins, which have been shown to reduce circulating angiogenic cell apoptosis [237]. Our data however, to our knowledge, are the first to demonstrate the *ex vivo* manipulation of LEPCs derived from a group at high risk of cardiovascular disease, resulting in rescued vascular regenerative function. Our data, therefore, provides an important proof of principle because, as discussed in the introduction, many disease states, including insulin resistant syndromes, are associated with progenitor cell dysfunction. Therefore, if progenitors such as LEPCs are to be taken from patients with cardiovascular disease, with a view to being utilised as part of an autologous cell therapy strategy, it is likely that *ex vivo* manipulation will first be necessary. We have demonstrated that this type of manipulation prior to administration is feasible, although clearly there are many remaining hurdles in the process of translation to the clinic.

7.2.4 Akt signalling, vascular regeneration and insulin resistance

As discussed in the introduction, insulin resistance is thought to play a major role in the increased CVD seen in South Asian populations. This, coupled with our findings of relative insulin resistance in our South Asian cohort, provides a basis for the inference that insulin resistance may underlie the abnormalities in SA LEPC function that have been observed. We have

causally implicated diminished Akt activity by improving SA LEPC function after increasing Akt activity. This observation is strengthened by our data, showing that silencing of Akt in WE LEPCs recreates the *in vivo* and *in vitro* dysfunction apparent in SA LEPC. When considering a link between perturbed Akt signalling and insulin resistance it is important to note that although Akt signalling is central to a diverse array of cellular processes, disturbed PI3-K/Akt signalling is a defining characteristic of vascular insulin resistance [13]. Unfortunately it is not possible to more accurately define the impact of insulin resistance on SA LEPC dysfunction with our data. Although human studies are invaluable in terms of clinical relevance and translation to the clinical setting, a significant obstacle is encountered when probing mechanisms responsible for observed abnormalities. This is due to the obvious technical and ethical limitations that are part of scientific study in humans. It is because of this that we decided to probe the effect of insulin resistance on vascular regeneration with a murine model and the context of the findings generated will be discussed further here.

7.2.5 Studies of vascular regeneration in the IRKO mouse

Work carried out in our lab has previously identified that when compared with WT mice, the IRKO mouse has delayed endothelial repair and regeneration. This was previously assessed in a femoral arterial injury model, and demonstrated that insulin resistance is associated with abnormal endothelial repair [155]. This study also identified that VEGF induced mobilisation of CPCs is impaired in the IRKO mouse. Although distinct processes, there is a degree of overlap in the steps required for both endothelial repair and

vascular regeneration, including endothelial cell migration and proliferation. Therefore our findings that vascular regeneration is impaired in the IRKO complement, and significantly expand, previous findings from our lab. The previous observation that VEGF induced CPC mobilisation is perturbed in the IRKO is pertinent, and offers a potential mechanism by which vascular regeneration may be impaired in the IRKO also. The only other available data that has addressed angiogenesis in insulin receptor knockout models comes from the work of Kondo *et al*. These authors found that mice with holoinsufficiency of the insulin receptor in vascular endothelium had reduced retinal neovascularisation in retinopathy of prematurity model [157]. This finding was associated with a blunted rise in angiogenic mediators VEGF, eNOS and endothelin-1. Again, in one respect, our findings are in keeping with this data, with angiogenesis in response to ischaemia being diminished, albeit in a different tissue bed, in different conditions, and in a mouse with systemic insulin receptor haploinsufficiency rather than holoinsufficiency confined to the endothelium. However we have found that VEGF expression is increased, rather than decreased, in the muscle of the IRKO mice, and our *in vitro* studies demonstrate that functional responses to VEGF, such as migration, are also reduced. Western blots also suggest that signalling in endothelial cells from IRKO mice, in response to VEGF exposure may be blunted. An explanation for these differences is not obvious, but important differences between models and methods used in our study and the Kondo could certainly have profound effects. Specifically, the animal model used by Kondo *et al* has a complete lack of insulin receptors in the endothelium, therefore insulin signalling in every other tissue in this model is preserved.

They also examined a different tissue bed using different experimental techniques.

Our *in vivo* findings of reduced vascular regeneration in the IRKO are complemented by *in vitro* data also. VEGF induced angiogenesis was reduced in the aortic ring endothelial sprouting assay. This assay, whilst allowing the study of isolated substances, such as growth factors, in relation to angiogenesis, also allows observation of the interaction between endothelial cells and supporting cells such as pericytes and fibroblasts, which are important in maintaining vessel integrity. Kahn *et al* have presented data from our lab on aortic rings explanted from IRKO mice previously [155]. They found in this small sample that there was a very small, non-significant reduction in mean sprout length from the IRKO mice compared with the WT mice. We believe that our data enhances this work because our sample size is bigger, but also our assay is much more refined and suited for identifying endothelial sprouts. The method utilised in this thesis, previously published by Baker *et al* [238], requires staining with the relatively specific endothelial marker, BS 1 lectin. This is important as it allows accurate identification of endothelial sprouts. As mentioned, one of the advantages of this assay is that it involves a heterogenous population of cells, but without the described staining it is more difficult to define what is an endothelial sprout versus, for example, fibroblast outgrowth. Our assay also looked at VEGF stimulated growth, the aortic rings in the study of Kahn *et al* were cultured in complete endothelial cell growth medium, which contains numerous growth factors and a higher percentage of FCS.

These findings were recapitulated in another *in vitro* assay, the assessment of vascular network formation on Matrigel by PECs. Therefore a reduction in VEGF stimulated angiogenesis has been demonstrated in two *in vitro* assays utilising tissue from the IRKO mouse. Further functional assays offer insight as to potential mechanisms responsible for the reduction in angiogenesis and vascular regeneration that we have observed. In particular, our observation that endothelial cells from the IRKO mice display impaired migratory response to VEGF compared with cells from WT mice, is an important one. As outlined in the discussion, an appropriate response to migratory cues is a crucial part of sprouting angiogenesis. It can be appreciated that an impaired ability to respond adequately to a VEGF concentration gradient is likely to negatively affect the rate at which angiogenesis occurs and ultimately could lead to a delay in tissue reperfusion, as has been demonstrated in our *in vivo* studies. It is important to interpret the data from our studies of PEC migration using the scratch wound method in the context of our dataset as a whole. The other functional assays that we have utilised observe angiogenic processes in response to VEGF exposure. *In vivo*, angiogenesis occurs in response to ischaemia in the setting of growth factor gradients, with areas of tissue that are most ischaemic producing more angiogenic mediators, such as VEGF. Considering, the scratch wound assay is perhaps more representative of the cell migration required following endothelial injury. In fact, our data demonstrating a similar capacity for PECs from both WT and IRKO mice to migrate and ‘heal’ a scratch wound, are compatible with the suggestion of

Kahn *et al*, that impaired endothelial regeneration in the IRKO mouse may be a CPC mediated phenomenon [155].

Our finding of increased VEGF-A expression in the ischaemic adductor muscle of IRKO in comparison to WT mice is potentially indicative of functional VEGF resistance. Our data from western blot studies also suggests VEGF resistance. Functional resistance to VEGF has previously been demonstrated by Waltenberger *et al* in monocytes obtained from patients with diabetes. Migration to VEGF was impaired in these cells, whilst migration to other chemotactic stimulants was preserved [176]. Recent work from Warren *et al* highlights how, mechanistically, VEGF resistance can arise in the setting of diabetes. They found that post-translational modification of VEGFR2 by ROS, generated as a result of hyperglycaemia, leads to impaired VEGFR2 trafficking to the cell membrane and progressively muted responses to VEGF [153]. Work from Mehra *et al* also supports the notion of VEGF resistance accompanying insulin resistance. These authors have demonstrated that by high fat feeding mice, a recognised method of inducing insulin resistance *in* vivo, they can induce VEGF resistance [239]. This was demonstrated by infusing VEGF into the aortae of both high fat fed and chow fed mice; eNOS phosphorylation was reduced in the aortae of the high fat fed mice. They also found that treating endothelial cells with palmitate rendered them resistant to VEGF. Mechanistically, they found that an increase in free fatty acids results in VEGF resistance as ceramide induces Protein phosphatase 2A (PP2A), which in turn dephosphorylates the molecular substrates of VEGF signalling and results in VEGF resistance. The

authors were able to restore VEGF signalling in high fat fed mice by genetically reducing serine palmitoyltransferase expression, the enzyme that facilitates the conversion of palmitate to ceramide. Our data complements and advances this growing body of evidence that insulin resistance is associated with VEGF resistance. Other work has been published that contrasts these findings however, for instance, Kajiwara *et al* found that the levels of expressions of angiogenic factors, including VEGF, were reduced in the ischaemic muscles of Zucker diabetic rats [177]. Importantly, these data were obtained from animal models of diabetes, and our observations are the first in which insulin resistance rather than diabetes, hyperglycaemia and dyslipidaemia can be implicated.

7.3 Limitations

As with any scientific study, our data has strengths and weaknesses. To fully understand the implications of our data it is important to appreciate the limitations of our data.

7.3.1 Defining ethnicity

As highlighted in the discussion, the definition of ethnicity is difficult. Although the method employed to define ethnicity in our study is typical, and is the method commonly employed throughout the literature, it is arbitrary and does not take into account many factors. These factors such as religion, culture and diet are responsible for the dramatic heterogeneity within ethnic groups and are also likely to impact significantly on cardiovascular risk. Furthermore,

with increasing globalisation there is increasing migration and commonly used ethnic descriptors apply to fewer people. These limitations are not easily overcome, and we have employed such a method to replicate prior studies.

7.3.2 Sample size

A factor with potential implications for the study is the relatively small sample size. Because of this it is important to note that where we have found no difference, it is not possible to be conclusive in this regard. During this study we have collected a relatively large amount of data for a small sample size, which is more effective with regards hypotheses generation, than collecting small amounts of data from a large sample size. Importantly, funds available for consumables during this project were finite, and the development and application of the described assays required significant expenditure of money and time. Where relevant, we have attempted to identify situations where sample size may influence our conclusions.

7.3.3 Origin and definition of LEPCs

As highlighted in the introduction of this thesis, difficulty and debate exists regarding the definition and classification of ‘EPC’s. The possibility exists that LEPCs can acquire and lose properties during cell culture or even that LEPCs are a cell that only exist in cell culture. In many ways, at least with regards to this thesis, this debate is academic, as we, along with other groups, have shown that LEPCs can augment vascular regeneration. They

have therefore been demonstrated to possess potential, in terms of benefitting patients as a ‘cell therapy’.

7.3.4 Lentiviral vector use and oncogenic potential

LEPCs have the potential to proliferate substantially and are capable of significant expansion. The capacity for LEPCs to partake in tumour angiogenesis when transfused as a cell therapy is therefore theoretically possible. This is however, speculation and further evaluation is required. Increased tumour growth has not been observed where LEPCs have been utilised in animal studies, but these studies are of course short and longer periods of follow-up would be required before the safety profile of LEPCs as a cell therapy can be more accurately defined. This issue is clouded further when lentiviruses are used to introduce a putative oncogene (E17KAkt) in order to permanently promote the angiogenic potential of these cells. Lentiviral vectors do have an advantage over some other methods of gene delivery as they integrate the target cell genome in a stable fashion, even in non-dividing cells [240]. The use of these vectors does raise biosafety issues, namely the risk of insertional oncogenesis, and the risk of recombination between the transfer vector and packaging constructs to create a replication competent lentivirus [240]. In an attempt to circumvent these issues, self inactivating (SIN) lentivectors have been developed [241]. As commented on above, studies utilising animals are short-term and there is therefore not enough longer term safety data to appraise the potential for harm when using lentiviral vectors to deliver gene therapy.

Further problems may be anticipated to arise from persistent constitutive Akt activation in LEPC. Indeed, there is evidence to suggest that chronic hyperactivation of Akt signalling can be detrimental, and work by Wang *et al* associates excess Akt signalling with increased endothelial cell senescence, reduced vascular network formation on Matrigel, and reduced proliferation. They also found that EPC mobilisation was reduced in response to ischaemia and VEGF stimulation [242]. This relationship between chronic Akt activation and senescence has been demonstrated by other groups also, Nishi *et al* found that endothelial cells with VEGFR1 deletion became prematurely senescent and they found excess Akt activation to be responsible for this [243]. Moreover, Akt over expression has been associated with oncogenesis [244, 245], and as described in the methods section, the mutation in Akt used in this thesis to augment Akt activity in SA LEPCs was originally identified in human breast, colorectal and ovarian cancers [228], this immediately raises concern with regards oncogenesis. Due to these issues it seems that alternative means of transiently promoting Akt activity in LEPC will be desirable before conducting clinical trials of modified LEPC.

7.3.5 Mechanistic insight

The fact that it is difficult to probe molecular mechanisms in human studies has already been highlighted. Much of the work done to probe signalling pathways in animal models, involves genetic modification via the production of gene knock-out/knock-in models, siRNA administration or the administration of novel drugs or inhibitors. This obviously cannot be done in

human studies, and the lack of a more precise molecular mechanism is therefore an unavoidable limitation of this study. It would be beneficial to ascertain a more precise molecular mechanism for the diminished Akt activity seen in our SA cohort, and this would perhaps allow us to dissect the contribution of insulin resistance from ethnicity, or indeed other factors on the observed SA LEPC dysfunction. If a more detailed molecular mechanism were known, signalling pathways might be modulated in a more subtle way than the method that we have employed, and so there may be more potential for human translation. Potential avenues for future exploration will be discussed later, and we have also gone on to utilise an animal model to probe the effects of insulin resistance on vascular regeneration in more general terms, but issues with human studies that I have outlined cannot be overcome.

7.3.6 Animal model of insulin resistance

As with all animal models, our murine model of insulin resistance has certain limitations, and caution should be exercised when extrapolating the findings to the setting of human insulin resistant syndromes. Insulin resistance seen in human disease is complex and is acquired via modification of insulin signalling pathways at multiple levels. This occurs due to the effects of several co-existing pathological factors, such as hyperglycaemia, lipotoxicity and inflammation. The murine model used in this project, the IRKO mouse, has haploinsufficiency of the insulin receptor and thus rendered insulin resistant. The manner by which insulin resistance is created is not necessarily analogous to the processes that result in insulin resistance seen

in human disease. However, this model also has advantages, we have demonstrated that it recapitulates ‘prediabetic’ vascular dysfunction [154], and for this project, the ability to separate the effects of systemic insulin resistance from hyperglycaemia and observe the impact this has on vascular regeneration, is important.

7.3.7 Limitations of methods employed

The murine hindlimb ischaemia model is a widely used assay to assess vascular regeneration. It has benefits and allows the study of arteriogenesis and collateral recruitment in proximal muscle groups as well as angiogenesis in distal muscle groups. With the development of laser Doppler imaging it also allows for the collection of a complete data set over a period of weeks from one mouse, removing the need for sacrifice at different time points to allow analysis. One limitation of this assay is the acute nature of ischaemia induction. Peripheral vascular disease usually progresses chronically in humans, which allows for the development of collaterals. When assessing therapies have we have done with SA LEPCs it is important to bear in mind that the pathological processes resulting in ischaemia are very different.

Although the data obtained from our studies of hindlimb ischaemia are interesting, it would perhaps be more compelling if other *in vivo* methods to assess vascular regeneration and angiogenesis were also utilised. Many other assays exist and some of these will be discussed in the future plans section.

Again, as with most, if not all, of the methods employed during scientific study, there are limitations in the *in vitro* assays used to determine the impact of insulin resistance on angiogenesis. It is important not to take the results of these *in vitro* assays in isolation and our data compliment our *in vivo* findings. When using cultured and expanded cells, such as PECs, it is possible that the cellular phenotype could be altered by such processes. Furthermore, angiogenesis is a complex process requiring the coordinated function of many different cell types. The tube forming assay used in this thesis is an assay that highlights these shortcomings. This assay uses one cell type that is expanded *ex vivo* and assesses angiogenesis in 2 dimensions only. The limitations of this assay are mitigated in our dataset by the use of another assay, the aortic ring sprouting assay. This assay allows the observation of angiogenesis in 3 dimensions, with a mixed cell population, that have not been expanded *ex vivo*. Using a number of *in vitro* methods to complement our *in vivo* findings results in a more robust dataset.

Just as with aspects of the data obtained during our SA LEPC studies, the use of a relatively small sample size in some of the experiments with the IRKO leads to certain limitations. The scratch wound assay and the proliferation assay, both of which used PECs from IRKO and WT mice, are affected by this factor. For example, we found no difference between IRKO and WT PECs when investigating migration in the scratch wound assay. This experiment was done using cells from 4 IRKO mice and 3 WT mice. The findings were almost identical but it is not possible to categorically state that there is no difference and in order to be entirely confident in this a bigger

sample would be necessary. This project was subject to time and resource constraint and it is our opinion that it was more productive to investigate a larger range of processes rather than focussing time and resources on fewer experiments, this allowed the observation of a wider range of processes. The same issue applies to our observation of proliferative capacity in the IRKO and WT PECs. In order to firmly state that a reduction in endothelial cell proliferative capacity is not contributing to the phenotype observed in the IRKO mice a more extensive assessment is probably required. Again, the sample size is relatively small, and the fraction of proliferating cells that was observed was surprisingly low in PECs from both the IRKO and WT mice. Investigation of proliferation at earlier passage, using different stimuli, and alternative methodologies would therefore be desirable, and this will be discussed further in the future studies section.

7.4 Future plans

7.4.1 Further work in SA LEPC

As outlined, the manipulation of Akt raises safety concerns when considering use in humans. Contributors to this concern are the potential negative impact of chronic Akt activation and potential for oncogenesis. It is for this reason that future work could be directed to more accurately define the mechanistic basis for reduced Akt signalling in SA LEPCs. If this were better understood, there is potential to modulate Akt signalling in a more subtle fashion, therefore tempering some of the safety concerns raised during discussions within this thesis. Previous work from our lab has highlighted that although there was a significant reduction in the level of Akt protein in SA LEPCs compared with WE LEPCs, mRNA levels of Akt1 were similar, implicating

translational or post-translational issues in our observations [156]. A possible explanation for this finding is that Akt is more rapidly degraded in the SA cells. The regulation of Akt activity and stability involves many complex signalling pathways [246]. To investigate if and how Akt stability is altered in SA LEPCs compared with WE LEPCs, it would be necessary to identify any differences in the levels of molecules that are known to regulate Akt degradation. There is an exhaustive list of signalling intermediaries that can affect the stability of Akt. Our strategy for investigating this would be to evaluate for levels of key mediators of Akt stability, for example, Pin1; a protein that has been shown to protect Akt from degradation [247]. This could be quantified by western blotting. Another process known to regulate protein activity, including kinases such as Akt, is the reversible acetylation and deacetylation of lysine residues by histone acetyltransferases and histone deacetylases, respectively [248]. Acetylation of the pleckstrin homology domain of Akt has been shown to reduce its activity, and conversely deacetylation by SIRT1 has been shown to increase its activity by allowing the binding of PIP_3 and subsequent membrane localisation, a critical step in Akt activation [249]. Interestingly, unpublished, pilot work from our laboratory suggests that reduced levels of the histone deacetylase, SIRT1, may be a factor in SA LEPC dysfunction. Future work would aim to robustly quantify SIRT1 levels in SA LEPC with PCR and/or western blotting. If a decrease in SIRT1 was confirmed in SA LEPC, treatment of cells to increase SIRT1 could be trialled. One such substance, capable of increasing SIRT1 activity is Resveratrol, a naturally occurring phenol, found in the skin of red

grapes and other fruits [250]. Manipulation of cells in this manner may carry with it less safety concern. Currently, however, this is of course speculative.

7.4.2 Further work in the IRKO mouse

Further work to define the role of insulin resistance on vascular regeneration and angiogenesis is planned. Additional *in vivo* experiments will be conducted in IRKO and WT mice. Another *in vivo* model that will be developed is the retinal angiogenesis model. This assay, involves the dissection of mouse pup retinas, and takes advantage of the fact that vascularisation of the mouse retina occurs post-natally. Further *in vivo* data will advance and add strength to our current observations. The retinal angiogenesis model allows the observation of development angiogenesis, as opposed to pathological angiogenesis seen in hind limb ischaemia studies. It also allows the visualisation of neovascularisation in a single plane. This has advantages with regards to our experimental needs. This assay will allow a more in depth interrogation of angiogenesis, such as, the observation of differences in vessel branching, filopodia formation and the recruitment of mural cells. All these processes are critical to normal angiogenesis, and although we have established that vascular regeneration is impaired in the IRKO, with the hindlimb ischaemia data, we cannot comment on these aforementioned processes. Potentially, responsible mechanisms for the reduced vascular regeneration observed could include, for example, abnormalities in mural cell function. This is, of course, speculative but this is an example of processes that the retinal angiogenesis model can help to define.

Other work that would strengthen our current *in vivo* data would be done to increase sample size in the scratch wound experiments and the proliferation experiments. The proliferation experiments will also be carried out at various time points to be sure that there is no difference in the proliferative state of endothelial cells from the IRKO mouse compared with WT. It is important to note that scratch wound experiments were carried out in complete growth medium. All our functional observations in the PECs from the IRKO have been made in the context of VEGF exposure. The scratch wound experiments will be repeated in a similar environment, i.e. in media containing VEGF only, rather than an array of other growth factors.

We have some interesting preliminary data from western blots but before firm conclusions can be drawn there is a clear need to increase the numbers of samples in which these studies are done. We will also look at other proteins, specifically, total levels of Akt, eNOS, ERK and levels of phospho ERK. These molecules however, represent a tiny fraction of the potential molecular candidates responsible for our findings. A method that could be employed to 'screen' for candidate molecules of importance is phosphoproteomic analysis. This method allows the analysis of the entire complement of phosphorylated proteins within a cell. Therefore, if analysis is carried out before and after stimulation with a growth factor, in our case VEGF, it may facilitate the identification of proteins important in relevant signalling pathways, and that are therefore worthy of further investigation. Ultimately, it is hoped, that elucidation of an underlying mechanism will yield targets by

which, we can attempt to reverse the vascular impairment we have seen in the insulin resistant setting.

Another interesting issue to address is the role that the endothelium has in the phenotype that we have observed. This could be investigated with the use of murine models with insulin resistance confined to the endothelium, for example, using mice haploinsufficient for the insulin receptor in the endothelium. Indeed work by Kondo *et al*, involving the study of this mice with endothelial holoinsufficiency of the insulin receptor (VENIRKO), has already been discussed in this thesis [157]. This work has demonstrated that absolute endothelial insulin resistance impacts retinal neovascularisation in a relative hyperoxia model, a model used to mimic retinopathy of prematurity. Another murine model with endothelial insulin resistance can be generated by expressing Thr^{1134} mutant human insulin receptors, under the control of the Tie2 promoter/enhancer. This results in the generation of endothelial specific mutant insulin receptor over expression (ESMIRO) mice [251]. The ESMIRO mouse has already been studied by our group and therefore for practical reasons this model is more likely to be used than the VENIRKO model. Repeating the experiments outlined in this thesis in the ESMIRO mouse will help to define the role of the endothelium. The systemically insulin resistant model that we have employed is more analogous to insulin resistance that is seen in human disease, but it does not separate, for example, the effects of hepatic insulin resistance from vascular insulin resistance on vascular regeneration. Another method of assessing the impact of the endothelium would be to restore insulin sensitivity specifically in

the endothelium. This could be achieved by crossing IRKO mice with mice that over express the insulin receptor in the endothelium, the human insulin receptor endothelial cell over expressing (HIRECO) mouse to create mice that are resistant to insulin systemically but sensitive to its effects in the endothelium (IRKO x HIRECO).

7.5 Study implications

As outlined at the outset of this thesis, diabetes and pre-diabetic, insulin resistant states, are responsible for significant morbidity and mortality worldwide. Whilst it is not possible to directly translate the findings of this thesis to the clinical setting, our data is the first to demonstrate LEPC dysfunction in samples derived from a group at high risk of diabetes and cardiovascular disease. Furthermore, our data is the first to demonstrate a critical proof of principle – restoration of the function of these cells *ex vivo*, resulting in increased efficacy when transfused into mice as a cell therapy. There are significant steps to be taken before similar steps are taken in humans, but nevertheless our findings are important and have the potential to indirectly impact many patients in the future. Our data demonstrating that insulin resistance negatively impacts vascular regeneration is also novel. This fundamental observation is far from being translated into the clinical arena and directly impacting patient care. It does, however, open an interesting avenue of investigation that could ultimately lead to the identification of novel therapeutics. This novel finding, therefore, has the potential to impact a very large number of patients that experience pre-diabetes, diabetes and vascular disease.

7.6 Concluding remarks

At the outset of this project, our initial aim was to assess the capacity of LEPCs from SA donors to augment vascular regeneration. We have presented data, confirming that we have met this aim. LEPCs derived from SA donors do not augment vascular regeneration in the same manner that those derived from WE donors do. Furthermore we have implicated deficient Akt signalling in this dysfunction; increasing Akt activity in SA LEPCs rescued their angiogenic function. This improved function has been demonstrated with an array of *in vivo* and *in vitro* studies. Further *in vitro* studies have shown that this improved function with increased Akt activity may be an NO mediated phenomenon. Interestingly, the dysfunction exhibited by SA LEPCs was recreated in WE LEPCs by diminishing Akt expression using siRNA, strengthening the negative association between impaired Akt signalling and LEPC dysfunction.

Our SA cohort was relatively insulin resistant, an interesting finding, as insulin resistance is thought to be a key factor underlying the increased cardiovascular risk seen in this population. Perturbations in the PI3K/Akt signalling node are also thought to be central to the pathogenesis of insulin resistance. However, beyond making these observations, it is not possible to further attribute the SA LEPC dysfunction we have demonstrated to insulin resistance or to separate the impact of insulin resistance from ethnicity.

We have gone on to demonstrate, however, that insulin resistance *per se* does negatively impact vascular regeneration. We have presented data from an array of *in vivo* and *in vitro* experiments to support this conclusion. Insulin resistant IRKO mice have impaired limb reperfusion following critical limb ischaemia, compared with their WT littermates. This is associated with defective angiogenesis; capillary density was reduced in ischaemic muscle from the IRKO compared with WT. Moreover, we have demonstrated impaired *in vitro* angiogenesis and impaired functional responses to VEGF, such as, impaired migration. Mechanistically we have data to support a potential role for VEGF resistance; this is supported by PCR data from ischaemic muscle demonstrating increased VEGF expression in samples from the IRKO, which had not translated into superior perfusion or capillary density compared with the WT. Further support is provided by preliminary data from western blot studies that suggest impaired Akt and eNOS phosphorylation in response to VEGF exposure in IRKO PECs.

A critical point to be highlighted from this thesis is that, to our knowledge, our data is the first to demonstrate that *ex vivo* manipulation of LEPCs, taken from a group at high risk of CVD, can restore their function when used as a cell based therapy. This is an important proof of principle, as manipulation of cells prior to administration as a therapeutic tool, is likely to be necessary if cell based techniques for CVD are to enter the clinical arena in the future.

This thesis also presents novel insight into the effects of insulin resistance on vascular regeneration. Although there is work to be done before these

findings are of direct benefit to patients with insulin resistant syndromes, our findings along with future research will help lead to the identification of therapeutic targets that could eventually aid many patients worldwide.

References

1. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. **27**(5): p. 1047-53.
2. Gu, K., C.C. Cowie, and M.I. Harris, *Mortality in adults with and without diabetes in a national cohort of the U.S. population, 1971-1993.* Diabetes Care, 1998. **21**(7): p. 1138-45.
3. Seshasai, S.R., et al., *Diabetes mellitus, fasting glucose, and risk of cause-specific death.* N Engl J Med, 2011. **364**(9): p. 829-41.
4. Booth, G.L., et al., *Relation between age and cardiovascular disease in men and women with diabetes compared with non-diabetic people: a population-based retrospective cohort study.* Lancet, 2006. **368**(9529): p. 29-36.
5. Cubbon, R.M., et al., *Temporal trends in mortality of patients with diabetes mellitus suffering acute myocardial infarction: a comparison of over 3000 patients between 1995 and 2003.* Eur Heart J, 2007. **28**(5): p. 540-5.
6. Barr, E.L., et al., *Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab).* Circulation, 2007. **116**(2): p. 151-7.
7. Ovbiagele, B., D. Markovic, and G.C. Fonarow, *Recent US Patterns and Predictors of Prevalent Diabetes among Acute Myocardial Infarction Patients.* Cardiol Res Pract, 2011. **7**(145615): p. 145615.

8. *Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria.* Arch Intern Med, 2001. **161**(3): p. 397-405.

9. Cubbon, R.M., et al., *Diabetes mellitus is associated with adverse prognosis in chronic heart failure of ischaemic and non-ischaemic aetiology.* Diab Vasc Dis Res, 2013. **10**(4): p. 330-6.

10. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.

11. Balletshofer, B.M., et al., *Endothelial dysfunction is detectable in young normotensive first-degree relatives of subjects with type 2 diabetes in association with insulin resistance.* Circulation, 2000. **101**(15): p. 1780-4.

12. Williams, I.L., et al., *Effect of fat distribution on endothelial-dependent and endothelial-independent vasodilatation in healthy humans.* Diabetes Obes Metab, 2006. **8**(3): p. 296-301.

13. Kim, J.A., et al., *Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms.* Circulation, 2006. **113**(15): p. 1888-904.

14. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases.* Cell, 2010. **141**(7): p. 1117-34.

15. Shaw, L.M., *The insulin receptor substrate (IRS) proteins: at the intersection of metabolism and cancer.* Cell Cycle, 2011. **10**(11): p. 1750-6.

16. Bevan, P., *Insulin signalling.* J Cell Sci, 2001. **114**(Pt 8): p. 1429-30.

17. Kim, J.K., et al., *Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7522-7.

18. Itani, S.I., et al., *Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha.* Diabetes, 2002. **51**(7): p. 2005-11.

19. Itani, S.I., et al., *Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity.* Diabetes, 2000. **49**(8): p. 1353-8.

20. Yu, C., et al., *Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle.* J Biol Chem, 2002. **277**(52): p. 50230-6.

21. Straczkowski, M., et al., *Increased skeletal muscle ceramide level in men at risk of developing type 2 diabetes.* Diabetologia, 2007. **50**(11): p. 2366-73.

22. Moro, C., et al., *Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals.* J Clin Endocrinol Metab, 2009. **94**(9): p. 3440-7.

23. Furukawa, S., et al., *Increased oxidative stress in obesity and its impact on metabolic syndrome.* J Clin Invest, 2004. **114**(12): p. 1752-61.

24. Gao, Z., et al., *Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex.* J Biol Chem, 2002. **277**(50): p. 48115-21.

25. Hirosumi, J., et al., *A central role for JNK in obesity and insulin resistance.* Nature, 2002. **420**(6913): p. 333-6.

26. Nishikawa, T., et al., *Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage.* Nature, 2000. **404**(6779): p. 787-90.

27. Kim, J.K., *Endothelial nuclear factor kappaB in obesity and aging: is endothelial nuclear factor kappaB a master regulator of inflammation and insulin resistance?* Circulation, 2012. **125**(9): p. 1081-3.

28. Miele, C., et al., *Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism.* J Biol Chem, 2003. **278**(48): p. 47376-87.

29. Wautier, M.P., et al., *Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE.* Am J Physiol Endocrinol Metab, 2001. **280**(5): p. E685-94.

30. Hirashima, Y., et al., *Insulin down-regulates insulin receptor substrate-2 expression through the phosphatidylinositol 3-kinase/Akt pathway.* J Endocrinol, 2003. **179**(2): p. 253-66.

31. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.* Nature, 1997. **389**(6651): p. 610-4.

32. Hoehn, K.L., et al., *IRS1-independent defects define major nodes of insulin resistance.* Cell Metab, 2008. **7**(5): p. 421-33.

33. Hoehn, K.L., et al., *Insulin resistance is a cellular antioxidant defense mechanism.* Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17787-92.

34. Swift, M.R. and B.M. Weinstein, *Arterial-venous specification during development.* Circ Res, 2009. **104**(5): p. 576-88.

35. Shi, Q., et al., *Proof of fallout endothelialization of impervious Dacron grafts in the aorta and inferior vena cava of the dog.* J Vasc Surg, 1994. **20**(4): p. 546-56.

36. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.

37. Hagensen, M.K., et al., *Circulating endothelial progenitor cells do not contribute to plaque endothelium in murine atherosclerosis.* Circulation, 2010. **121**(7): p. 898-905.

38. Hagensen, M.K., et al., *Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury.* Cardiovasc Res, 2012. **93**(2): p. 223-31.

39. Hagensen, M.K., et al., *Flanking recipient vasculature, not circulating progenitor cells, contributes to endothelium and smooth muscle in murine allograft vasculopathy.* Arterioscler Thromb Vasc Biol, 2011. **31**(4): p. 808-13.

40. Potente, M., H. Gerhardt, and P. Carmeliet, *Basic and therapeutic aspects of angiogenesis.* Cell, 2011. **146**(6): p. 873-87.

41. Eble, J.A. and S. Niland, *The extracellular matrix of blood vessels.* Curr Pharm Des, 2009. **15**(12): p. 1385-400.

42. Majmundar, A.J., W.J. Wong, and M.C. Simon, *Hypoxia-inducible factors and the response to hypoxic stress.* Mol Cell, 2010. **40**(2): p. 294-309.

43. Thurston, G., I. Noguera-Troise, and G.D. Yancopoulos, *The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth.* Nat Rev Cancer, 2007. **7**(5): p. 327-31.

44. Phng, L.K. and H. Gerhardt, *Angiogenesis: a team effort coordinated by notch.* Dev Cell, 2009. **16**(2): p. 196-208.

45. Hellberg, C., A. Ostman, and C.H. Heldin, *PDGF and vessel maturation.* Recent Results Cancer Res, 2010. **180**: p. 103-14.

46. Lindahl, P., et al., *Pericyte loss and microaneurysm formation in PDGF-B-deficient mice.* Science, 1997. **277**(5323): p. 242-5.

47. Senger, D.R., et al., *Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid.* Science, 1983. **219**(4587): p. 983-5.

48. Olofsson, B., et al., *Vascular endothelial growth factor B, a novel growth factor for endothelial cells.* Proc Natl Acad Sci U S A, 1996. **93**(6): p. 2576-81.

49. Joukov, V., et al., *A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases.* Embo J, 1996. **15**(2): p. 290-98.

50. Achen, M.G., et al., *Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4).* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 548-53.

51. Maglione, D., et al., *Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9267-71.

52. Shweiki, D., et al., *Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis.* Nature, 1992. **359**(6398): p. 843-5.

53. Lee, S., et al., *Autocrine VEGF signaling is required for vascular homeostasis.* Cell, 2007. **130**(4): p. 691-703.

54. de Vries, C., et al., *The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor.* Science, 1992. **255**(5047): p. 989-91.

55. Quinn, T.P., et al., *Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium.* Proc Natl Acad Sci U S A, 1993. **90**(16): p. 7533-7.

56. Pajusola, K., et al., *FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines.* Cancer Res, 1992. **52**(20): p. 5738-43.

57. Olsson, A.K., et al., *VEGF receptor signalling - in control of vascular function.* Nat Rev Mol Cell Biol, 2006. **7**(5): p. 359-71.

58. Benedito, R., et al., *Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling.* Nature, 2012. **484**(7392): p. 110-4.

59. Leung, D.W., et al., *Vascular endothelial growth factor is a secreted angiogenic mitogen.* Science, 1989. **246**(4935): p. 1306-9.

60. Connolly, D.T., et al., *Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis.* J Clin Invest, 1989. **84**(5): p. 1470-8.

61. Ferrara, N. and W.J. Henzel, *Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells.* Biochem Biophys Res Commun, 1989. **161**(2): p. 851-8.

62. Keck, P.J., et al., *Vascular permeability factor, an endothelial cell mitogen related to PDGF.* Science, 1989. **246**(4935): p. 1309-12.

63. Lange, T., et al., *VEGF162, a new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells.* J Biol Chem, 2003. **278**(19): p. 17164-9.

64. Bates, D.O. and S.J. Harper, *Regulation of vascular permeability by vascular endothelial growth factors.* Vascul Pharmacol, 2002. **39**(4-5): p. 225-37.

65. Woolard, J., et al., *VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression.* Cancer Res, 2004. **64**(21): p. 7822-35.

66. Koch, S., et al., *Signal transduction by vascular endothelial growth factor receptors.* Biochem J, 2011. **437**(2): p. 169-83.

67. Fujisawa, H. and T. Kitsukawa, *Receptors for collapsin/semaphorins.* Curr Opin Neurobiol, 1998. **8**(5): p. 587-92.

68. Kitsukawa, T., et al., *Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs.* Development, 1995. **121**(12): p. 4309-18.

69. Yuan, L., et al., *Abnormal lymphatic vessel development in neuropilin 2 mutant mice.* Development, 2002. **129**(20): p. 4797-806.

70. Fong, G.H., et al., *Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium.* Nature, 1995. **376**(6535): p. 66-70.

71. Kappas, N.C., et al., *The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching.* J Cell Biol, 2008. **181**(5): p. 847-58.

72. Hiratsuka, S., et al., *Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis.* Cancer Res, 2001. **61**(3): p. 1207-13.

73. Beck, H., et al., *VEGFR-1 signaling regulates the homing of bone marrow-derived cells in a mouse stroke model.* J Neuropathol Exp Neurol, 2010. **69**(2): p. 168-75.

74. Shalaby, F., et al., *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.* Nature, 1995. **376**(6535): p. 62-6.

75. Sakurai, Y., et al., *Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice.* Proc Natl Acad Sci U S A, 2005. **102**(4): p. 1076-81.

76. Dumont, D.J., et al., *Cardiovascular failure in mouse embryos deficient in VEGF receptor-3.* Science, 1998. **282**(5390): p. 946-9.

77. Haiko, P., et al., *Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos.* Mol Cell Biol, 2008. **28**(15): p. 4843-50.

78. Foteinos, G., et al., *Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice.* Circulation, 2008. **117**(14): p. 1856-63.

79. Vasa, M., et al., *Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease.* Circulation, 2001. **103**(24): p. 2885-90.

80. Peichev, M., et al., *Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors.* Blood, 2000. **95**(3): p. 952-8.

81. Werner, N., et al., *Circulating endothelial progenitor cells and cardiovascular outcomes.* N Engl J Med, 2005. **353**(10): p. 999-1007.

82. Aicher, A., et al., *Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells.* Nat Med, 2003. **9**(11): p. 1370-6.

83. Heissig, B., et al., *Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand.* Cell, 2002. **109**(5): p. 625-37.

84. George, A.L., et al., *Endothelial progenitor cell biology in disease and tissue regeneration.* J Hematol Oncol, 2011. **4**(24): p. 1756-8722.

85. Ii, M., et al., *Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity.* Circulation, 2005. **111**(9): p. 1114-20.

86. Iwakura, A., et al., *Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived*

endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. Circulation, 2006. **113**(12): p. 1605-14.

87. Basile, D.P. and M.C. Yoder, *Circulating and tissue resident endothelial progenitor cells.* J Cell Physiol, 2014. **229**(1): p. 10-6.

88. Hur, J., et al., *Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis.* Arterioscler Thromb Vasc Biol, 2004. **24**(2): p. 288-93.

89. Prokopi, M. and M. Mayr, *Proteomics: a reality-check for putative stem cells.* Circ Res, 2011. **108**(4): p. 499-511.

90. Urbich, C., et al., *Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells.* Circulation, 2003. **108**(20): p. 2511-6.

91. Urbich, C., et al., *Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells.* J Mol Cell Cardiol, 2005. **39**(5): p. 733-42.

92. Hill, J.M., et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk.* N Engl J Med, 2003. **348**(7): p. 593-600.

93. Hirschi, K.K., D.A. Ingram, and M.C. Yoder, *Assessing identity, phenotype, and fate of endothelial progenitor cells.* Arterioscler Thromb Vasc Biol, 2008. **28**(9): p. 1584-95.

94. Rohde, E., et al., *Immune cells mimic the morphology of endothelial progenitor colonies in vitro.* Stem Cells, 2007. **25**(7): p. 1746-52.

95. Hur, J., et al., *Identification of a novel role of T cells in postnatal vasculogenesis: characterization of endothelial progenitor cell colonies.* Circulation, 2007. **116**(15): p. 1671-82.

96. Yoder, M.C., et al., *Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals.* Blood, 2007. **109**(5): p. 1801-9.

97. Ingram, D.A., et al., *Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood.* Blood, 2004. **104**(9): p. 2752-60.

98. Brunt, K.R., et al., *Ex vivo Akt/HO-1 gene therapy to human endothelial progenitor cells enhances myocardial infarction recovery.* Cell Transplant, 2012. **21**(7): p. 1443-61.

99. Tura, O., et al., *Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow.* Stem Cells, 2013. **31**(2): p. 338-48.

100. Galeano, M., et al., *Raxofelast, a hydrophilic vitamin E-like antioxidant, stimulates wound healing in genetically diabetic mice.* Surgery, 2001. **129**(4): p. 467-77.

101. Sarkar, K., et al., *Adenoviral transfer of HIF-1alpha enhances vascular responses to critical limb ischemia in diabetic mice.* Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18769-74.

102. Abaci, A., et al., *Effect of diabetes mellitus on formation of coronary collateral vessels.* Circulation, 1999. **99**(17): p. 2239-42.

103. Desouza, C.V., *Does drug therapy reverse endothelial progenitor cell dysfunction in diabetes?* J Diabetes Complications, 2013. **25**(13): p. 00075-5.

104. Alberti, K.G., P. Zimmet, and J. Shaw, *The metabolic syndrome--a new worldwide definition.* Lancet, 2005. **366**(9491): p. 1059-62.

105. Yao, E.H., et al., *Losartan improves the impaired function of endothelial progenitor cells in hypertension via an antioxidant effect.* Hypertens Res, 2007. **30**(11): p. 1119-28.

106. Suzuki, R., et al., *Effects of an Angiotensin II Receptor Blocker on the Impaired Function of Endothelial Progenitor Cells in Patients With Essential Hypertension.* Am J Hypertens, 2013. **7**: p. 7.

107. Liu, X., et al., *Lacidipine improves endothelial repair capacity of endothelial progenitor cells from patients with essential hypertension.* Int J Cardiol, 2013. **168**(4): p. 3317-26.

108. Chen, D.D., et al., *Endothelin 1 activation of endothelin A receptor/NADPH oxidase pathway and diminished antioxidants critically contribute to endothelial progenitor cell reduction and dysfunction in salt-sensitive hypertension.* Hypertension, 2012. **59**(5): p. 1037-43.

109. Michel, F., et al., *Aldosterone enhances ischemia-induced neovascularization through angiotensin II-dependent pathway.* Circulation, 2004. **109**(16): p. 1933-7.

110. Kolovou, G.D., K.K. Anagnostopoulou, and D.V. Cokkinos, *Pathophysiology of dyslipidaemia in the metabolic syndrome.* Postgrad Med J, 2005. **81**(956): p. 358-66.

111. Pellegatta, F., et al., *In vitro isolation of circulating endothelial progenitor cells is related to the high density lipoprotein plasma levels.* Int J Mol Med, 2006. **17**(2): p. 203-8.

112. Ma, F.X., et al., *Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase.* J Lipid Res, 2006. **47**(6): p. 1227-37.

113. Imanishi, T., et al., *Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction.* Clin Exp Pharmacol Physiol, 2004. **31**(7): p. 407-13.

114. Van Belle, E., et al., *Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines.* Circulation, 1997. **96**(8): p. 2667-74.

115. Couffinhal, T., et al., *Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE-/- mice.* Circulation, 1999. **99**(24): p. 3188-98.

116. Haddad, P., et al., *Nox2-derived reactive oxygen species contribute to hypercholesterolemia-induced inhibition of neovascularization: effects on endothelial progenitor cells and mature endothelial cells.* Atherosclerosis, 2011. **217**(2): p. 340-9.

117. Noor, R., et al., *High-density lipoprotein cholesterol regulates endothelial progenitor cells by increasing eNOS and preventing apoptosis.* Atherosclerosis, 2007. **192**(1): p. 92-9.

118. Petoumenos, V., G. Nickenig, and N. Werner, *High-density lipoprotein exerts vasculoprotection via endothelial progenitor cells.* J Cell Mol Med, 2009. **13**(11-12): p. 4623-35.

119. Sumi, M., et al., *Reconstituted high-density lipoprotein stimulates differentiation of endothelial progenitor cells and enhances ischemia-induced angiogenesis.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 813-8.

120. Huang, C.Y., et al., *Moderate to high concentrations of high-density lipoprotein from healthy subjects paradoxically impair human endothelial progenitor cells and related angiogenesis by activating Rho-associated kinase pathways.* Arterioscler Thromb Vasc Biol, 2012. **32**(10): p. 2405-17.

121. Romeo, G.R., J. Lee, and S.E. Shoelson, *Metabolic syndrome, insulin resistance, and roles of inflammation--mechanisms and therapeutic targets.* Arterioscler Thromb Vasc Biol, 2012. **32**(8): p. 1771-6.

122. Kim, Y.W., X.Z. West, and T.V. Byzova, *Inflammation and oxidative stress in angiogenesis and vascular disease.* J Mol Med, 2013. **91**(3): p. 323-8.

123. Tousoulis, D., et al., *Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: therapeutic implications for cardiovascular diseases.* Atherosclerosis, 2008. **201**(2): p. 236-47.

124. Shintani, S., et al., *Mobilization of endothelial progenitor cells in patients with acute myocardial infarction.* Circulation, 2001. **103**(23): p. 2776-9.

125. Seeger, F.H., et al., *p38 mitogen-activated protein kinase downregulates endothelial progenitor cells.* Circulation, 2005. **111**(9): p. 1184-91.

126. Fujii, H., et al., *C-reactive protein alters antioxidant defenses and promotes apoptosis in endothelial progenitor cells.* Arterioscler Thromb Vasc Biol, 2006. **26**(11): p. 2476-82.

127. Valgimigli, M., et al., *CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure.* Circulation, 2004. **110**(10): p. 1209-12.

128. Grisar, J., et al., *Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis.* Circulation, 2005. **111**(2): p. 204-11.

129. Deng, Y. and P.E. Scherer, *Adipokines as novel biomarkers and regulators of the metabolic syndrome.* Ann N Y Acad Sci, 2010. **10**(05875).

130. Landskroner-Eiger, S., et al., *Proangiogenic contribution of adiponectin toward mammary tumor growth in vivo.* Clin Cancer Res, 2009. **15**(10): p. 3265-76.

131. Shibata, R., et al., *Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of amp-activated protein kinase signaling.* J Biol Chem, 2004. **279**(27): p. 28670-4.

132. Ouchi, N., et al., *Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells.* J Biol Chem, 2004. **279**(2): p. 1304-9.

133. Shibata, R., et al., *Adiponectin promotes endothelial progenitor cell number and function.* FEBS Lett, 2008. **582**(11): p. 1607-12.

134. Nakamura, N., et al., *Adiponectin promotes migration activities of endothelial progenitor cells via Cdc42/Rac1.* FEBS Lett, 2009. **583**(15): p. 2457-63.

135. Wolk, R., et al., *Leptin receptor and functional effects of leptin in human endothelial progenitor cells.* Atherosclerosis, 2005. **183**(1): p. 131-9.

136. Schroeter, M.R., et al., *Leptin enhances the recruitment of endothelial progenitor cells into neointimal lesions after vascular injury by promoting integrin-mediated adhesion.* Circ Res, 2008. **103**(5): p. 536-44.

137. Bouloumie, A., et al., *Leptin, the product of Ob gene, promotes angiogenesis.* Circ Res, 1998. **83**(10): p. 1059-66.

138. Anagnostoulis, S., et al., *Human leptin induces angiogenesis in vivo.* Cytokine, 2008. **42**(3): p. 353-7.

139. Mu, H., et al., *Adipokine resistin promotes in vitro angiogenesis of human endothelial cells.* Cardiovasc Res, 2006. **70**(1): p. 146-57.

140. Robertson, S.A., C.J. Rae, and A. Graham, *Induction of angiogenesis by murine resistin: putative role of PI3-kinase and NO-dependent pathways.* Regul Pept, 2009. **152**(1-3): p. 41-7.

141. Sun, Y., et al., *Effect of visfatin on the function of endothelial progenitor cells in high-fat-fed obese rats and investigation of its mechanism of action.* Int J Mol Med, 2012. **30**(3): p. 622-8.

142. Kim, S.R., et al., *Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2.* Biochem Biophys Res Commun, 2007. **357**(1): p. 150-6.

143. Lovren, F., et al., *Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis.* Am J Physiol Endocrinol Metab, 2009. **296**(6): p. 7.

144. Loomans, C.J., et al., *Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes.* Diabetes, 2004. **53**(1): p. 195-9.

145. Tepper, O.M., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-6.

146. Chen, Y.H., et al., *High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms.* Diabetes, 2007. **56**(6): p. 1559-68.

147. Yoon, C.H., et al., *High glucose-induced jagged 1 in endothelial cells disturbs notch signaling for angiogenesis: A novel mechanism of diabetic vasculopathy.* J Mol Cell Cardiol, 2013. **17**(13): p. 00355-6.

148. Salis, M.B., et al., *Nerve growth factor supplementation reverses the impairment, induced by Type 1 diabetes, of hindlimb post-ischaemic recovery in mice.* Diabetologia, 2004. **47**(6): p. 1055-63.

149. Ebrahimian, T.G., et al., *NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes.* Am J Pathol, 2006. **169**(2): p. 719-28.

150. Kobayashi, K., et al., *The db/db mouse, a model for diabetic dyslipidemia: molecular characterization and effects of Western diet feeding.* Metabolism, 2000. **49**(1): p. 22-31.

151. Yan, J., et al., *Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: roles of endothelial nitric oxide synthase and endothelial progenitor cells.* J Vasc Surg, 2009. **50**(6): p. 1412-22.

152. Sawada, N., et al., *Endothelial PGC-1alpha Mediates Vascular Dysfunction in Diabetes.* Cell Metab, 2014. **19**(2): p. 246-58.

153. Warren, C.M., et al., *A Ligand-Independent VEGFR2 Signaling Pathway Limits Angiogenic Responses in Diabetes.* Sci Signal, 2014. **7**(307): p. 2004235.

154. Wheatcroft, S.B., et al., *Preserved glucoregulation but attenuation of the vascular actions of insulin in mice heterozygous for knockout of the insulin receptor.* Diabetes, 2004. **53**(10): p. 2645-52.

155. Kahn, M.B., et al., *Insulin resistance impairs circulating angiogenic progenitor cell function and delays endothelial regeneration.* Diabetes, 2011. **60**(4): p. 1295-303.

156. R M Cubbon, et al., *A Gene-based restoration of Akt activity in endothelial progenitor cells from human subjects at high cardiovascular risk rescues vascular reparative capacity.* Heart, 2012. **98**(Suppl 1): p. A1.

157. Kondo, T., et al., *Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization.* J Clin Invest, 2003. **111**(12): p. 1835-42.

158. He, Z., et al., *Regulation of vascular endothelial growth factor expression and vascularization in the myocardium by insulin receptor*

and PI3K/Akt pathways in insulin resistance and ischemia. Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 787-93.

159. Jiang, B.H., et al., *Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells.* Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1749-53.
160. Chavakis, E., et al., *Phosphatidylinositol-3-kinase-gamma is integral to homing functions of progenitor cells.* Circ Res, 2008. **102**(8): p. 942-9.
161. Ackah, E., et al., *Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis.* J Clin Invest, 2005. **115**(8): p. 2119-27.
162. Schleicher, M., et al., *The Akt1-eNOS axis illustrates the specificity of kinase-substrate relationships in vivo.* Sci Signal, 2009. **2**(82): p. 2000343.
163. Cubbon, R.M., et al., *Human exercise-induced circulating progenitor cell mobilization is nitric oxide-dependent and is blunted in South Asian men.* Arterioscler Thromb Vasc Biol, 2010. **30**(4): p. 878-84.
164. Brieger, K., et al., *Reactive oxygen species: from health to disease.* Swiss Med Wkly, 2012. **17**(142): p. 13659.
165. Ceriello, A. and E. Motz, *Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited.* Arterioscler Thromb Vasc Biol, 2004. **24**(5): p. 816-23.
166. Ingram, D.A., et al., *Clonogenic endothelial progenitor cells are sensitive to oxidative stress.* Stem Cells, 2007. **25**(2): p. 297-304.

167. Yasuda, M., et al., *Stimulation of in vitro angiogenesis by hydrogen peroxide and the relation with ETS-1 in endothelial cells.* Life Sci, 1999. **64**(4): p. 249-58.

168. Garrido-Urbani, S., et al., *Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPARalpha mediated mechanism.* PLoS One, 2011. **6**(2): p. 0014665.

169. Tojo, T., et al., *Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia.* Circulation, 2005. **111**(18): p. 2347-55.

170. Schroder, K., et al., *Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase.* Circ Res, 2012. **110**(9): p. 1217-25.

171. Tuttle, J.L., et al., *Impaired collateral artery development in spontaneously hypertensive rats.* Microcirculation, 2002. **9**(5): p. 343-51.

172. Kobayashi, N., et al., *Effect of eplerenone on endothelial progenitor cells and oxidative stress in ischemic hindlimb.* Am J Hypertens, 2010. **23**(9): p. 1007-13.

173. Rocic, P., et al., *Optimal reactive oxygen species concentration and p38 MAP kinase are required for coronary collateral growth.* Am J Physiol Heart Circ Physiol, 2007. **292**(6): p. 16.

174. Kim, Y.W. and T.V. Byzova, *Oxidative stress in angiogenesis and vascular disease.* Blood, 2014. **123**(5): p. 625-31.

175. Ostman, A., et al., *Regulation of protein tyrosine phosphatases by reversible oxidation.* J Biochem, 2011. **150**(4): p. 345-56.

176. Waltenberger, J., J. Lange, and A. Kranz, *Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: A potential predictor for the individual capacity to develop collaterals.* Circulation, 2000. **102**(2): p. 185-90.

177. Kajiwara, H., et al., *A hypoxic inducible factor-1 alpha hybrid enhances collateral development and reduces vascular leakage in diabetic rats.* J Gene Med, 2009. **11**(5): p. 390-400.

178. Senthil, D., et al., *The type 2 vascular endothelial growth factor receptor recruits insulin receptor substrate-1 in its signalling pathway.* Biochem J, 2002. **368**(Pt 1): p. 49-56.

179. Al-Mahmood, S., et al., *Potent in vivo antiangiogenic effects of GS-101 (5'-TATCCGGAGGGCTCGCCATGCTGCT-3'), an antisense oligonucleotide preventing the expression of insulin receptor substrate-1.* J Pharmacol Exp Ther, 2009. **329**(2): p. 496-504.

180. Maeno, Y., et al., *Inhibition of insulin signaling in endothelial cells by protein kinase C-induced phosphorylation of p85 subunit of phosphatidylinositol 3-kinase (PI3K).* J Biol Chem, 2012. **287**(7): p. 4518-30.

181. Assmus, B., et al., *Transcoronary transplantation of progenitor cells after myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1222-32.

182. Assmus, B., et al., *Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI).* Circulation, 2002. **106**(24): p. 3009-17.

183. Wollert, K.C., et al., *Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial.* Lancet, 2004. **364**(9429): p. 141-8.

184. Leistner, D.M., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy.* Clin Res Cardiol, 2011. **100**(10): p. 925-34.

185. Schachinger, V., et al., *Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1210-21.

186. Lunde, K., et al., *Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1199-209.

187. Bolli, R., et al., *Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial.* Lancet, 2011. **378**(9806): p. 1847-57.

188. Malliaras, K., et al., *Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction).* J Am Coll Cardiol, 2014. **63**(2): p. 110-22.

189. Nowbar, A.N., et al., *Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis.* Bmj, 2014. **28**(348).

190. Leeper, N.J., A.L. Hunter, and J.P. Cooke, *Stem cell therapy for vascular regeneration: adult, embryonic, and induced pluripotent stem cells.* Circulation, 2010. **122**(5): p. 517-26.

191. Yoon, C.H., et al., *Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases.* Circulation, 2005. **112**(11): p. 1618-27.

192. Dubois, C., et al., *Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction.* J Am Coll Cardiol, 2010. **55**(20): p. 2232-43.

193. Burchard, E.G., et al., *The importance of race and ethnic background in biomedical research and clinical practice.* N Engl J Med, 2003. **348**(12): p. 1170-5.

194. Pais, P., et al., *Risk factors for acute myocardial infarction in Indians: a case-control study.* Lancet, 1996. **348**(9024): p. 358-63.

195. Wild, S. and P. McKeigue, *Cross sectional analysis of mortality by country of birth in England and Wales, 1970-92.* Bmj, 1997. **314**(7082): p. 705-10.

196. *Health survey for England: The health of minority ethnic groups.* London: The Stationary Office, 2004.

197. Enas, E.A., et al., *Coronary heart disease and its risk factors in first-generation immigrant Asian Indians to the United States of America.* Indian Heart J, 1996. **48**(4): p. 343-53.

198. Ahmad, N. and R. Bhopal, *Is coronary heart disease rising in India? A systematic review based on ECG defined coronary heart disease.* Heart, 2005. **91**(6): p. 719-25.

199. Gupta, M. and S. Brister, *Is South Asian ethnicity an independent cardiovascular risk factor?* Can J Cardiol, 2006. **22**(3): p. 193-7.

200. Silbiger, J.J., et al., *Coronary artery disease in South Asian immigrants living in New York City: angiographic findings and risk factor burdens.* Ethn Dis, 2013. **23**(3): p. 292-5.

201. Yusuf, S., et al., *Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study.* Lancet, 2004. **364**(9438): p. 937-52.

202. McKeigue, P.M., G.J. Miller, and M.G. Marmot, *Coronary heart disease in south Asians overseas: a review.* J Clin Epidemiol, 1989. **42**(7): p. 597-609.

203. England, H.S.f., *The Health of Minority Ethnic Groups.* 2004.

204. Kestila, P., et al., *Socioeconomic status, cardiovascular risk factors, and subclinical atherosclerosis in young adults: the cardiovascular risk in Young Finns Study.* Arterioscler Thromb Vasc Biol, 2012. **32**(3): p. 815-21.

205. Harding S, M.R., *Differencein mortality of migrants. health inequalities: decennial supplement.* London, the stationary office, 1997.

206. Britton, A., et al., *Does access to cardiac investigation and treatment contribute to social and ethnic differences in coronary heart disease? Whitehall II prospective cohort study.* Bmj, 2004. **329**(7461): p. 5.

207. Shaukat, N., et al., *First myocardial infarction in patients of Indian subcontinent and European origin: comparison of risk factors, management, and long term outcome.* Bmj, 1997. **314**(7081): p. 639-42.

208. Shaukat, N., D.P. de Bono, and J.K. Cruickshank, *Clinical features, risk factors, and referral delay in British patients of Indian and European origin with angina matched for age and extent of coronary atheroma.* Bmj, 1993. **307**(6906): p. 717-8.

209. Farooqi, A., et al., *Attitudes to lifestyle risk factors for coronary heart disease amongst South Asians in Leicester: a focus group study.* Fam Pract, 2000. **17**(4): p. 293-7.

210. Feder, G., et al., *Ethnic differences in invasive management of coronary disease: prospective cohort study of patients undergoing angiography.* Bmj, 2002. **324**(7336): p. 511-6.

211. Pinto, R.J., et al., *Coronary artery disease in premenopausal Indian women: risk factors and angiographic profile.* Indian Heart J, 1992. **44**(2): p. 99-101.

212. Bhopal, R., et al., *Heterogeneity of coronary heart disease risk factors in Indian, Pakistani, Bangladeshi, and European origin populations: cross sectional study.* Bmj, 1999. **319**(7204): p. 215-20.

213. Kulkarni, K.R., et al., *Increased prevalence of smaller and denser LDL particles in Asian Indians.* Arterioscler Thromb Vasc Biol, 1999. **19**(11): p. 2749-55.

214. Banerji, M.A., et al., *Body composition, visceral fat, leptin, and insulin resistance in Asian Indian men.* J Clin Endocrinol Metab, 1999. **84**(1): p. 137-44.

215. McKeigue, P.M., B. Shah, and M.G. Marmot, *Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians.* Lancet, 1991. **337**(8738): p. 382-6.

216. Barker, D.J., et al., *Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth.* Diabetologia, 1993. **36**(1): p. 62-7.

217. C, F., *Fetal and early life origins of cardiovascular disease in South Asians in The epidemic of coronary heart disease in south asians: causes and consequences.* 2011: p. 74-80.

218. McKeigue, P.M., et al., *Association of early-onset coronary heart disease in South Asian men with glucose intolerance and hyperinsulinemia.* Circulation, 1993. **87**(1): p. 152-61.

219. Thorne, S., et al., *Early endothelial dysfunction in adults at risk from atherosclerosis: different responses to L-arginine.* J Am Coll Cardiol, 1998. **32**(1): p. 110-6.

220. Chambers, J.C., et al., *Abnormalities of vascular endothelial function may contribute to increased coronary heart disease risk in UK Indian Asians.* Heart, 1999. **81**(5): p. 501-4.

221. Guzik, T.J., et al., *Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and*

endothelial nitric oxide synthase. Circulation, 2002. **105**(14): p. 1656-62.

222. Shepherd, P.R. and B.B. Kahn, *Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus.* N Engl J Med, 1999. **341**(4): p. 248-57.

223. Chaturvedi, N., P.M. McKeigue, and M.G. Marmot, *Relationship of glucose intolerance to coronary risk in Afro-Caribbeans compared with Europeans.* Diabetologia, 1994. **37**(8): p. 765-72.

224. Dhawan, J. and C.L. Bray, *Asian Indians, coronary artery disease, and physical exercise.* Heart, 1997. **78**(6): p. 550-4.

225. Murphy, C., et al., *Vascular dysfunction and reduced circulating endothelial progenitor cells in young healthy UK South Asian men.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 936-42.

226. Health, D.o., *United Kingdom Department of Health. A practical guide to ethnic monitoring*

in NHS and social care. Department of Health, London, UK, January 2012.

227. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-9.

228. Carpten, J.D., et al., *A transforming mutation in the pleckstrin homology domain of AKT1 in cancer.* Nature, 2007. **448**(7152): p. 439-44.

229. Demaison, C., et al., *High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector*

containing an internal spleen focus forming virus promoter. Hum Gene Ther, 2002. **13**(7): p. 803-13.

230. Accili, D., et al., *Early neonatal death in mice homozygous for a null allele of the insulin receptor gene.* Nat Genet, 1996. **12**(1): p. 106-9.
231. Kido, Y., et al., *Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2.* J Clin Invest, 2000. **105**(2): p. 199-205.
232. Sobczak, M., J. Dargatz, and M. Chrzanowska-Wodnicka, *Isolation and culture of pulmonary endothelial cells from neonatal mice.* J Vis Exp, 2010. **14**(46).
233. Danial, N.N. and S.J. Korsmeyer, *Cell death: critical control points.* Cell, 2004. **116**(2): p. 205-19.
234. Murohara, T., et al., *Nitric oxide synthase modulates angiogenesis in response to tissue ischemia.* J Clin Invest, 1998. **101**(11): p. 2567-78.
235. Timmermans, F., et al., *Endothelial progenitor cells: identity defined?* J Cell Mol Med, 2009. **13**(1): p. 87-102.
236. Cubbon, R.M., et al., *Restoring Akt1 activity in outgrowth endothelial cells from South Asian men rescues vascular reparative potential.* Stem Cells, 2014. **10**(10).
237. Mangialardi, G., et al., *Nitric oxide-donating statin improves multiple functions of circulating angiogenic cells.* Br J Pharmacol, 2011. **164**(2b): p. 570-83.
238. Baker, M., et al., *Use of the mouse aortic ring assay to study angiogenesis.* Nat Protoc, 2011. **7**(1): p. 89-104.

239. Mehra, V.C., et al., *Ceramide-activated phosphatase mediates fatty acid-induced endothelial VEGF resistance and impaired angiogenesis.* Am J Pathol, 2014. **184**(5): p. 1562-76.

240. Logan, A.C., et al., *Integrated self-inactivating lentiviral vectors produce full-length genomic transcripts competent for encapsidation and integration.* J Virol, 2004. **78**(16): p. 8421-36.

241. Miyoshi, H., et al., *Development of a self-inactivating lentivirus vector.* J Virol, 1998. **72**(10): p. 8150-7.

242. Wang, C.Y., et al., *Increased vascular senescence and impaired endothelial progenitor cell function mediated by mutation of circadian gene Per2.* Circulation, 2008. **118**(21): p. 2166-73.

243. Nishi, J., et al., *Vascular endothelial growth factor receptor-1 regulates postnatal angiogenesis through inhibition of the excessive activation of Akt.* Circ Res, 2008. **103**(3): p. 261-8.

244. Roy, H.K., et al., *AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis.* Carcinogenesis, 2002. **23**(1): p. 201-5.

245. Sun, M., et al., *AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells.* Am J Pathol, 2001. **159**(2): p. 431-7.

246. Liao, Y. and M.C. Hung, *Physiological regulation of Akt activity and stability.* Am J Transl Res, 2010. **2**(1): p. 19-42.

247. Liao, Y., et al., *Peptidyl-prolyl cis/trans isomerase Pin1 is critical for the regulation of PKB/Akt stability and activation phosphorylation.* Oncogene, 2009. **28**(26): p. 2436-45.

248. Finkel, T., C.X. Deng, and R. Mostoslavsky, *Recent progress in the biology and physiology of sirtuins.* Nature, 2009. **460**(7255): p. 587-91.

249. Sundaresan, N.R., et al., *The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy.* Sci Signal, 2011. **4**(182): p. 2001465.

250. Ljubicic, V., et al., *Resveratrol induces expression of the slow, oxidative phenotype in mdx mouse muscle together with enhanced activity of the SIRT1-PGC-1alpha axis.* Am J Physiol Cell Physiol, 2014. **23**: p. 23.

251. Duncan, E.R., et al., *Effect of endothelium-specific insulin resistance on endothelial function in vivo.* Diabetes, 2008. **57**(12): p. 3307-14.