Enhancing vascular endothelial repair in the setting of insulin resistance: effects of insulin-like growth factor binding protein-1

PhD Thesis

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The candidate confirms that the work submitted is his own, except where work which has formed 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.

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Review article which forms part of Chapter 1 of my thesis. I drafted the manuscript which was then edited by my co-author.

Kahn MB, Yuldasheva NY, Cubbon RM, Smith J, Rashid ST, Viswambharan H, Imrie H, Abbas A, Rajwani A, **Aziz A**, Baliga V, Sukumar P, Gage M, Kearney MT, Wheatcroft SB. Insulin resistance impairs circulating angiogenic progenitor cell function and delays endothelial regeneration. *Diabetes. 2011 Apr;60(4):1295-303. doi: 10.2337/db10-1080. Epub 2011 Feb 11.*

Rajwani A, Ezzat V, Smith J, Yuldasheva NY, Duncan ER, Gage M, Cubbon RM, Kahn MB, Imrie H, Abbas A, Viswambharan H, **Aziz A**, Sukumar P, Vidal-Puig A, Sethi JK, Xuan S, Shah AM, Grant PJ, Porter KE, Kearney MT, Wheatcroft SB. Increasing circulating IGFBP1 levels improves insulin sensitivity, promotes nitric oxide production, lowers blood pressure, and protects against atherosclerosis. *Diabetes. 2012 Apr;61(4):915-24. doi: 10.2337/db11-0963. Epub 2012 Feb 22.*

Original research publications resulting from the primary author's studies. I assisted in some experimental work. The results from these two papers have been included and form part of Chapter 1 of my thesis, with acknowledgement given to the primary authors.

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Abstract

Insulin resistance (IR) leads to the development of type 2 diabetes mellitus and predisposes to cardiovascular disease (CVD) through its link with endothelial dysfunction. Cardiovascular risk factors and iatrogenic damage lead to biochemical and structural injury to the endothelium. Endogenous repair mechanisms are in place to regenerate injured endothelium. Insulin resistance has recently been shown to impair endothelial repair. The endogenously produced circulating insulin-like growth factor binding protein-1 (IGFBP-1) is potentially protective in the vasculature by stimulating nitric oxide production and enhancing insulin signalling in the endothelium. Cross-sectional studies have shown an association between low IGFBP-1 levels and CVD. This raises the possibility of exploiting IGFBP-1 therapeutically to prevent CVD in patients with diabetes. This project investigated whether IGFBP-1 enhances vascular endothelial repair in insulin resistant mice *in vivo* and probed potential molecular mechanisms by examining the effects of IGFBP-1 on human endothelial cells (EC) and angiogenic progenitor cells (APCs) *in vitro*. Endothelial regeneration was enhanced following arterial endothelium-denuding injury in IRKO mice by over-expressing human IGFBP-1. This was not explained by altered abundance or function of APCs. Incubation with IGFBP-1 significantly enhanced the ability of human EC to adhere to and regenerate denuded human vein *ex vivo.* In EC, IR was mimicked by the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) which significantly inhibited EC migration and proliferation *in vitro*. Co-incubation with IGFBP-1 restored the migratory and proliferative capacity of EC. IGFBP-1 significantly increased FAK phosphorylation, induced rapid activation of RhoA, and increased expression of $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins in EC. These multifactorial effects of IGFBP-1 on EC responses and acceleration of endothelial regeneration in mice raise the possibility that manipulating IGFBP-1 could be a strategy to enhance endothelial repair in humans with IR.

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Glossary

AcLDL – Acetylated Low Density Lipoprotein

Akt – Protein kinase B

APC - Angiogenic Progenitor Cell

BH_4 – Tetrahydrobiopterin

BM – Bone marrow

BSA – Bovine serum albumin

CD – Cluster differentiation marker

CFU – Colony forming unit

CVD – Cardiovascular disease

CXCR4 – Chemokine X receptor-4 (SDF receptor)

DiI - 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EBM – Endothelial basal medium

EDTA – Ethylenediaminetetraacetic acid

ECGM – Endothelial cell growth medium

ELISA – Enzyme linked immuno-sorbent assay

eNOS – Endothelial nitric oxide synthase

FcR – Crystallisable fragment receptor

FITC – Fluorescein isothiocyanate

FACS – Fluorescence activated cell sorting

FCS – Foetal calf serum

GLUT – Glucose transporter

HCAEC – Human coronary artery endothelial cell

HDL – High-density-lipoprotein

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP – Horseradish peroxidase

HOMA-IR – Homeostasis model assessment of insulin resistance

HUVEC – Human umbilical vein endothelial cell

IGF-1 – Insulin-like growth factor-1

IGFBP – Insulin-like growth factor binding protein

IHD – Ischaemic Heart Disease

IR – Insulin resistance

IRKO – Insulin receptor knockout

KDR – Kinase domain receptor (or VEGF receptor-2)

L-NMMA – Levo-N-monomethyl arginine

MAPK – Mitogen activated protein kinase

MMP – Matrix metalloproteinase

NADPH – Nicotine adenine dinucleotide phosphate

NO – Nitric oxide

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PI3-K - Phosphatidyl Inositol-3 kinase

PBMC – Peripheral blood mononuclear cell

PE - Phycoerythrin

ROS – Reactive oxygen species

SDF-α – Stromal cell-derived factor-alpha

TAE - Tris-acetate-EDTA

TBS – Tris buffered saline

T2DM – Type 2 Diabetes Mellitus

TNF-α – Tumour necrosis factor alpha

VEGF – Vascular endothelial growth factor

V – Volt

W – Watt

WT – Wild-Type

1 Introduction

1.1 Type 2 Diabetes Mellitus and insulin resistance

Diabetes mellitus is a chronic multifactorial disease characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or both. Chronic hyperglycaemia is associated with long-term damage to vital organs such as the heart, blood vessels and kidneys, leading to cardiovascular disease (CVD) and chronic kidney disease. Two main types of diabetes mellitus are described, reflecting two distinct patho-physiological processes involved in diabetes development. Type 1 diabetes mellitus (T1DM) is characterised by autoimmune destruction of the β-cells of the pancreas leading to insulin deficiency; whereas type 2 diabetes mellitus (T2DM) is caused by abnormalities leading to resistance in the actions of insulin[1–3]. Other types of diabetes[4], for example gestational diabetes (occurring during pregnancy), neonatal diabetes and maturity onset of diabetes in the young[5] (MODY) – a hereditary form of diabetes, are much lower in prevalence.

The prevalence of T2DM, representing 90-95% of all cases of diabetes[6] is rising globally. It is estimated that 382 million people (4% of the world's population) are affected by diabetes currently and it is predicted that this will increase to 592 million people by 2035[6]. The management of patients with diabetes incurs a significant burden on healthcare resources around the world. In 2010-2011, the cost of T2DM in the UK was estimated to be approximately £21.8bn[7], which equated to 21.2% of the total NHS budget.

Insulin resistance (IR) is an imbalance of insulin-glucose homeostasis in which insulin's ability to stimulate glucose uptake in peripheral tissues (e.g. skeletal muscle and adipose tissue) or to inhibit hepatic gluconeogenesis is reduced[8]. IR is an important metabolic abnormality in obesity, metabolic syndrome and T2DM, where CVD is the most significant cause of morbidity and mortality. IR develops as a result of combined genetic, lifestyle and environmental factors. The most likely reason for the recent increase in the global prevalence of IR is the 'Westernisation' of the world, i.e. increased calorie intake with decreased physical exercise[9]. Diabetes develops

gradually over a number of years, in which the evolution of hyperglycaemia is preceded by a slowly progressive decline in insulin-sensitivity. The decline in insulin-sensitivity is associated with compensatory hyperinsulinaemia – a state which is often termed 'pre-diabetes'[10] (Figure 1.1) in which fasting blood glucose levels are maintained at near-normal concentrations for many years, with mild elevation of post-feed levels. Diabetes is diagnosed when relative deficiency in pancreatic insulin secretion allows hyperglycaemia to develop[11].

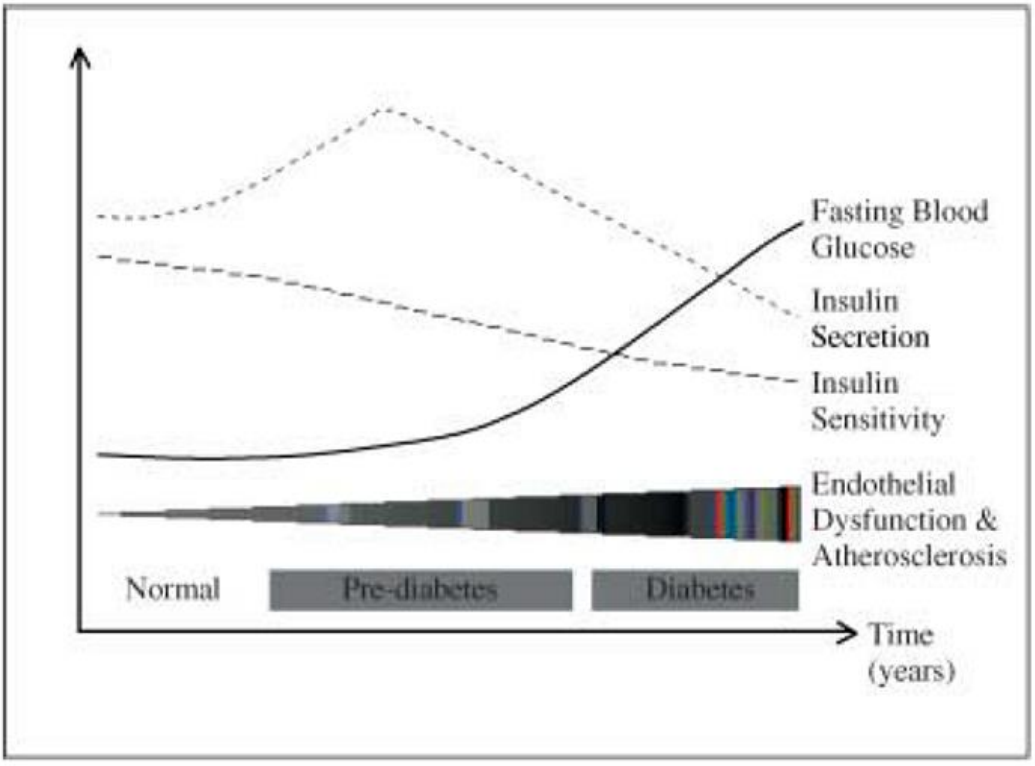


Figure 1-1 Representative diagram of the evolution of diabetes.
The pre-diabetes phase increases cardiovascular risk before diabetes is present clinically. Reproduced with permission from Wheatcroft *et al*, Diabetic Medicine, 20, 255-268.

1.1.1 Insulin resistance (metabolic) syndrome, T2DM and cardiovascular disease

Diabetes can affect multiple organs, however vascular complications predominate and can be broadly categorised as microvascular (e.g. retinopathy, neuropathy and nephropathy) or macrovascular (e.g. stroke, peripheral arterial disease and coronary heart disease). Macrovascular complications represent the most common cause of death in individuals with diabetes.

Insulin resistance (metabolic) syndrome was first described in 1988 by Reaven as "a cluster of risk factors for diabetes and cardiovascular disease"[10] comprising obesity (especially abdominal), hypertension, glucose intolerance, dyslipidaemia, hyperinsulinaemia, endothelial dysfunction with insulin resistance being at the core of the syndrome[12]. IR and the multiple components of the metabolic syndrome contribute to CVD development[10,13–15].

CVD represents the most common cause of morbidity and mortality in individuals with T2DM. The Framingham study was one of the earliest studies to demonstrate an increased risk of cardiovascular mortality in people with diabetes (mainly T2DM)[16]. Another observational study based in Finland showed that patients with T2DM, without a previous history of CVD have as high a risk of having a myocardial infarction as patients who had a previous myocardial infarction but had no history of T2DM[17].

T2DM is often preceded by a long period of IR, during which normal blood glucose levels are maintained by compensatory hyperinsulinaemia. During this period, subtle abnormalities of glucose regulation can arise - which are observed clinically as impaired glucose tolerance or impaired fasting glycaemia[6,12]. This asymptomatic pre-diabetic period of IR is linked with increased cardiovascular risk[18] and the population affected by pre-diabetes is increasing**.** In a recent population based cross-sectional study, the prevalence of pre-diabetes in adults in the UK increased dramatically from

11.6% in 2003 to 35.3% in 2011[19]. The San Antonio Heart Study observed 2569 individuals who were non-diabetic at baseline. Over 5 years of follow-up, 187 subjects experienced a cardiovascular event, with individuals in the highest quintile of IR having an approximately 2.5-fold increased incidence of CVD[20]. In the Botnia study, 3606 non-diabetic individuals were observed over a 7 year period. Metabolic syndrome increased the risk of CVD by threefold and metabolic syndrome was seen in 78% of women and 84% of men with T2DM[21]. A recent meta-analysis of 65 cohort/nested case-control studies showed that IR, measured by Homeostasis Model Assessment (HOMA), was a better predictor of cardiovascular events than fasting levels of glucose or insulin in adults without DM[22]. These studies suggest that diabetes leads to a CV mortality risk similar to that attributable to a prior diagnosis of coronary artery disease.

1.2 Endothelium

1.2.1 Endothelium in health

The vascular system is lined by a continuous monolayer of cells termed the endothelium (Figure 1.2) which provide a physical barrier between the vessel wall and lumen, but importantly play a major role in regulating vascular homeostasis. Endothelial cells (EC) secrete vasoactive substances which modulate vascular tone, vascular smooth muscle function (proliferation and migration), inflammatory cell adhesion, platelet aggregation, thrombosis and fibrinolysis[23,24]. The endothelium functions in paracrine, autocrine and endocrine manners. Vascular homeostasis is maintained through the secretion of vasodilators such as Nitric Oxide (NO) (a key anti-atherogenic molecule which will be discussed in more detail) and bradykinin and vasoconstrictors (endothelin-1, thromboxane A2, angiotensin II and certain reactive oxygen species)[23].

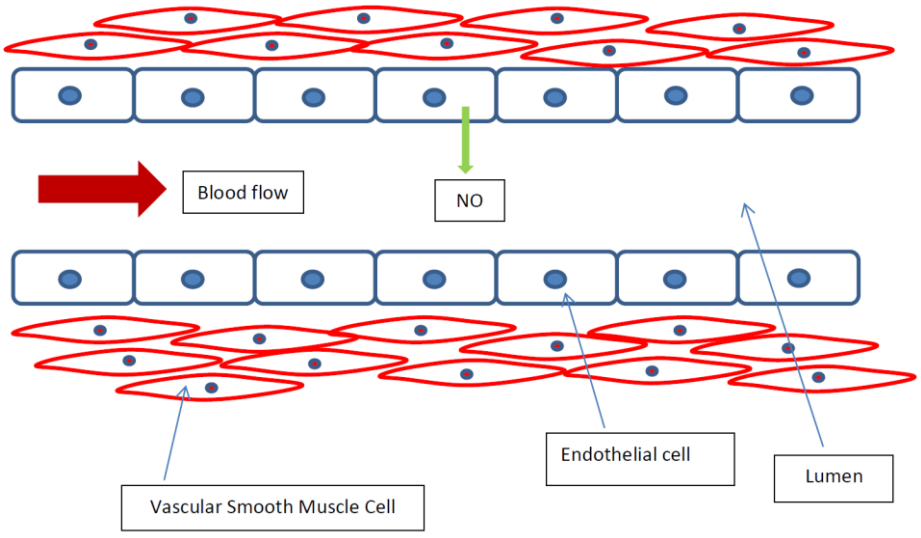


Figure 1-2 The endothelium in health.
Endothelial cells form a monolayer and secrete vasoactive molecules such as Nitric Oxide (NO) to maintain vascular homeostasis.

1.2.2 Endothelial dysfunction

Vascular homeostasis is maintained through secretion of vasoactive molecules such as NO and endothelin-1. Endothelial dysfunction occurs when there is an imbalance in the bioavailability of these vasoactive molecules. Reduced bioavailability of NO, in particular, promotes thrombosis, occlusion, vasospasm and increases adhesion molecule expression (e.g. E-selectin, P-selectin, vascular-cell adhesion molecule 1 and intercellular adhesion molecule 1)[25]. Endothelial dysfunction promotes uptake of inflammatory cells into the arterial wall and also leads to vascular smooth muscle cell proliferation and migration[26]. Not only does damaged endothelium disturb the balance of vasodilatory and vasoconstrictory substances, there is also an increase in inflammatory cytokine expression such as tumour necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6)[27].

Endothelial dysfunction commonly arises as a consequence of biochemical damage attributable to exposure to cardiovascular risk factors such as IR, smoking, hyperlipidaemia and hypertension. More importantly longitudinal studies have shown that endothelial dysfunction leads to impaired

endothelial repair, predisposes to atherosclerosis and is predictive of future cardiovascular disease[28–30].

Endothelial dysfunction can also occur through mechanical damage to EC. Physical trauma to the endothelium resulting in loss of endothelial integrity is an inevitable consequence of clinical revascularisation procedures (e.g. angioplasty, stent insertion and coronary artery bypass surgery). Loss of EC integrity is a potent driver for adverse vascular remodelling and leads to atherosclerosis, bypass graft failure, restenosis and stent thrombosis.

1.2.3 Nitric oxide and eNOS

Endothelium-Derived Relaxing Factor (EDRF) was discovered in the 1980s by the pioneering experiments of Furchgott and Zawadzki[31] and was later identified as nitric oxide (NO)[32,33]. Since the 1980s numerous studies exhibited the important roles NO plays in the human body[34,35]. NO is the major compound secreted by EC and is also the most widely studied. This anti-atherogenic signalling molecule possesses vasodilator, anti-platelet, anti-inflammatory and antioxidant properties[36] (Figure 1.3), these will be discussed in greater detail later.

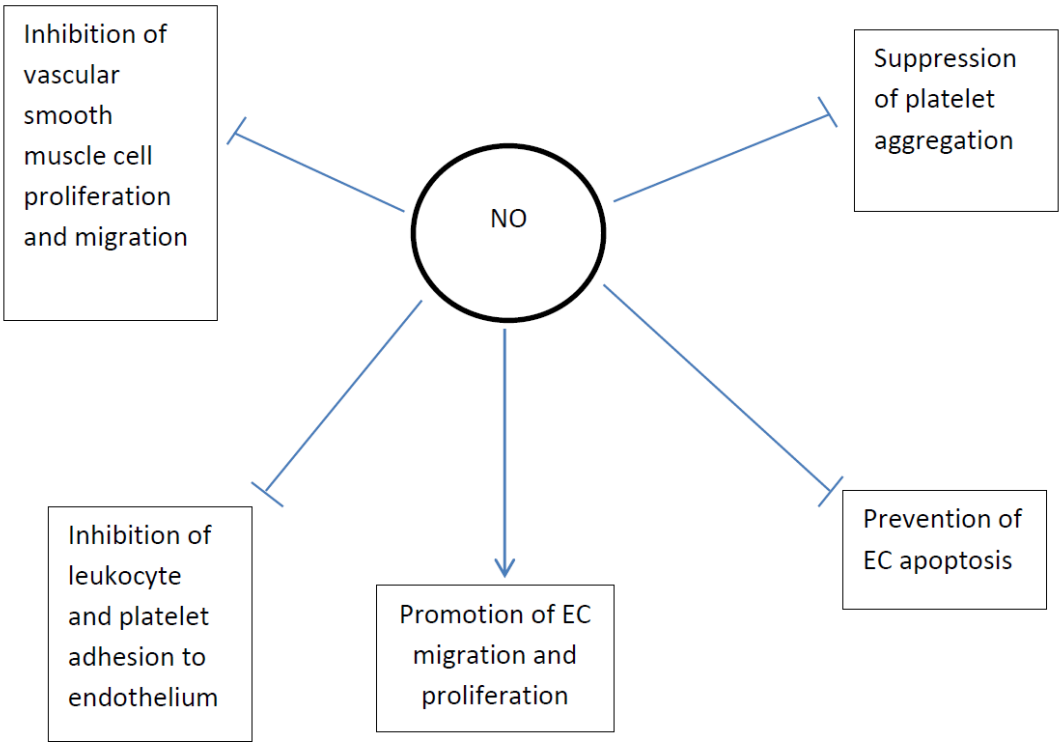


Figure 1-3 The vasculoprotective effects of Nitric Oxide.
NO inhibits platelet aggregation; prevents EC apoptosis whilst promoting EC migration and proliferation; inhibits leukocyte and platelet adhesion to the endothelium and finally inhibits vascular smooth muscle cell proliferation and migration.

ECs produce NO from L-arginine in a reaction catalysed by eNOS (endothelial isoform of nitric oxide synthase). NO diffuses locally within the vascular wall to regulate vascular tone, inflammatory cell adhesion, platelet aggregation and vascular smooth muscle cell proliferation[23]. Bioavailability of NO is dependent upon its rate of production and inactivation by binding to reactive oxygen species (ROS) such as superoxide[37].

The effects of NO on its target tissues are mediated through guanylate-cyclase-mediated increases in cyclic guanosine monophosphate. In vascular smooth muscle cells[27,38], this results in vascular smooth muscle relaxation and vasodilatation, which are often used as functional markers of the health of the endothelium.

NO is generated by three isoforms of its synthetic enzyme, NOS, namely neuronal (nNOS), inducible (iNOS) and endothelial (eNOS), all of which are expressed in the CV system and eNOS is the major isoform in ECs. eNOS expression was originally thought to be constitutive, however studies show that it's expression levels is regulated in response to mechanical stimulation, cytokines and growth[39].

eNOS is a bi-domain enzyme comprising a C-terminal reductase domain which binds nicotinamide adenine dinucleotide phosphate (NADPH), the flavins adenine dinucleotide (FAD) and mononucleotide (FMN); an N-terminal oxidase domain which binds heme, zinc, calmodulin and tetrahydrobiopterin (BH_4)[39]. BH_4, FAD and FMN are essential cofactors and calcium-loaded calmodulin stimulates enzyme activity and phosphorylation[40]. Also, where there is either L-arginine or BH_4 deficiency e.g. in diabetes, eNOS may produce superoxide, hence reduced NO bioavailability may partly be due to eNOS-mediated superoxide production[41,42].

1.2.4 Reactive oxygen species

Reactive oxygen species (ROS) are a natural by-product of oxygen metabolism. Examples of ROS include oxygen ions, peroxide, hydrogen peroxide and superoxide. ROS are classically generated at sites of inflammation and are a cause of oxidative stress. At high concentrations, ROS can cause cell injury and death. Generation of ROS, such as superoxide, plays a major role in endothelial dysfunction and is a risk factor in IR associated vasculopathy[43,44]. Superoxide reduces NO bioavailability by reacting quickly with NO to produce peroxynitrite, which itself may exert toxicity by nitrosylating cellular proteins[45,46]. Superoxide is also involved in the regulation of platelet aggregation[47], smooth muscle proliferation[48] and endothelial activation leading, for example, to monocyte attachment to the endothelium[8,49]. Potential sources of superoxide within the vascular system include lipoxygenases, xanthine oxidase, mitochondrial oxidases, cytochrome P450 and eNOS in the presence of low tetrahydrobiopterin

levels[43]. More recently, a major source of superoxide in blood vessels has been found to be a phagocyte-type NADPH oxidase[50,51], this plays an important part in endothelial dysfunction in the insulin resistant setting[52].

The effects of ROS on the endothelium are the result of its overproduction and/or a deficiency of antioxidant mechanisms, put simply “oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of pro-oxidant versus anti-oxidant reactions”[53].

1.3 Insulin and the endothelium

1.3.1 Insulin signalling and the endothelium

Insulin's actions are not limited to canonical insulin responsive tissues (liver, skeletal muscle and adipocytes). Indeed, the presence of Insulin receptors on ECs implies insulin may play an important role in endothelial cell function[12]. It has been elucidated there is a complete biochemical signalling pathway which links the insulin receptor to eNOS activation in the vascular endothelium. Insulin stimulates EC NO production through a similar signalling pathway to which it promotes glucose uptake in skeletal muscle[54–57]. The pathway (Figure 1.4) involves insulin receptor phosphorylation of Insulin Receptor Substrate-1 (IRS-1), which binds and activates Phosphatidyl Inositol-3 kinase (PI3-K), this leads on to phosphorylation of PDK-1/2 and then protein kinase B which finally leads to endothelial Nitric Oxide Synthase (eNOS) phosphorylation and NO production. Insulin also activates a separate pathway, the ras-mitogen activated protein kinase (MAPK) signalling cascade pathway which is pro-mitogenic. In this pathway, binding to the insulin receptor initiates a signalling pathway involving intermediaries (Shc, SOS, Ras and GRB2), which results in activation of MAPKs[58]. This is important for the mitogenic effects of insulin which include smooth muscle cell proliferation and migration, endothelial cell activation, stress responses and expression of endothelin, a potent vasoconstrictor.

IR results in reduced NO bioavailability due to selective inhibition of the PI3-kinase(PI3-k)/Akt signalling[59,60]. In contrast to this, the Mitogen-activated Protein Kinase (MAPK) pathway remains largely unaffected by IR, causing an imbalance in the activation states of both signalling pathways. Furthermore, because IR is accompanied by hyperinsulinaemia, insulin signalling leads to an increase in MAPK activity whilst the PI3-k/Akt pathway remains unresponsive. As a result enhancement of the mitogenic pathway promotes a pro-inflammatory and pro-atherogenic environment[61].

The multi-faceted insulin signalling pathway has multiple components at different levels which may be direct targets of IR. Experimental models have shown that defects in several components of the insulin signalling pathway can induce IR. However, the relative contribution of these models to cases of human IR are not fully clarified. Proposed theories include down-regulation of tyrosine phosphorylation of the insulin receptor, PI3-kinase, IRS proteins and reduced amount of these proteins[45,62,63].

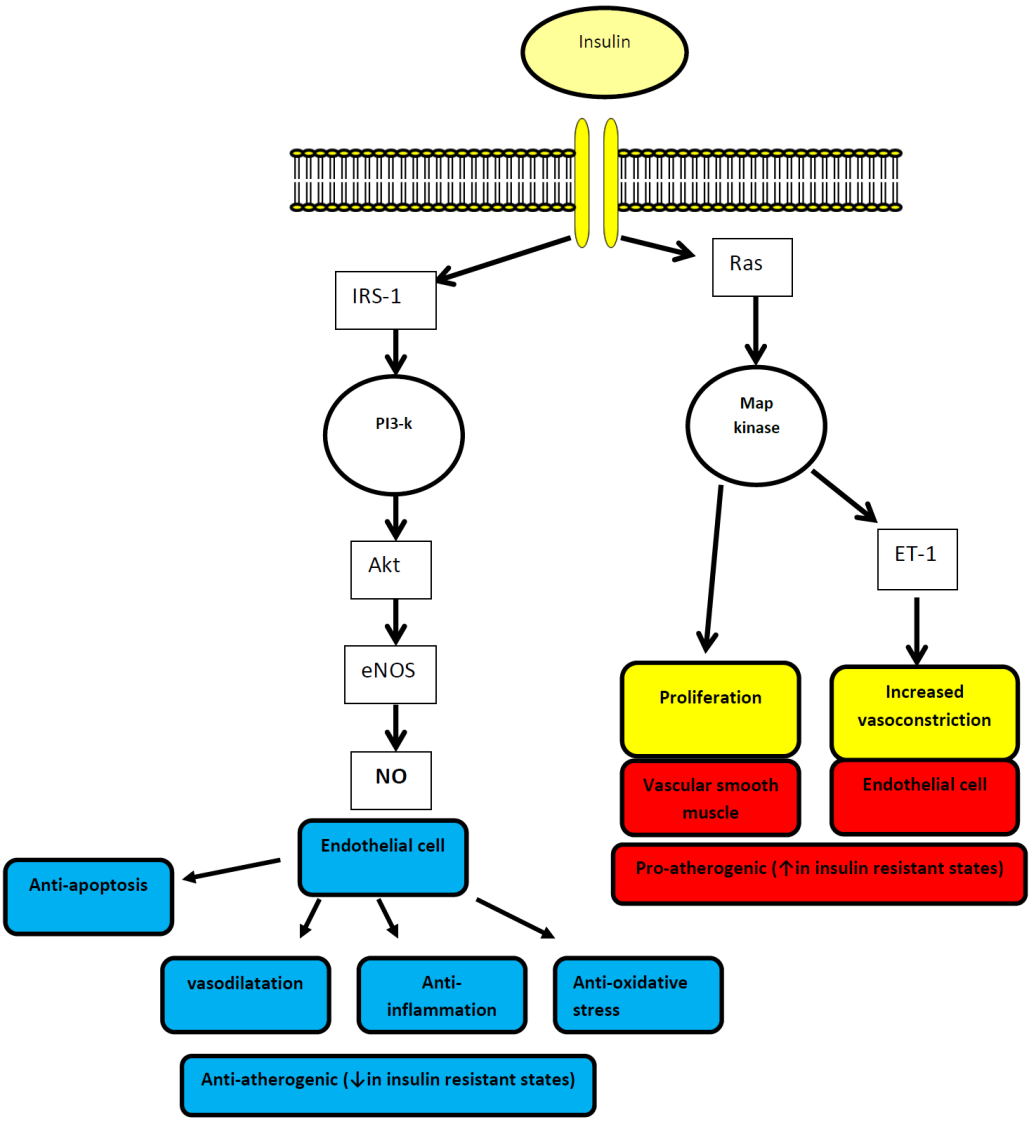


Figure 1-4 Schematic drawing of insulin signalling pathways in EC and VSMC.
Activation of either PI3-kinase or MAP kinase (MAPK) can mediate insulin's actions, with the former stimulating anti-atherogenic effects and the latter stimulating atherogenic actions. IR is associated with selective down regulation of the PI3-k pathway whereas MAP-kinase pathway is enhanced and therefore promotes atherosclerosis.

1.3.2 Insulin resistance and endothelial dysfunction

There is an important pathophysiological reciprocal relationship between IR and endothelial dysfunction which has been demonstrated in numerous studies[61,64,65], including those in subjects with T2DM[66] or obesity[64]. Mechanisms that contribute to IR and endothelial dysfunction include inflammation, lipotoxicity and glucotoxicity.

In healthy young men of South Asian origin, IR is associated with reduced NO bioavailability and decreased shear stress-induced NO production elicited by flow-mediated brachial artery dilatation[67]. The relationship of IR with endothelial dysfunction and other cardiovascular risk factors has led to the 'common soil' hypothesis, in which genetic factors may predispose individuals to the development of IR and CVD[68]. In addition to these genetic influences, a range of biochemical and signalling alterations have been discovered that are mechanistically implicated in the association between obesity, IR and endothelial dysfunction, which have been addressed by comprehensive research in models of IR (human and animal) and cell culture studies[69].

In animal models, it has been shown that disrupting insulin signalling leads to decreased PI3-k/Akt and phospho-eNOS activation which results in endothelial dysfunction, adverse vascular remodelling and atherosclerosis[70]. Mice with hemizygous deletion of insulin receptors and exhibit normal glucose levels but are hyperinsulinaemic have a significant reduction in NO bioavailability, hypertension and increased ROS production[71]. These findings are consistent with the concept of endothelial dysfunction being induced by IR. However the proposed mechanism that this is dependent upon down regulation of PI3K/Akt signalling has come under debate. One study used mice with genetic ablation of insulin receptors and Akt1 to investigate the mechanism of obesity-related endothelial dysfunction[72]. This study confirmed that obesity leads to endothelial dysfunction characterised by impaired insulin-induced eNOS phosphorylation, but this may be

attributable to free fatty acid inhibition on eNOS phosphorylation rather than defective upstream signalling via Akt[69].

A murine model with endothelial-specific expression of a dominant negative human insulin receptor (ESMIRO), in which glucoregulation is intact, demonstrated that endothelium-selective IR results in impaired NO release and increased ROS production[70,73]. Another study looking at a mouse model where insulin receptors were selectively deleted in ECs (VENIRKO) showed reduced eNOS expression and susceptibility to hypertension and atherosclerosis[74]. In summary, these studies provide evidence that even without any systemic effects of IR, IR in ECs is sufficient to promote endothelial dysfunction and damage with reduced NO bioavailability and increased ROS production.

Oxidative stress as a result of increased ROS generation is believed to play a role in linking IR, endothelial dysfunction and impaired endothelial regeneration. The enzymes most strongly linked with endothelial dysfunction associated with T2DM and obesity are NADPH oxidase and uncoupled eNOS. Normally eNOS produces NO, however if there is deficiency in the essential cofactor tetrahydrobiopterin, eNOS uncoupling results in superoxide generation[75].

T2DM is associated with reduced bioavailability of tetrahydrobiopterin, the diabetic vasculature is therefore exposed to the proatherosclerotic combination of reduced NO production and increased superoxide production by eNOS in EC[76]. Overall, there is convincing evidence that insulin resistance in endothelial cells contributes to endothelial dysfunction.

1.3.3 Unifying mechanisms underlying IR and endothelial dysfunction

As mentioned there is a reciprocal relationship between IR and endothelial dysfunction, however the question of whether they are directly linked with each other or represent manifestations of a common underlying pathology

remains uncertain[27]. This section will discuss potential mechanisms that link IR and endothelial dysfunction.

1.3.3.1 Intracellular signalling pathways

In IR, individuals exhibit resistance to the metabolic and to some of the vascular actions of insulin. As mentioned, the signalling pathways involved are similar for NO production and glucose uptake stimulated by insulin. Insulin binds to cell membrane insulin receptors which promotes GLUT4 translocation in skeletal muscle and increases eNOS activity in the endothelium through the PI-3-kinase/Akt signalling cascade[77]. In the insulin resistant setting and diabetes, insulin-mediated eNOS activation via PI-3-kinase/Akt is down-regulated, whilst MAPK pathway remains intact[60] which promotes endothelin production (a potent vasoconstrictor and mitogen)[78], vascular smooth muscle production[79] and to the expression of pro-inflammatory adhesion molecules e.g. ICAM-1[27], which may promote vascular disease[27,80].

Human studies support these mechanisms. In healthy humans, insulin stimulates vasodilatation and increases blood flow in peripheral tissues which is endothelial mediated, but this effect is reduced in patients with DM and IR[65,81]. Hyperinsulinaemia accompanies IR and is also an independent risk factor for CVD[82]. In the early phase of IR, there is a hyperinsulinaemic phase to maintain normal glucose levels. Montagnani *et al* designed a number of investigations mimicking metabolic IR by blocking the PI3-kinase pathway and exposing ECs to increasing levels of insulin. They found that PI3-kinase inhibition blocked insulin stimulated eNOS expression with no alteration to the MAP-kinase pathway and enhanced mitogenic activity of ECs[80].

1.3.3.2 Cytokines and inflammation

EC can be activated by pro-inflammatory molecules such as tumour necrosis factor-alpha (TNF-α) and C-reactive protein which promotes endothelial dysfunction and an atherogenic phenotype[83,84], this then promotes expression of adhesion molecules which accelerates atherosclerosis, and in turn leads to decreased eNOS expression and reduced NO bioactivity[85–87]. Adipose tissue is a major source of circulating pro-inflammatory cytokines such as interleukin-6[88] and TNF-α[89]. TNF-α expression is increased in muscle and adipose tissue[89] samples in insulin resistant humans. Cross-talk between adipose depots and blood vessels is mediated, at least in part, by TNF-α which reduces the bioavailability of NO in ECs and impairs endothelium-dependent dilatation in human and animal studies[76,90]. Other proposed molecular mechanisms for the detrimental effects of TNF-α on blood vessels include increased superoxide production by vascular NADPH oxidase and TNF-α-mediated decrease in the half-life of eNOS mRNA[91].

1.3.3.3 Oxidative stress

As mentioned earlier, oxidative stress plays a major role in the pathogenesis of endothelial dysfunction and has been shown to be associated with insulin resistant states in human and animal models[92,93]. Arterial tissue exposure to hyperglycaemia or increased free fatty acid concentrations induces superoxide production and impairs NO bioavailability in the endothelium[94,95]. In murine models with whole body or endothelium specific IR, Kearney *et al* demonstrated the presence of significant oxidative stress and reduced NO bioavailability[73,96]. NADPH oxidase and dysfunctional eNOS are seen as the major ROS sources in the vascular wall[42].

1.4 Endothelial damage and repair

Since ECs play an essential role in maintaining vascular health, when the endothelium is damaged, repair mechanisms are essential to institute timely and effective regeneration of functional endothelium. Endothelial damage occurs due to a number of reasons: exposure to risk factors leading to

endothelial dysfunction and endothelial cell apoptosis, mechanical loss of endothelial cells following injury such as coronary interventional procedures or coronary artery bypass grafting and following acute inflammation associated with sepsis[97].

Long-term exposure to vascular risk factors, e.g. IR, causes exhaustion of the endogenous anti-inflammatory systems within EC. Endothelial dysfunction ensues, along with loss of structural integrity, senescence, apoptosis and ultimately detachment of EC from the extracellular matrix into the circulation (Figure 1.5). Endothelial microparticles are derived from such cells and are used as a marker of EC apoptosis. Exposure to risk factors such as atherosclerotic disease, chronic inflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis leads to an increase in endothelial microparticle release, which in turn is evidence of continuing EC loss[29,98].

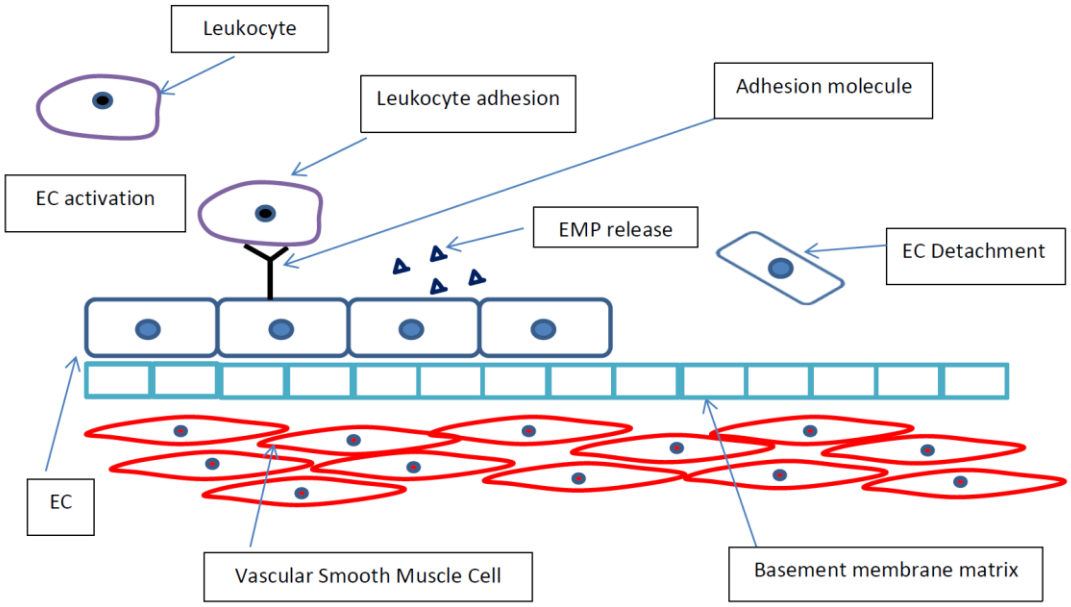


Figure 1-5 Endothelial damage mechanisms.
EC express adhesion molecules such as VCAM-1 or ICAM-1, this allows leukocytes to adhere, migrate through the EC and initiate an inflammatory cascade reaction and release of endothelial microparticles. ECs become senescent and detach from the basement membrane matrix.

Arterial wall injury following angioplasty, stent insertion and coronary artery bypass surgery leads to loss of ECs either through direct trauma, apoptosis

or necrosis. The endothelial cell loss triggers a number of local responses including vascular smooth muscle proliferation and migration, resulting in neointimal hyperplasia[99]. The exposed basement membrane matrix attracts leukocytes and platelets[100–102], predisposing to thrombosis. Accordingly, loss of EC integrity acts as a potent driver for a range of vascular pathologies including atherosclerosis[103], bypass graft failure[104], post-angioplasty restenosis[105], in-stent restenosis and stent thrombosis[106].

Release of chemotactic factors and mitogens from platelets occurs which affects VSMC. In addition, angioplasty causes stretching of the vessel and cell lysis of EC and VSMC, this then causes proliferation and migration of VSMC plus synthesis of the ECM and hence neo-intimal hyperplasia leading to restenosis especially post-stent insertion[100,107,108].

It has long been recognised that damaged endothelium is ‘repaired’ by the regeneration of the endothelial monolayer[109–111]. Endothelial repair (Figure 1.6) is believed to occur through a series of interactions between native ECs (migration and proliferation of EC to sites of damage)[110–112] and circulating bone-marrow derived angiogenic progenitor cells (APCs)[113,114] which are recruited to the diseased vessel. Studies conducted in the 1970s and early 1980s suggested a dominant role for EC mitosis and migration in mediating endothelial regeneration after vascular damage[110–112,115,116]. Following arterial injury[110,116–118] local endothelial cell migration and proliferation from adjacent non-injured endothelium has been highlighted as the central mechanism in endothelial regeneration.

Endothelial regrowth following arterial injury or angioplasty occurs through EC replicating to restore endothelial continuity. Migration and proliferation of ECs is initiated by loss of contact inhibition and growth factor secretion by vascular smooth muscle cells, ECs and APCs. Regeneration of the injured endothelium commonly starts from the leading edge of the denuded area[119] begins within 24 hours and ceases within 6-10 weeks. One study observed that the quicker the endothelium recovered following balloon injury, the less neo-intimal hyperplasia occurred[120]. Therefore, facilitating rapid endothelial

regeneration after injury would be expected to be helpful in the clinical setting as this would reduce the incidence of restenosis.

It has also been noted that following widespread denudation, EC are unable to re-endothelialise completely[121,122] and the regenerated endothelium shows abnormal morphology such as close cell-to-cell contacts, ECs not aligned with the direction of blood flow and the presence of polygonal shapes and irregular size[109,119,122–125].

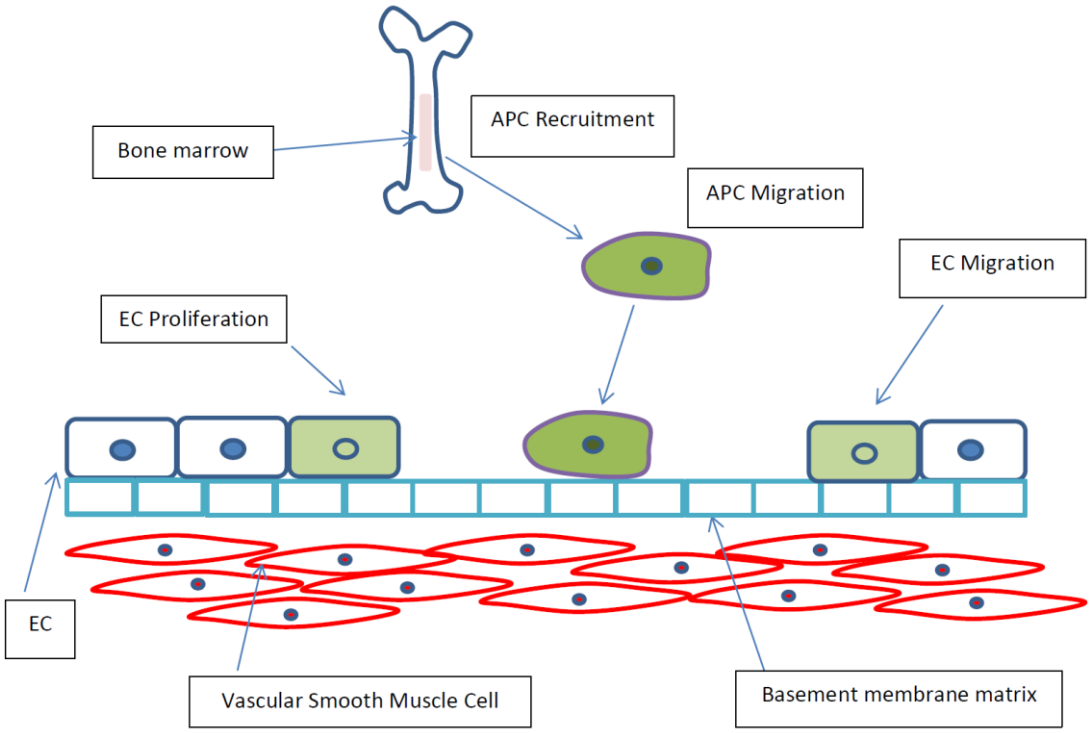


Figure 1-6 Endothelial repair.
Damaged and denuded endothelium leads to EC migration and proliferation, leading to the restoration of the endothelium. Angiogenic cytokines induce APC mobilisation from bone marrow and migrate to the damaged endothelium and integrate into the EC layer.

The release of growth factors from the vessel wall plays an important role in endothelial regeneration. One example is Fibroblast Growth Factor (FGF), which is formed and stored by ECs and VSMCs[126–129]. Angioplasty leads to FGF release from injured cells[130,131] and human studies have observed raised FGF levels in blood post-angioplasty. VEGF is also observed to have increased levels following human angioplasty. VEGF is synthesised by VSMC and binds exclusively to ECs[108,132]. Over-expression of VEGF in

mice led to accelerated endothelial repair and reduced neo-intimal formation following arterial wire injury[133]. In the following sections, aspects of molecular biology and cell biology pertinent to EC regeneration will be discussed in more detail before introducing the contribution of APCs to vascular repair.

1.4.1 Endothelial cell adhesion

Endothelial cell adhesion to the extracellular matrix (ECM) plays a critical role in regulating EC migration, proliferation and survival. Integrins and Rho GTPases are essential in mediating cellular responses downstream of ECM engagement[134].

Integrins are a family of cell adhesion molecules, which interact with the ECM or with adhesion molecules on other cells[135]. In all, 24 integrins have been identified and each is a heterodimer of an α and a β subunit[135]. Integrins exhibit both inside-out/outside-in (bidirectional) signalling, i.e. signals within the cell cause integrins to change which leads to integrin activation and therefore an increased affinity for extracellular ligands. In the opposite direction, integrins binding to their ligands can initiate change to their cytoplasmic domains and activate multiple signalling pathways[136]. An ECM protein such as fibronectin can engage several integrins. The most commonly expressed fibronectin-binding integrins are $\alpha_V\beta_3$ and $\alpha_5\beta_1$ - both bind to the Arg-Gly-Asp (RGD) tripeptide sequence within fibronectin[134]. The RGD sequence is the major cell attachment site for a large number of proteins and the majority of integrins recognise the RGD sequence[137].

Rho GTPases are a family of small (21 kDa) signalling G proteins. Rho GTPases play an essential role in transmitting integrin-mediated responses and by regulating the actin cytoskeleton dynamics, they provide a key-signalling link through which migration and adhesion are controlled[134]. The most studied Rho GTPases regulating cell adhesion are Rho, Rac and Cdc42[138] which will be discussed later.

1.4.2 Endothelial cell migration

EC migration is essential in the re-endothelialisation in arteries after injury, angioplasty and coronary artery bypass grafting. ECs migrate as a consequence of three principal mechanisms: chemotaxis, haptotaxis and mechanotaxis[139]. Chemotaxis is the migrational direction of cells toward a

gradient of soluble chemoattractants; haptotaxis is the migrational direction of cells toward a gradient of immobilised ligands; and mechanotaxis is the migrational direction of cells generated by mechanical forces[140]. Endothelial cell chemotaxis is typically driven by growth factors such as VEGF and basic fibroblast growth factor and haptotaxis is associated with increased EC migration activated in response to integrins binding to extra cellular matrix (ECM) component[139]. Due to the location of EC at the inner face of the vessel wall, they are continuously in contact with shear stress. This contributes to activate migratory pathways by initiating mechanotaxis and modulates migration steps which includes leading edge extension, adhesion to the matrix and adhesion release at the rear[139,140]. EC migration is a mechanically integrated process which involves fluid changes in signal transduction, cell adhesion and cytoskeletal organisation.

1.4.2.1 Role of focal adhesion kinases in endothelial cell migration and cell proliferation

A coordinated process is required for cell migration to occur that involves rapid changes in actin filament dynamics, together with the formation and disassembly of cell adhesion sites[141]. External stimuli that affects cell migration are transduced into intracellular biochemical signals through the interaction of transmembrane integrins that bind to the ECM. Integrin activation allows there to be a functional connection between focal adhesions and actin cytoskeleton that is needed for cell migration[139].

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is a converging signalling point between Rho and integrins and it controls the assembly/disassembly of focal adhesions that is required with actin polymerisation regulation for EC migration[142].

FAK was identified in 1992 in normal cells as a highly tyrosine-phosphorylated protein that localised to integrin-enriched cell adhesion sites known as focal adhesions[141]. Focal adhesions are formed at ECM-integrin junctions and bring together cytoskeletal and signalling proteins during the

processes of cell migration and adhesion. Early studies showed that FAK was activated by growth factors and phosphorylation of FAK is a rapid event associated with focal adhesion formation[143]. In keeping with a critical role for FAK in cell migration, it has been shown FAK-deficient cells spread more slowly on ECM, exhibited an increased number of prominent focal adhesions and migrated poorly in response to haptotactic and chemotactic signals[144–147].

FAK regulates cell migration through a number of pathways but in particular through the Rho subfamily of small GTPases and the assembly/disassembly of actin cytoskeleton[148]. FAK deletion in fibroblasts leads to increased RhoA activity, increased cell spreading and reduced cell migration – observations which can be rescued by FAK re-expression in these cells[149–151].

EC proliferation is one of the hallmarks of endothelial regeneration. However, these cells have a finite proliferative capacity. In vitro studies have shown that focal adhesion kinase appears to regulate not only cell migration, focal adhesion disassembly, and cytoskeletal rearrangements, but also endothelial cell proliferation in vivo and endothelial cell permeability[152–155].

1.4.2.1 Rho Family GTPase and endothelial cell migration

Over twenty Rho family proteins have been identified in humans[156]. The Rho family GTPases RhoA, Rac and Cdc42 are most studied and modulate EC migration by regulating actin cytoskeleton rearrangement[140,157] (Figure 1.7). These members act as ‘molecular switches,’ cycling between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state to activate the GTPase[158]. RhoA induces the formation of actin stress fibres and focal adhesions and stimulates cell contraction through downstream effectors ROCK and mDia. Rho-kinase (Rho-associated coiled-coil-containing protein kinase {ROCK}) is an effector of GTPase RhoA[159] whilst Rac and Cdc42 regulate the polymerisation of actin to form peripheral lamellipodial and filopodial protrusions[158].

Cdc42 and Rac operate at the leading edge of the migrating cell. Cdc42 generates filopodia through inducing actin polymerisation. Rac generates a protrusive force through localised actin polymerisation[160].

RhoA activity is associated with focal adhesion assembly, cell contractility and rear end retraction. The Rho target ROCK, which stimulates actin:myosin filament assembly, is essential for rear cell detachment[158].

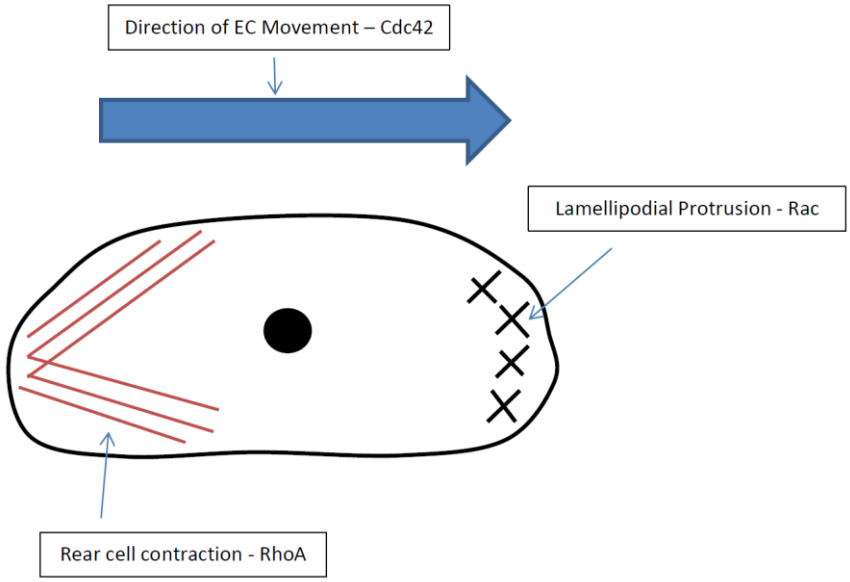


Figure 1-7 A migrating endothelial cell.
Migrating endothelial cells perform coordinated steps in order to move. Cdc42 regulates cell direction. Rac induces lamellipodial protrusion at the front of the cell, this is through actin polymerization stimulation and integrin/focal adhesion complexes. Finally RhoA induces actin/myosin contraction at the rear of the cell.

1.4.2.2 Actin Remodelling

Actin, composed of 43-kDa monomeric subunits (G-actin) that polymerise into filaments (F-actin), is the major cytoskeletal component of ECs. Remodelling of the actin cytoskeleton into filopodia, lamellipodia and stress fibres is essential for cell migration. Filopodia contain long parallel actin filaments that are arranged in tight bundles and are membrane projections which act as sensors of motile stimuli. Lamellipodia are cytoplasmic protrusions which form at the leading edge of migrating cells[161]. Stress fibres are actin filaments linked by α-actinin and myosin and distributed along contractile fibres[162], they are also linked to the cell membrane at focal adhesions. These three constructions are vital to drive the steps involved in actin-based endothelial cell motility.

The steps involved in endothelial cell migration (Figure 1.8) are

1. Sensing of the motogenic signal by filopodia
2. Formation and protrusion of lamellipodia and pseudopodia-like forward extension
3. Attachment of the protrusions to the ECM
4. Stress fibre-mediated contraction of the cell body to allow forward progress
5. Rear release
6. Recycling of adhesive and signalling molecules[139].

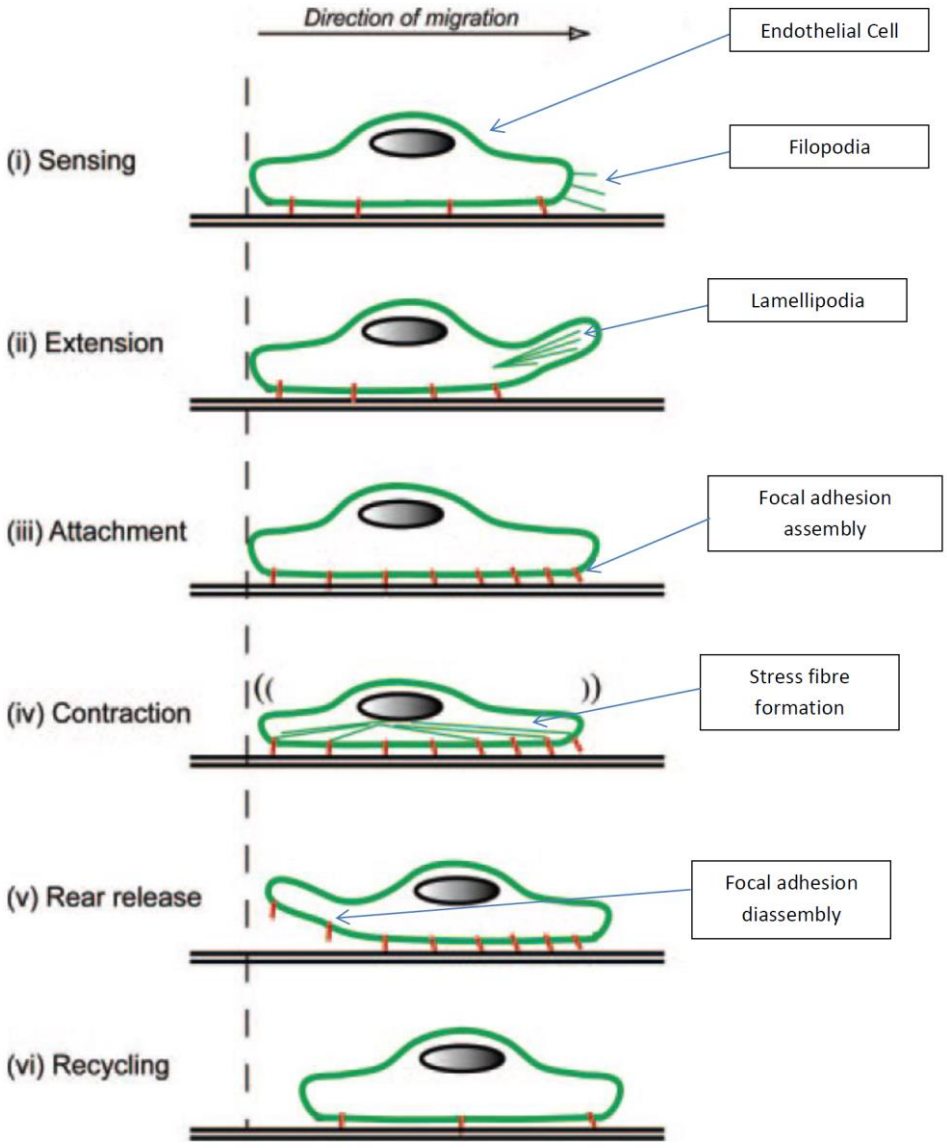


Figure 1-8 Steps involved in endothelial cell migration.
6 steps are involved (i) sensing of the motile stimuli by filopodia (ii) cellular extension of protruding lamellipodia (iii) lamellipodia attachment to the extracellular matrix at focal adhesions (iv) forward progression of the cell via stress fibre-mediated contraction (v) rear release of cell by stress fibre-mediated traction forces (vi) recycling of the adhesive and signalling components. Adapted with permission from Lamalice *et al*, Circulation Research, 2007; 100:782-794.

1.4.3 Angiogenic Progenitor Cells and endothelial repair

Angiogenic progenitor cells (APCs) represent a sub-population of circulating blood mononuclear cells which have been suggested to play a part in re-endothelialisation of injured arteries, ischaemia-induced neovascularisation and maintenance of endothelial function[163,164]. APCs may therefore represent an endogenous repair mechanism through which the integrity of the endothelial monolayer is maintained[165,166].

APCs are thought to contribute to endothelial regeneration by two main methods, either through release of pro-angiogenic molecules which stimulate proliferation of EC or other APCs or secondly by differentiating into mature endothelial cells that integrate into the damaged vessels. For this to occur, APCs "home" to angiogenic active sites and adhere to damaged/activated endothelial cells or to the extracellular matrix, therefore contributing to the endothelial repair process (Figure 1.6)[167].

APC mobilisation into the circulation occurs in response to chemokines, cytokines and angiogenic growth factors being released following particular stimuli, e.g. ischaemia or local trauma[167]. In ischaemic tissues, granulocyte macrophage-colony stimulating factor (GM-CSF) amplifies APC mobilisation and recruits APCs into the site of new blood vessel formation[168]. Hypoxia-inducible factor-1 alpha (HIF-1α), a transcriptional regulator of hypoxia-specific gene expression, induces the expression of stromal-derived factor-1-alpha (SDF-1α) and vascular endothelial growth factor (VEGF) in APCs or ECs and facilitates APC adhesion to the diseased endothelium[167]. SDF-1α (CXCL12) modulates haematopoiesis and angiogenesis[169]. SDF-1α and VEGF up-regulate bone marrow matrix metalloproteinase-9 (MMP-9) activity, which cleaves the progenitor cell membrane-bound kit ligand and allows APC mobilisation into the bone marrow vascular zone. VEGF-stimulated APC mobilisation occurs through S-nitrosylation of NO which activates MMP-9[170].

Several cardiovascular risk factors (e.g. T2DM, hypertension, hypercholesterolaemia, smoking) are associated with a reduction in APC numbers and function[167]. A correlation between reduced APC levels and endothelial dysfunction, suggests that impaired APC-mediated vascular repair promotes the progression of vascular disease[171].

Animal studies have shown that APCs contribute to endothelial regeneration following endothelium-denuding injury[172], bypass grafting[173] and hyperlipidaemia[174] and could also be exploited therapeutically as studies have shown that APCs enhance endothelial regeneration[166,175–179] and prevents neo-intimal formation[172]. APC infusion also reduces the development of endothelial dysfunction[163] and neointima[166]. Taken together, the evidence suggests a role for APCs in maintaining endothelial homeostasis and preventing atherosclerosis and restenosis.

1.4.3.1 APC definitions and classification

Two main methods are commonly cited in the literature for isolating and quantifying APCs; identification of cells expressing specific cell-surface markers by flow cytometry and enumeration of cells exhibiting typical functional properties in cell culture. Asahara *et al*[180] defined APCs as cells positive for the haematopoietic stem cell (HSC) marker CD34 and the vascular endothelial growth factor 2 (VEGFR2 or KDR in humans)[181]. However, at present there is no universal definition and no specific marker has been identified for murine or human APCs.

1.4.3.1.1 Flow cytometry

Flow cytometry is based on immunolabelling cells with antibodies targeted against specific cell surface antigens and is widely regarded to be the gold standard for obtaining quantitative data on circulating APCs as it is reproducible, sensitive and specific. However this method has a number of important limitations. Currently there is no fully specific marker available to characterise and quantify circulating APCs. The markers currently used

overlap with those of other cell lineages which make it difficult to standardise and compare APC quantification between published studies. In addition, defining circulating APCs by their expression of cell surface markers implies a presumed function is attributed to a simple antigenic phenotype[182].

In the absence of a specific marker, a combination of haematopoietic stem cell markers, e.g. CD34, CD117 (cKit) and CD133, and a marker for endothelial cells, e.g. KDR (vascular endothelial growth factor (VEGF) receptor 2) and Ve-cadherin is commonly used to identify APCs in humans[183]. Because expression of cell surface markers differ between species, APCs in mice are commonly classified as co-expressing the stem cell marker Sca-1 and the endothelial marker KDR[172].

A preferred approach to identify circulating APCs in the peripheral blood may be to include as many markers as possible. However this approach is limited by the low number of circulating APCs in the bloodstream, representing only 0.01 – 0.0001% of mononuclear cells (MNCs), and a number of other factors such as antibody affinity and the health of the subject[183]. Although sub-optimal, APCs are commonly defined in practice based on their expression of 2 or 3 cell surface antigens[182]. Even though there is no defining standard, interesting observations have emerged using flow cytometry to enumerate circulating APCs in healthy and diseased subjects.

Functional characterisation of APCs requires the expansion of MNCs by cell culture, as only this approach will provide the sufficient cell numbers to study their biological properties. Although APCs identified by flow cytometry are often considered similar to those enumerated in cell culture, it is important to recognise that these two assays are not necessarily measuring the same cells and the cells can demonstrate different phenotypes[182].

1.4.3.1.2 Cell culture

Functional characterisation of APCs derived from peripheral blood mononuclear cells or storage depots (e.g. bone marrow or spleen) requires an appropriate method of identification and expansion in cell culture.

Three distinct APC phenotypes derived from human peripheral blood mononuclear cells have been described in culture[181] (Figure 1.9). As with flow cytometry, differences in culture techniques and markers used to culture and identify APCs, makes comparison between studies difficult. The derivation and role for APCs generated by each of the three recognised techniques are summarised as follows:

After seeding mononuclear cells onto fibronectin coated cell culture plates and culturing in 'endothelial' medium, colonies of cells with an elongated and spindle shape are observed - similar to the APCs first reported by Asahara et al[181]. Cells may be identified within this population which demonstrate typical endothelial properties, including dual-staining for expression of endothelial-specific lectin and uptake of DiI-labelled acetylated low density lipoproteins (DiL-AcLDL)[184]. These cells are called 'early' APCs and typically die within 4 weeks. 'Early' APCs may display myeloid-like features and their ability to participate in vascular repair is thought to occur via secretion of pro-angiogenic growth factors and cytokines (e.g. VEGF and IGF-1) as opposed to their direct integration into the endothelium[185,186].

Another method of culturing 'early' APCs involves a pre-plating step. Following 48 hours of incubation of whole blood mononuclear cells, non-adherent cells are re-plated on fibronectin plates for a further 3 days[187] where APC colony-forming units emerge a few days later. This method avoids contamination of early adherent cells e.g. differentiated monocytes. Following re-plating, the colonies are composed of spherical-shaped cells in the centre with surrounding spindle shaped cells. These colony forming units also contain cells that stain for both DiI-AcLDL and lectin. However, Yoder *et al* demonstrated that these colony-forming units 'display

hematopoietic-restricted and macrophage-specific cellular proteins, possess limited hematopoietic colony-forming activity, and function as macrophages to ingest bacteria'[188]. These cells, therefore, may be regarded as different to traditional 'early' APCs which have a greater proliferative capacity and show greater endothelial morphology[182].

'Late outgrowth' APCs or endothelial colony forming cells (ECFCs) display a cobblestone morphology typical of endothelial cells after 3 weeks of culture and are capable of multiple passages[181]. To generate late outgrowth APCs, peripheral blood MNCs are plated onto fibronectin-coated plates. Non-adherent cells are discarded at intervals allowing late-outgrowth APC colonies to develop from the adherent cells after 2-3 weeks. Flow cytometry indicates that late outgrowth APCs are CD146+CD34+CD31+CD45-AC133-expressing cells - similar to mature circulating and resident ECs[181,189].

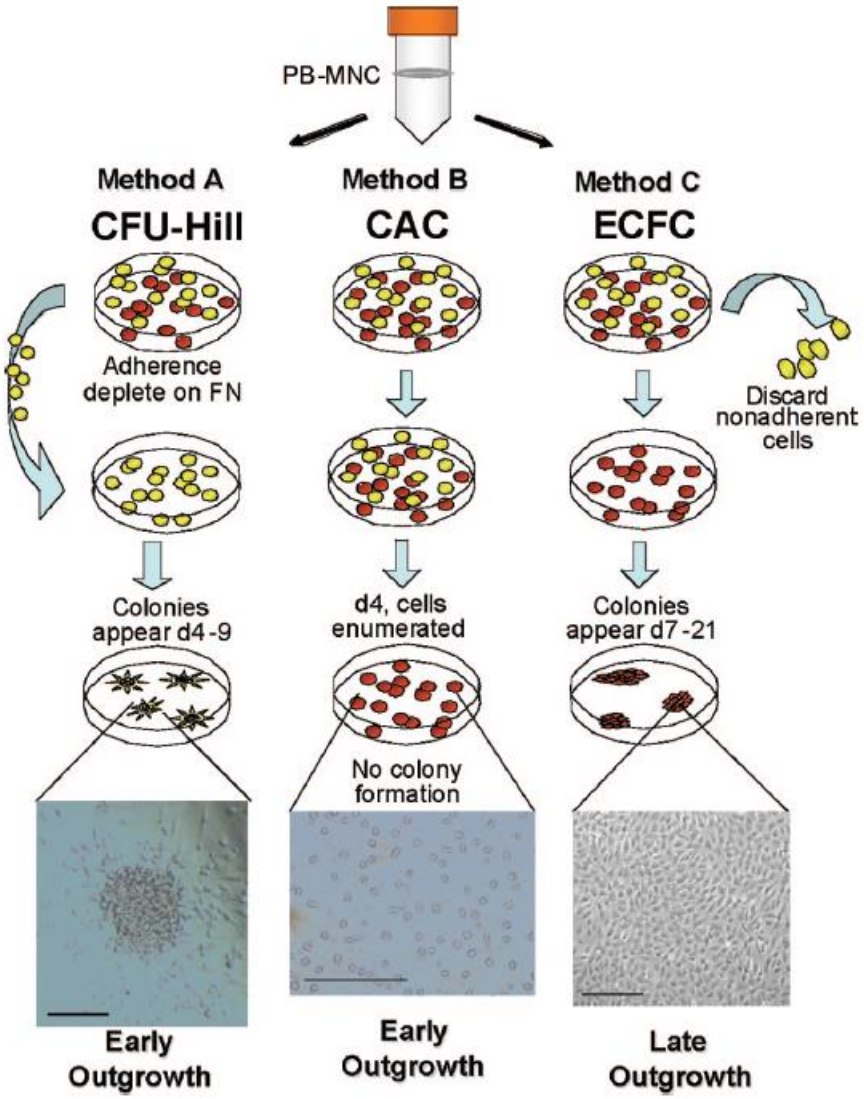


Figure 1-9 Differing methods of *in vitro* expansion of APCs.
Image taken with permission from Hirschi *et al* ATVB 2008; 28: 1584-95.

In vivo¸ all types of APCs show similar vasculogenic capacity[185], however 'late' APCs have a higher proliferative capacity, incorporate themselves into the endothelium and are considered by some to represent true endothelial progenitors[184]. Studies using 'Early' APC colonies from peripheral blood culture of MNCs should be interpreted with caution as it has recently been demonstrated that platelet-derived microparticles can be taken up by MNCs, which can, as a consequence, artefactually acquire EC characteristics[190]. In summary, there is no single, universally accepted definition for the identification of APCs, resulting in the use of different markers and culture assays used to isolate and measure APCs. Late-outgrowth APCs are very

difficult to culture from mice and only a few centres have succeeded in developing reproducible protocols[191].

1.4.3.2 APCs and Cardiovascular disease

An imbalance between endothelial damage and repair has been proposed as an important step in the development of atherosclerosis. In support of this suggestion, numerous reports indicate that reduced APC numbers and APC dysfunction are associated with an increased risk of CVD. In particular, atherosclerotic risk factors are linked inversely with the number of APCs in the blood *in vivo* and with APC function *in vitro*[169,183,187,192–196].

1.4.4 Insulin resistance and defective endothelial repair

Compelling evidence indicates a negative association between diabetes, IR and impairment in endothelial regeneration/repair. Diabetes is associated with a pronounced increase in the risk of vascular pathologies in which effective endothelial regeneration is required. In-stent restenosis, stent thrombosis and bypass graft failure are all much commoner in individuals with diabetes than in those without[197–199].

At the mechanistic level, diabetes is associated with decreased expression of SDF1-α[200] and CXCR4 in peripheral mononuclear cells[201], which may inhibit APC recruitment from the circulation. Diabetes not only impairs APC mobilisation, but is also associated with reduced numbers of APCs which are dysfunctional in vitro[202-203], APCs from individuals with diabetes display multiple functional impairments such as reduced proliferation, adhesion, migration and incorporation into tubular structures[204-205]. Li *et al*[175] conclusively demonstrated that T2DM is associated with impaired re-endothelialisation following arterial endothelial denudation in mice. They found that diabetic APC recruitment to the injured vessel *in vivo* was reduced and *in vitro*, diabetic APCs exhibited decreased cell adhesion and migration.

Animal studies reveal that IR per se is sufficient to impede endothelial repair in the presence of normal blood glucose concentrations[206]. In models of whole-body and endothelial-specific insulin resistance, PI-3 kinase/Akt signalling and NO bioavailability are downregulated[70,71]. These pathways play essential roles in APC mobilisation and endothelial regeneration[204,207]. Endothelial regeneration following wire-injury of the femoral artery is delayed significantly in insulin receptor knockout (IRKO) mice compared with wildtype (WT) mice (Figure 1.10)[206]. These findings were associated with reduced numbers of APCs and impaired APC mobilisation from the bone marrow in IRKO mice. Interestingly this paper demonstrated that endothelial regeneration is restored to normal by transfusing insulin-sensitive APCs or bone-marrow cells from wild type mice[206]. A recent study by Fu *et al*[208] investigated the effects of endothelial repair following femoral artery injury in non-obese diabetic and metabolic syndrome mice. The authors found that diabetic and metabolic syndrome mice had a 5-6 times increase in neointimal hyperplasia and a prolonged period of EC apoptosis compared to WT mice[208], hence confirming the theory that IR causes defective endothelial repair.

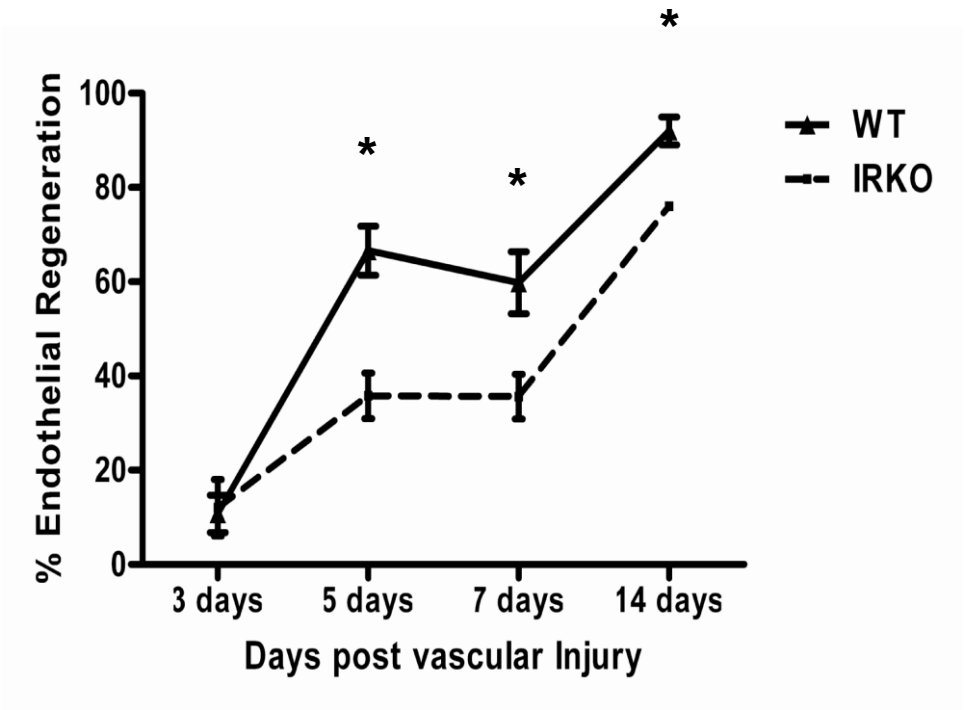


Figure 1-10. Time dependent endothelial regeneration following vascular injury in WT and IRKO mice.
IRKO mice show delayed and impaired endothelial regeneration compared to WT mice. (*P<0.05; n=5 mice per group). Reproduced with permission from Kahn *et al*[206].

1.5 Insulin-like growth factor binding protein-1

In the preceding sections of this chapter, the role of insulin on the endothelium and the effects of IR on endothelial regeneration have been discussed. However, there is recognised to be a close synergy between the actions of insulin and those of the related peptide Insulin-like Growth Factor-1 (IGF-1) which has similarly been implicated in IR, T2DM and CVD[209]. IGF-1 shares similar signalling functions to insulin, possibly due to their receptors on ECs sharing structural qualities[210] and more interestingly both insulin and IGF-1 play parallel roles in metabolic and vascular homeostasis[211].

Unlike insulin, in which bioactivity is modulated by rapid changes in plasma concentrations, the bio-availability of IGF-1 is dependent upon its interaction with a family of high affinity binding proteins (IGFBP). IGFBPs limit interaction of IGFs with their receptor and are generally considered to inhibit IGF bioavailability. However, certain IGFBPs are now recognised to act as signalling molecules in their own right independently of IGFs. IGFBP-1 is of particular interest as not only can it signal independently of IGF-1, but its plasma concentrations vary dynamically in response to nutritional cues allowing minute-by-minute regulation of IGF bioavailability.

1.5.1 Insulin-like growth factor-1

IGF-1 is produced mainly by the liver, with circulating IGF-1 levels largely regulated by growth hormone via a negative feedback mechanism[212]. In addition to its action as a growth factor, IGF-1 acts as an important hormone in regulating glucose metabolism. Like insulin, IGF-1 is capable of stimulating glucose uptake, glycogen synthesis, lipogenesis, increases insulin sensitivity and shares many components of the insulin signalling pathway[213].

IGF-1 circulates bound to IGF-binding proteins (IGFBPs). IGFBPs extend the half-life of IGFs and modulate their interaction with receptors thereby influencing their biological activity[214]. IGF-1 bioavailability is regulated tightly

by a family of 6 IGF-binding proteins (IGFBPs) which bind 99% of IGF-1 with high affinity[215]. IGFBPs functions are regulated by phosphorylation, proteolysis, polymerisation and cell or matrix association of the IGFBP[216]. All IGFBPs have shown to inhibit IGF-1 action, but IGFBP-1, -3 and -5 have also been shown to stimulate IGF-1 activity under certain experimental conditions[217]. IGFBPs also have IGF-1 independent effects, not only acting as carrier proteins but also as signalling molecules[218].

Most IGFBPs form a binary complex with IGF-1 which crosses freely into the extravascular space. Over 90% of IGF-1 circulates in a large ternary complex with IGFBP-3[219]. This complex is restricted to the intravascular space, therefore it has been thought the role of IGFBPs is to restrict IGF-bioavailability whilst prolonging its half-life.

1.5.2 Insulin-like Growth Factor Binding Proteins

IGFBPs are a family of homologous proteins, totalling 6 members all varying in molecular size, hormonal control and functional significance. All members of the IGFBP family have a conserved N-terminal cysteine-rich domain, including the IGFBP motif GCGCCXXC, but vary in the intermediate region and C-terminal domain of the protein[220]. The expression of IGFBPs is tissue- and developmental stage specific, and the concentrations in different compartments vary. IGFBPs functions are regulated by phosphorylation, proteolysis, polymerization and cell or matrix association of the IGFBP[220].

1.5.2.1 Structure

IGFBP secretion is mainly hepatic, however they are also secreted in kidneys and reproductive organs and most cell types express at least one member of the IGFBP family. The IGFBP family comprises 6 soluble proteins ranging in length from 216 to 280 amino acids[221] and mass from approximately 24 to 50kDa[222]. The IGFBPs share a similar overall 3-domain structure, with the structured N- and C-terminal domains connected by a less structured linker domain[222]. The N-terminal domain of 80-93 amino acids

shares approximately 60% similarity across the IGFBP family, with cysteines that contribute to configuration by forming disulphide bonds[223]. High-affinity binding to IGF is the only defined function shown for this domain[220,224]. The C-terminal is also vital for IGF binding. In addition this region contains individual motifs that can interact with biomolecules and therefore exert IGF-independent actions. For example, IGFBP-1 and IGFBP-2 contain an Arg-Gly-Asp (RGD) motif which can interact with integrins[225,226]. The least conserved region is the central linker domain. The linker domain comprises sites affected by post-translational modifications including proteolysis and phosphorylation and contain specific binding motifs that facilitate ECM associations and interactions with cell-surface proteins[227].

1.5.2.2 Function

The fundamental role of IGFBPs is to regulate IGF-1 bioavailability at the cellular level. Interestingly, IGFBPs can also interact with a wide spectrum of molecules such as plasminogen, transferrin, integrins and fibronectin[228] and demonstrate novel actions independent of IGF-1. Observational studies have shown altered levels of individual IGFBPs in certain diseases, alluding to a role for IGFBPs as potential biomarkers. Low concentrations of IGFBP-1, -3, -6 and increased concentration of IGFBP-2 predict increased neoplastic risk in breast and colorectal tissue; in addition IGFBP-3 is negatively associated with lung and prostate malignancy, and acute lymphocytic leukaemia[220,229]. Low concentrations of IGFBP-1, -2 and -3 are associated with disorders of metabolic homeostasis and cardiovascular disease[227].

1.5.3 Insulin-like Growth Factor Binding Protein-1

IGFBP-1 is a 25kDa protein produced mainly in the liver and kidney and with lesser expression in the reproductive system[227]. The crystal structure of the C-terminal domain of IGFBP-1 has been determined. The N- and C-terminal portions are highly conserved across the IGFBP family and are responsible for IGF binding (Figure 1.11). Although less than 5% of total bound IGF is

bound to IGFBP-1[227,230,231], IGFBP-1 has a prominent role in regulation of IGF-1 bioactivity as its plasma concentration is acutely and dynamically regulated in response to nutritional cues (e.g. fasting or post-prandial states)[227]. Inhibition of IGFBP-1 synthesis by changing hepatic portal insulin concentrations provides the connection between ambient insulin levels and the hypoglycaemic potential of IGF-1[12]. In addition to its regulatory role in IGF-1 bioactivity, IGFBP-1 can interact directly with cells through its Arg-Gly-Asp (RGD) integrin binding motif, which has functional significance independent of IGF-1[225].

IGFBPs predominantly control the access of IGFs to tissues and cell-surface IGF receptors. Of these, IGFBP-1 is the most likely candidate for acute regulation of IGF actions because of its acute down regulation by insulin and up regulation by other glucoregulatory hormones and cytokines and in catabolic states[227]. Furthermore, trans-endothelial movement of IGFBP-1 into the tissues is known to occur and to be enhanced by insulin. IGFBP-1 thus provides an important link between intermediary metabolism and regulation of IGF actions[227]. Low IGFBP-1 concentrations result in greater IGF bioavailability and promote IGF-induced effects on major blood vessels, myocardial tissue and lipid metabolism[227].

IGFBP-1 has both inhibitory and stimulatory effects on IGF-1 bioavailability. With respect to glucose regulation, it has been suggested that an overall inhibitory effect predominates and therefore diminishes the hypoglycaemic response to exogenous IGF-1[227].

Serum levels of IGFBP-1 vary considerably depending on the metabolic conditions and correlate inversely with both body mass and serum levels of insulin. Down-regulation of IGFBP-1 levels by insulin contributes to the metabolic response to food intake because a decrease in IGFBP-1 would increase the bioavailability of IGFs, which exert insulin-like metabolic functions. Overexpression of IGFBP-1 in mice has provided additional insights into the physiological role of IGFBP-1 in glucose metabolism. Wheatcroft *et al* found reduced blood pressure after a glucose challenge and

increased vascular production of nitric oxide in mice expressing human IGFBP-1 under the control of the native promoter and regulatory sequences[232]. Another study by the Wheatcroft group found that in IGFBP-1-overexpressing mice subjected to diet-induced obesity, there was improved insulin sensitivity and increased insulin-stimulated NO generation[233]. However some other data pertaining to the metabolic phenotype resulting from IGFBP-1 overexpression are conflicting. For example, transgenic mice overexpressing the IGFBP-1 gene under the control of different (constitutive) promoters showed impaired glucose tolerance and abnormalities of insulin action, suggesting that IGFBP-1 may participate in disruption of the physiological control of glucose homeostasis[234].

IGFBP-1 is post-translationally regulated by phosphorylation at multiple serine residues, which leads to an active form of the protein and this occurs in response to changes in the metabolic environment. The majority of circulating IGFBP-1 is in the phosphorylated form, which has a 10 fold higher binding affinity for IGF-1 than the dephosphorylated form. Phosphorylation of IGFBP-1 therefore represents a potential mechanism by which IGF-1 bioavailability is regulated[235].

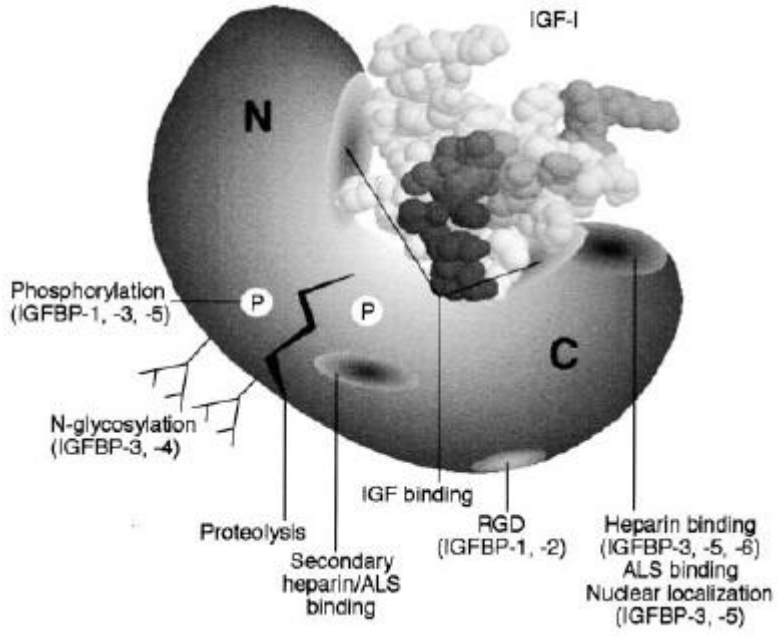


Figure 1-11 Generalised diagram of IGFBP structure showing proposed interaction with IGF-1 through both N and C domains.
Reproduced with permission from Firth et al[220].

1.5.3.1 IGF-dependent actions of IGFBP-1

Both inhibitory and stimulatory effects of IGF-1 activity by IGFBPs have been reported[217,236–238]. An inhibitory effect of IGFBP-1 on glucose regulation is consistent with the observation that administration of IGFBP-1 reduces the hypoglycaemic response to exogenous IGF-1[234,239]. IGFBP-1's affinity for IGF-1 exceeds the affinity of IGF-1 for the type-1 IGF-receptor, therefore IGFBP-1 reduces free IGF-1 levels and inhibits signalling via the IGF receptor. In the fasting state, IGFBP-1 levels are high due to the low inhibitory effect of insulin and the stimulatory effect of cortisol and glucagon on hepatic IGFBP-1 transcription. The high IGFBP-1 state limits IGF bioavailability and therefore decreases the insulin-like activity of IGF-1 on peripheral metabolism. In the post-fed state, the opposite occurs, carbohydrate loading causes insulin to rise and therefore IGFBP-1 levels fall rapidly, resulting in increased IGF-1 bioactivity which augments the insulin-like actions of IGF-1[227].

1.5.3.2 IGF-independent actions of IGFBP-1 on cells

IGFBP-1 can independently enhance migration in certain (non-vascular) cells. Jones *et* al[225] found that IGFBP-1 increased Chinese hamster ovary cell migration independently of IGF-1 through $\alpha_5\beta_1$ integrin activation. Integrin-mediated effects of IGFBP-1 have also been demonstrated in human trophoblast migration[240] and in human breast cancer cells[241]. IGFBP-1 can directly interact with cells by the Arg-Gly-Asp (RGD) motif in the C-terminus of IGFBP-1 with cell surface $\alpha_5\beta_1$ integrins, a mechanism which has been proposed to mediate IGF-independent effects[225,242]. It is also interesting to note that integrin signalling influences metabolic pathways. For example, FAK is activated after integrin engagement and interacts with multiple signalling pathways such as PI-3-kinase-Akt pathway[243,244].

1.5.3.3 Circulating Insulin-like Growth Factor Binding Protein-1 and Insulin resistance

There is overwhelming evidence supporting a strong association between IGFBP-1 and insulin-sensitivity. In particular, decreased IGFBP-1 levels are observed in individuals with insulin resistant states (metabolic syndrome, obesity, T2DM)[245–247].

In addition, low fasting circulating IGFBP-1 levels are found in obese children[248], adolescents[249] and adults[250] and low IGFBP-1 is recognised as a marker for the metabolic syndrome in population studies[245,251–253].

One cross-sectional study[254] looking at young healthy men in different ethnic groups found that fasting IGFBP-1 levels are a significant predictor of insulin sensitivity. A combination of low fasting IGFBP-1 levels and relative insulin resistance was shown in healthy, glucose tolerant Asian patients, which may partially explain their higher risk of developing diabetes and cardiovascular disease.

The IGF-1 system has been related to poor glucose control and low levels of IGF-1 are predictive of future T2DM[255]. In addition, *low* IGFBP-1 and *high*

IGFBP-3 relate to increased cardiovascular risk[214,256] and have all been independently associated with the presence of the metabolic syndrome and with insulin resistance[257].

Two prospective studies have identified low IGFBP-1 concentrations as a predictor of future development of diabetes. One long-term follow-up study identified that low IGFBP-1 and low IGF-1 levels predicted an increased risk of T2DM[255]. A Swedish longitudinal study found that low fasting IGFBP-1 concentrations predicted the development of T2DM over ten years[258]. Interestingly, in individuals who developed diabetes, IGFBP-1 levels increased by almost a third in this study[258]. Possible explanations were given for the rise in IGFBP-1 levels such as IGFBP-1 being a marker of decreasing β cell function and inflammatory cytokines playing an unspecified role.

1.5.3.4 Insulin-like Growth Factor Binding Protein-1 and Cardiovascular Disease

In addition to the links with metabolic regulation discussed above, circulating IGFBP-1 concentrations have also been associated with the risk of incident CVD and mortality. The Rancho Bernardo Study[259], a longitudinal study, demonstrated an association of low levels of IGFBP-1 with all-cause mortality. Low circulating IGFBP-1 levels were associated with high triglyceride, low HDL cholesterol levels and higher concentrations of glucose and insulin[259].

Several cross-sectional studies have shown plasma IGFBP-1 concentrations are negatively correlated with markers of insulin resistance, cardiovascular risk factors and carotid intima thickness[2,3,12,20,256,259–261]. These findings suggest that low levels of IGFBP-1 may be permissive for the development of CVD. However some groups have found conflicting conclusions[213,262]. Hu and colleagues[230] found a strong and significant association between higher IGFBP-1 and IGFBP-2 levels and mortality in the elderly. Paradoxically, despite the apparent association with increased mortality,

higher IGFBP-1 and IGFBP-2 levels were associated with favourable risk factors, including lower fasting glucose and lower fasting insulin levels. Mellbin *et al* investigated the prognostic importance of IGFBP-1 in patients with CVD and T2DM[263]. In this study, copeptin (a peptide which is activated by stress and plays pivotal roles in vascular tone and osmoregulation) was associated with IGFBP-1 levels. The investigators postulated the apparent association between IGFBP-1 and adverse outcomes observed in some studies may be explained by confounding through the association between IGFBP-1 and copeptin[263].

1.5.3.5 Insulin-like Growth Factor Binding Protein-1 and Endothelial function

Interest in a potential role of IGFBP-1 in vascular physiology was prompted by previous work in our laboratory, with the generation of a transgenic mouse which over-expresses human IGFBP-1. In this model, increased circulating IGFBP-1 concentrations were associated with increased basal vascular NO production and lower blood pressure[232]. Recently our laboratory has shown that IGFBP-1 directly upregulates NO generation in ECs, independently of IGF-1, via PI-3 kinase activation and eNOS phosphorylation. IGFBP-1 was also shown to favourably modulate vascular phenotype *in vivo*, specifically in the setting of insulin resistance. For example, in obese and non-obese mouse models of insulin resistance, IGFBP-1 over-expression protected against blood pressure elevation, rescued endothelial function and enhanced endothelial insulin-sensitivity (obese mouse model of insulin resistance only). Finally in a murine model of pro-atherogenesis, IGFBP-1 over-expression protected against the development of atherosclerosis.

In summary, this introduction has highlighted the increasing global prevalence of T2DM and IR. Recognition that these states impair the capacity for endothelial regeneration emphasises the importance of discovering potential new treatments to improve endothelial regeneration and prevent cardiovascular disease. IGFBP-1 is an endogenous protein

which is implicated inversely with IR and CVD. *In vivo* and *in vitro* studies have shown a protective role for IGFBP-1 in endothelial function and atherosclerosis, however the effects of IGFBP-1 on endothelial regeneration have not been investigated and form the focus of this thesis. Understanding the role of IGFBP-1 in endothelial regeneration is important in order to identify novel treatments to promote endothelial repair in insulin resistant patients.

2 Original hypothesis, objectives and experimental design

The fundamental hypothesis of this thesis is:

IGFBP-1 augments vascular endothelial repair in the setting of insulin resistance by promoting endothelial cell/angiogenic progenitor cell reparative functions.

2.1 Objectives

1. To investigate the effect of IGFBP-1 over-expression on endothelial repair *in vivo*.
2. To examine the effect of IGFBP-1 on the functional properties of human ECs implicated in endothelial repair *in vitro*.
3. To examine the effect of IGFBP-1 on the functional properties of APCs implicated in endothelial repair *in vitro*.
4. To identify the molecular mechanisms of functional effects determined in objectives 2 and 3.

2.2 Experimental design

The following complementary *in vivo* and *in vitro* approaches were employed to investigate endothelial-reparative effects of IGFBP-1:

1. To assess the impact of IGFBP-1 on endothelial regeneration, murine models with over-expression of human IGFBP-1 either in an insulin sensitive or insulin resistant setting were subjected to arterial injury resulting in complete denudation of the endothelium. The time-course and completeness of endothelial regeneration were then evaluated.
2. To investigate the effect of IGFBP-1 on APC abundance, the number of APCs in the mononuclear fraction of peripheral blood was quantified by flow cytometry in murine models with over-expression of human IGFBP-1 with/without insulin resistance.
3. To investigate the effect of IGFBP-1 on APC abundance and function, APCs were expanded from peripheral blood, bone marrow and spleen

from murine models with over-expression of human IGFBP-1 with/without insulin resistance..

4. To assess the effect of IGFBP-1 on endothelial cell functional properties implicated in endothelial repair, the influence of direct IGFBP-1 exposure on cell migration and proliferation was investigated in human ECs.
5. The effects of IGFBP-1 on ex-vivo angiogenesis and ex-vivo endothelial regeneration were also investigated using murine aorta and human saphenous vein samples respectively.
6. Investigations were carried out to identify the molecular mechanisms by which IGFBP-1 modulates endothelial repair, focussing on Focal adhesion kinase, Rho-kinase and integrins.

These 6 broad experimental approaches will be described in detail in chapters 4-7. Chapter 3 will outline generic methods used in this research project in terms of their general application.

3 General Methods

3.1 Bioreactive agents

Bioreactive agents that are used across a number of protocols are described here to detail their source and handling. Purified native human IGFBP-1 (Groprep, Adelaide, Australia) was reconstituted in 10mmol/L HCL and snap-frozen in liquid nitrogen. Qualitative analysis and mass spectrometry from the manufacturer confirmed the absence of other binding proteins and IGF-1. Recombinant cell-culture grade insulin (Sigma) was reconstituted in HEPES buffer when used with endothelial cells. VEGF (R&D, Minneapolis) was reconstituted in filter-sterile water. PBS and Trypsin/EDTA were obtained from Gibco, BRL. BSA was purchased from Sigma. FCS was from Biosera, East Sussex and was subject to in-house batch-testing.

3.2 Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) (Promocell, Heidelberg, Germany) were shipped at secondary passage in cryo-medium on dry ice and stored immediately in liquid nitrogen. To recover the cells, the cryo-vial was submerged in a water bath at 37°C until fully thawed, and transferred to 5 mL of Endothelial cell growth medium (20% FCS). Typical endothelial features such as cobblestone appearance and contact inhibition were confirmed on culture.

Endothelial cells were seeded either in a $25cm^3$ or $75cm^3$ tissue culture flask and were maintained in a humidified incubator with 5% CO_2 at 37°C. Growth medium was half-exchanged every 2 days. Once cells reached 90% confluence, cells were washed with warm PBS and then passaged at a ratio of 1:3 using Trypsin/EDTA at room temperature for 2 minutes. Endothelial cells were reseeded in either a tissue culture flask or a cell culture plate. In all cases, cells were used between passage number 4 - 7.

3.3 Animal husbandry

3.3.1 Gene manipulation in mice

Transgenesis of mice were not undertaken by the author, as breeding colonies were already established at the University of Leeds. Generation of these models have been described by investigators in previously published literature, and is briefly summarised below.

3.3.2 Insulin receptor knockout mice with over-expression of IGFBP-1

Mice with transgenic over-expression of human IGFBP-1 were originally generated by Crossey et al[234] at King's College London. A cosmid clone was purified which encompassed the human IGFBP-1 structural gene including all the cis sequences necessary for tissue specific regulation. Mice with knockout of the insulin receptor were originally generated by Accili et al[264] at the National Institute of Health, Bethesda, USA. Mice for use in this project were supplied by the Medical Research Council Mammalian Genetics Unit, Harwell, Oxfordshire and backcrossed to C57BL6 for 12 generations. Mice with heterozygous knockout of the insulin receptor (IRKO) are viable, however mice homozygous for the null allele ($IR^{-/-}$) die as neonates from ketoacidosis. IGFBP-1 and IRKO mice were intercrossed to generate offspring of the following 4 genotypes: WT, IRKO, IGFBP-1 and IRKO*IGFBP-1.

3.3.3 Housing and experimental conditions

As mandated by the Experimental Animals (Scientific Procedures) Act 1988, experiments were conducted in full compliance with Home Office UK regulations. Experiments were sanctioned under personal licence PIL 40/9839 and project licence numbers 40/2988 up until 24/7/2011 and 40/3523 from 25/7/2011 onwards. Mice were housed in the University of Leeds animal facility, under standard laboratory conditions in humidity (55%) and temperature (21°C) controlled conditions with a 12-hour light-dark cycle,

and free access to food and water. Offspring were weaned to standard chow diet (B&K Universal Ltd, Hull) at age 3-4 weeks, at this point littermates were separated by gender. Ear-notching by punch-biopsy in conscious mice allowed numerical identification, the ear notches were subsequently used for DNA analysis. Littermates were housed together with same sex siblings in groups of up to four animals per cage. Housing of single animals was avoided wherever possible.

Male mice aged 8-16 weeks were used for experimental procedures. Oestrogen is well known to significantly affect APC biology, therefore female mice were not studied due to their cyclical hormonal changes.

For animal euthanasia, mice were left unconscious by 4 minutes of CO_2 exposure followed by blunt dislocation of the cervical spine, except in endothelial regeneration studies where terminal anaesthesia with perfusion fixation using paraformaldehyde was employed before femoral artery extraction.

3.3.4 Breeding conditions

Breeding pairs or trios (1 male with up to 2 females on an IRKO background) were established with only the male carrying the IGFBP-1 transgene. This was to avoid the potentially confounding influence of maternal *in utero* IGFBP-1 over-expression. Mating programmes required mice of at least 6 weeks of age and ideally less than 6 months of age. Mice were allowed free access to standard chow diet and water, and offspring were separated from parents after weaning.

3.4 Blood sampling from mice

3.4.1 Animal handling

Prior to experiments mice were acclimatised for at least 10 minutes in an undisturbed quiet room at a controlled ambient temperature of 21°C.

Conscious mice were gently placed in a restraining device that allowed free air flow. If mice became unduly agitated during a blood testing session the experiment was discontinued and repeated the next day.

3.4.2 Blood sampling

Blood was required from mice for flow cytometry and cell culture experiments. For flow cytometry assessments, conscious mice were placed in a cylindrical restraining device head-first, whilst the right leg was gripped between the operator's left thumb and index finger. Typically, mice had already been acclimatised to restraining devices through prior training sessions. The limb was removed of hair with hair removal cream, which exposed the lateral saphenous vein, this was them lightly punctured using a 14G needle. Up to 200µL of blood was collected into capillary blood collection tubes coated with powdered lithium heparin (Sarstedt, Numbrecht) and stored on ice before further processing. Haemostasis at the puncture site was achieved with light pressure. When repeated sampling was required, light disturbance of thrombus at the puncture site was sufficient to provide fresh whole blood. For cell culture experiments, up to 1mL was obtained from the *vena cava* under terminal anaesthesia. Mice were anaesthetised with isofluorane (see below) before a vertical abdominal incision was made. The *vena cava* was exposed and up to 1mL of blood was aspirated using a 29G needle.

3.5 General anaesthesia

Invasive murine studies (arterial injury, vessel harvest, blood collection for APC culture) required a general anaesthetic (isoflurane). The anaesthetic apparatus consists of tubes running from an isoflurane vaporiser to a carbon filter with valves that either open or close the tube's circulation. One tube controls the flow of isoflurane and oxygen to the induction chamber unit of the isoflurane set up (this is where the mouse is initially exposed to the anaesthesia and is sedated) and another tube controls the flow of isoflurane

and oxygen to the mask portion of the isoflurane set up, (this tubing has a nose cone attached and is eventually fitted to the mouse) (Figure 3.1).

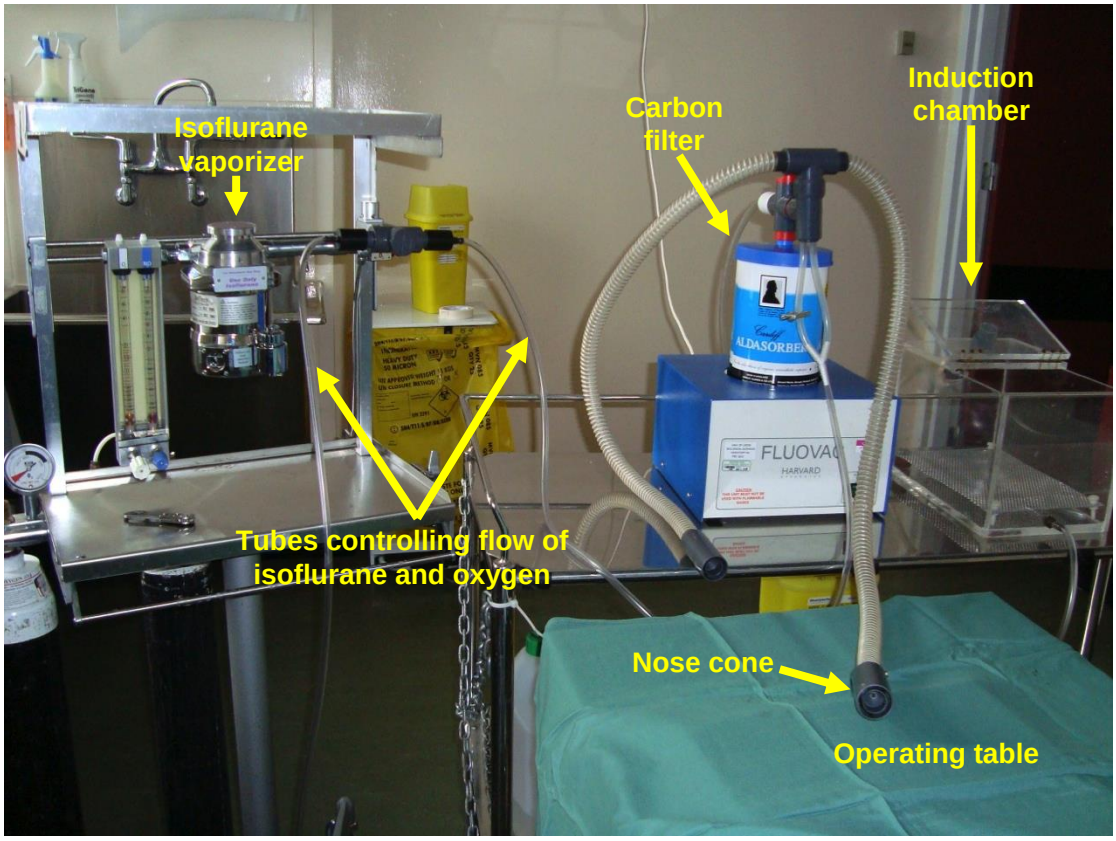


Figure 3-1 Apparatus for general anaesthesia.
(With permission from Kahn MB PhD Thesis, University of Leeds).

Once the system is checked to ensure there are adequate amounts of O_2 and isoflurane for the duration of the procedure, the system was set to flow towards the induction chamber and the O_2 supply was turned on. The mouse was placed into the induction chamber (this housing unit is ventilated with oxygen and isoflurane) and the isoflurane vaporiser was turned on and adjusted to 2.5-3.5%. The oxygen was then turned on and adjusted to at least 1 litre per minute (this level of oxygen was maintained throughout the entire procedure). Once unconscious, the system was switched to flow to the nose cone, providing isoflurane to the mouse nose cone (at this point the gas to the induction chamber was turned off). Tubing connected to the isoflurane vaporiser and oxygen was taped to the operating table and the mouse was then removed from the chamber and the head positioned in the nosecone and the body of the mouse was secured to a warmed table pad

(38°C) with tape. Maintenance anaesthesia was kept between 1.5-2.5%; if an animal started responding, the mouse was gently restrained in the nosecone until fully anesthetized again. Respiration and response to stimulation during the procedure were monitored and the anaesthetic adjusted accordingly. On completion of the procedure, the vaporiser was turned off and the mouse was allowed to breathe O_2 until it awoke. The mouse was then placed in a recovery area with thermal support until fully recovered. Mice received post-operative analgesia (buprenorphine 0.25mg/kg s.c.).

3.6 PCR genotyping

3.6.1 DNA extraction

Mice were genotyped by amplification of DNA from ear notch digests by the polymerase chain reaction (PCR). Ears were marked by cutting of small notches in the periphery of the pinna according to a standardized numbering protocol.

100µL of NaOH+EDTA was added to the sample and placed on heat block (pre-heated to 95°C) for 20 minutes. After 20 minutes, samples were taken off the heat block and 100µL of 40mM Tris-HCl were added. Samples were then vortexed and placed in the fridge to cool down for 30 minutes to 1 hour for immediate PCR or stored at 4°C until required.

3.6.2 Conventional polymerase chain reaction

PCR was performed in 25µL reaction volumes using a thermal cycler (MJ Research). This was performed under a laminar flow hood to prevent any contamination, with pipetting via DNAase-free tips. Oligonucleotide primers were prepared commercially (Invitrogen), with sequences derived from published literature. Sequences complementary to the human IGFBP-1 transgene were GCA TCA TGG GCA GCT GGT TTC AC (forward) and CAA GGG TAG ACG CAC CAG CAG AG (reverse). The sequences complementary to the alleles encoding InsR null and wildtype functional InsR

were TTA AGG GCC AGC TCA TTC CT CC (forward, inside neomycin cassette), AGC TCT GCA CTT CCC TGC TC AC (forward, inside endogenous receptor allele) and TCT TTG CCT GTG CTC CAC TCT CA (reverse, inside endogenous receptor allele). The PCR products were maintained at 4°C after thermal cycling was complete.

Table 1: Reagents quantities (µl) for 25µl reaction volumes and PCR amplification profiles as optimised with specific primers

	IGFBP-1	IRKO
Buffer	2.5	2.5
MgCl2	1.5	2
dNTPS	0.4	0.5
Primer 1	0.5	0.5
Primer 2	0.5	0.5
Primer 3	-	0.5
Taq polymerase	0.125	0.5
ddH20	18.475	17
DNA solution	1	1
Amplification profile (hold; 3-stage cycle; hold)	95°C 5 mins; 95°C 1 min, 63°C 1 min, 72°C 1 min; 72°C 4 mins	94°C 4 mins; 94°C 1 min, 62°C 1 min, 72°C 1 min; 72°C 4 mins
Cycles	x 35	x 31
Band size (base-pairs)	197 (transgenic)	232 (wildtype) 255 (Insr null)

PCR product abundance was determined by electrophoresis on a 1.5% agarose gel (with ethidium bromide). Under ultraviolet imaging bands were detected and assessed using a software package (Chemilmager 5.5). PCR

products molecular weight was estimated by running a 100 base pair ladder on the same gel.

3.6.3 DNA gel-resolution and detection

Using a 1.2% agarose gel, PCR products were visualised by electrophoresis. 1.2% agarose gel was made by denaturing 1.2g agarose (Bioline, London) in 120mL TAE buffer (50X solution: 242g TRIS base, 750mL H_20, 57.1mL glacial acetic acid, 100mL 0.5M EDTA pH 8 made up to 1L with ddH_20 (diluted to a 1X solution with ddH_20)) in an 800W microwave for 1 minute and 30 seconds, using an autoclaved beaker. 5µL ethidium bromide (Sigma) was added after cooling to 40°C, and this viscous solution was left to cool for 2-3 minutes and then poured into a running tray. A comb was placed prior to setting in the running tray, to form indentations into which 21µL of each PCR reaction solution could subsequently be pipetted. PCR product was then separated by electrophoresis by conducting 100V across TAE buffer bathing the gel for 60 minutes. The PCR product was visualized in a light cabinet using AlphaEase software (Alpha Innotech Corp, CA) and identified by reference to a 100 base-pair ladder.

3.7 Statistical analysis and data presentation

Graphpad Prism v5 (Graphpad software, San Diego, CA, USA) was used to perform statistical and graphical analysis. Unless stated otherwise, continuous data are presented as arithmetic mean and standard error of the mean and n is the number of replicates. 2-tailed unpaired t-test was used for comparing continuous data for differences in means. Repeated measures one-way ANOVA with post-hoc Newman-Keuls was used for analysis for experiments with multiple treatments. $p<0.05$ was accepted as statistically significant.

4 The effects of IGFBP-1 on endothelial regeneration following vascular injury in relation to Insulin resistance

4.1 Introduction

Arterial wall injury following angioplasty, stent insertion and coronary artery bypass surgery leads to loss of endothelial cells either through direct trauma, apoptosis or necrosis and triggers a number of responses such as smooth muscle proliferation and migration (resulting in neointimal hyperplasia)[99] and recruitment of leucocytes and platelets (resulting in inflammation and thrombosis) Adverse arterial remodelling resulting from endothelial cell loss contributes to atherosclerosis[103], bypass graft failure[104], restenosis[105] and stent thrombosis[106].

Endothelial repair following injury occurs through a series of interactions between native EC (migration and proliferation of EC to sites of damage) and circulating bone-marrow derived angiogenic progenitor cells (APCs)[113,114] which are recruited to the diseased vessel. Studies conducted in the 1970s observed the dominant role of EC mitosis and migration in endothelial regeneration after vascular damage[110–112,115,116]. Recent data have shown that APCs promote endothelial regeneration after vascular injury[166,175], have an athero-protective effect[174] and prevent neointima formation[172]. Numerous human and animal studies have shown diabetes and IR to be associated with reduced number and function of APCs with delayed endothelial regeneration following arterial injury[175,206,265,266].

Interest in a potential role of IGFBP-1 in vascular physiology was prompted by previous work in our laboratory, with the generation of a transgenic mouse which over-expresses human IGFBP-1. In this model increased circulating IGFBP-1 concentrations were associated with increased basal vascular NO production and lower blood pressure[232]. IGFBP-1 directly upregulated NO generation in ECs independently of IGF-1, via a signalling pathway comprising PI-3 kinase activation and eNOS phosphorylation[267]. Because endothelial repair is critically dependant on NO bioavailability, one may postulate that IGFBP-1 may have a positive role in endothelial repair by increasing NO production.

To assess the effects of IGFBP-1 on endothelial regeneration in an insulin sensitive and insulin resistant setting, a well-established model of arterial injury was performed. Several models of arterial injury have been described including extra-luminal approaches such as simple arterial ligature[268], perivascular electric current[269] and peri-arterial cuff placement[270]. A more clinically relevant experimental model is endothelial denudation of the carotid artery in mice. However, this experimental model is technically challenging and can cause significant complications to the animal[271]. In this chapter, a well-validated model of femoral arterial injury was used successfully in our laboratory[206]. This model is clinically relevant and results in complete endothelial denudation along with neointimal hyperplasia. Other advantages of this technique include a short operative time, low morbidity and mortality rates in comparison to the carotid artery injury technique, and the feasibility of performing bilateral studies[272].

4.2 Aims

The aims of the investigations described in this chapter were as follows:

1. To acquire skills in order to establish a reproducible murine model of femoral arterial wire-injury which results in complete endothelial denudation.
2. To assess if over-expression of human IGFBP-1 increases endothelial regeneration after vascular injury in insulin sensitive (WT) and in insulin resistant (IRKO) murine models *in vivo*.
3. To assess the regenerative capacity of human IGFBP-1 on murine thoracic aorta samples using an aortic ring angiogenesis assay *ex vivo*.

4.3 Methods

4.3.1 Breeding colonies and genotype

A breeding colony of IRKO*IGFBP-1 mice was established (as described in section 3.3.2) and the genotype of male offspring was determined by polymerase chain reaction of ear-notch tissue digests (as described in section 3.6.2). Male IGFBP-1, IRKO, IRKO*IGFBP-1 mice aged 12-16 weeks were compared with age- and sex-matched wild-type littermate controls.

4.3.2 Arterial injury technique

Two comparisons were performed in the arterial injury experiments, the first investigating the effects of human IGFBP-1 over-expression in metabolically healthy mice (i.e. WT vs IGFBP-1) and secondly, investigating the effects of human IGFBP-1 over-expression in insulin-resistant mice (i.e. IRKO vs IRKO*IGFBP-1). The above murine models were subjected to femoral artery endothelial denuding wire-injury between 12-16 weeks of age.

Surgery was performed using a dissecting microscope (Zeiss OPMI 1-FC, Germany). Mice were anaesthetised (isoflurane 2-3%) before being positioned supine and secured to a table pad (warmed to 38°C) with tape (as described in section 3.5). Mice were fixed in position with upper paws fixed on the nosecone and lower extremities abducted and extended. Hair was removed from the lower abdomen and groins with hair removal cream. The lower abdomen and groins were cleaned with providone-iodine solution (0.75%).

An incision was made in the mid-thigh and extending towards the midline. After blunt dissection of the subcutaneous tissues, the fat pad across the vessel (which marks the point of departure of the epigastric artery) was identified. To avoid damage to the epigastric artery the fat pad was handled carefully as this artery perfuses the limb when the main artery is ligated.

Overlying fascia was removed which revealed the neurovascular bundle. The femoral artery was carefully separated from the nerve and vein immediately distal to the epigastric branch. An 8/0 suture was loosely tied around the femoral artery at this point and a clamp placed across the neurovascular bundle in the proximal operative field at the level of the inguinal ligament. To prevent vasospasm, a drop of 1% lignocaine was pipetted onto the artery. An arteriotomy was performed using iris scissors (World Precision Instruments) and then advancing a 0.36mm angioplasty guide wire with tapered tip (Hi-torque Cross-it 200XT, Abbott-Vascular, Illinois, USA) into the femoral artery using an operating microscope. The angioplasty wire was advanced 3cm (clamp being removed at this point). 3 passages in total were performed per mouse which resulted in complete arterial denudation (confirmed using Evans blue staining as described below). The angioplasty wire was removed completely and suture tightened quickly. The vessel was tied off and skin closed with continuous sutures. The contralateral artery underwent an identical operation without passage of the wire, a ‘sham’ procedure.

The mouse was then placed in an incubator with thermal support until fully recovered. Mice received post-operative analgesia (buprenorphine 0.25mg/kg s.c.) and were monitored for signs of distress. All surgical operations were performed by Dr. Nadira Yuldasheva.

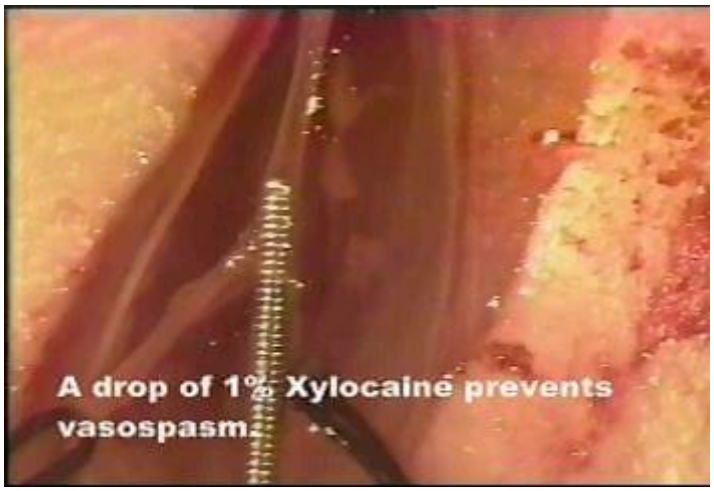


Figure 4-1 Femoral artery denudation.
The angioplasty guide wire is advanced 3cm into the femoral artery. (With permission from Kahn MB PhD Thesis, University of Leeds).

4.3.3 Endothelial Regeneration

After 5 days the mice were anaesthetised (this time point was selected based on previous data from our laboratory)[206]. 50 μL 5% Evans blue dye was injected into the inferior vena cava. After 10 minutes, the mice were perfusion fixed with formaldehyde before the femoral arteries were harvested. Evans blue dye is an azo dye which has a very high affinity for albumin. The dye binds only to exposed inter-cellular matrix when endothelial cells are denuded because albumin cannot cross the endothelial monolayer. Therefore, absence of endothelium appears blue when damaged and this marks the denuded area of the femoral artery. Perfusion fixation was carried out via a 23G needle placed into the left ventricle under physiological pressure with 4% paraformaldehyde (pH 7.2). Paraformaldehyde was infused until the efflux (from a small incision made in the right atrium) ran less blue or the muscles began to fasciculate. Vessels were excised of injured and non-injured femoral arteries up to level of the aortic bifurcation and then stored in 4% paraformaldehyde.

Under a dissecting microscope (Olympus Dissecting Stereo Microscope 5761), the arteries were then cleaned from the overlying fascia and opened up longitudinally and images obtained using a digital camera (QiCam Olympus digital camera). The stained and unstained areas in blue were

measured microscopically in the injured area 5mm from the proximal suture and the percentage areas were calculated using ImagePro Plus 6.2 software (Media Cybernetics, Bethesda, USA). This was done automatically by the program once the region of interest was outlined and any blue areas identified (Figure 4.2). Endothelial regeneration was quantified separately by two investigators blinded to genotype.

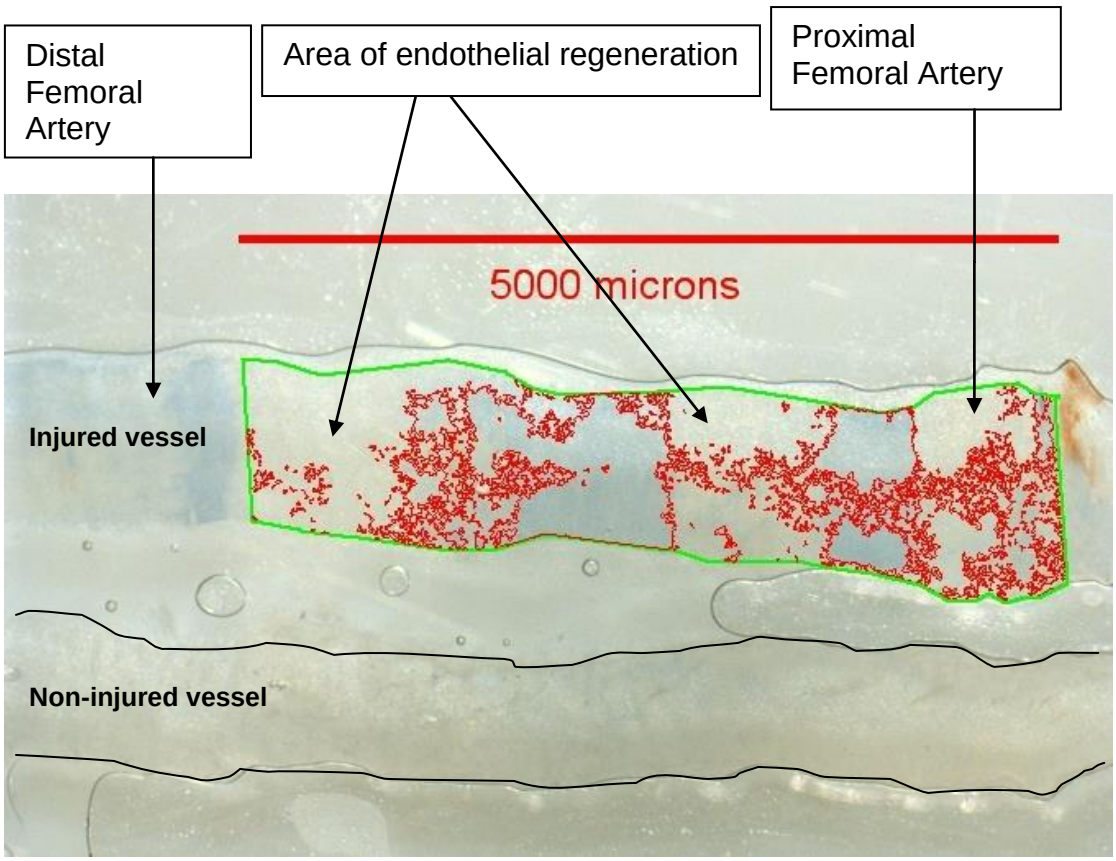


Figure 4-2 Calculation of endothelial regeneration using ImagePro Plus 6.2 software.
The green border represents the region of interest. The red borders represents patches of blue indicating denuded endothelium. Compare the 2 vessels and note the patches of blue present on the injured vessel indicating denuded endothelium however the non-injured vessel is completely clear indicating an intact endothelial monolayer.

4.3.4 Aortic ring angiogenesis assay

An aortic ring angiogenesis assay was performed to assess the contribution of native mature endothelial cells and surrounding non-endothelial cells to angiogenesis in the absence of circulating progenitor cells. Immediately after euthanasia of WT mice, the chest cavity was opened and overlying organs excised. Using fine Vannas scissors (World Precision Instruments, Sarasota), descending thoracic aortae were carefully excised, flushed with ice-cold PBS until blood free. The thoracic aorta was then placed in cold Krebs-Henselheit solution. Using a dissecting microscope, surrounding fibroadipose tissue was dissected free and the aorta sectioned into 1-mm rings. 24 well culture plates were then coated with 200 µL per well of growth factor reduced Matrigel (BD Bioscience) and allowed to polymerize for 30 minutes at 37°C. After 30 minutes, rings were then placed on growth factor reduced Matrigel and incubated with control vehicle or IGFBP-1 (500ng/mL) in 10% FBS Endothelial Cell Growth Medium), which was replaced on alternate days. Quantitative analysis of endothelial sprouting was performed using images using an Olympus CKX-41 microscope from day 7, sprouting analysis could occur up to 14 days from initial plating, however it has been known that peak sprouting occurs at day 6 and by day 9 sprouts become variable[273]. The greatest distance from the aortic-ring body to the end of the vascular sprouts was measured at 3 distinct points per ring and in 3 different rings per mouse as described previously[274].

4.4 Results

4.4.1 Endothelial regeneration following vascular injury

4.4.1.1 Wildtype v. IGFBP-1 transgenic mice

In order to investigate the effects of IGFBP-1 over-expression on endothelial repair in insulin sensitive animals, transgenic and WT mice underwent femoral artery denuding wire injury. Evans blue staining of whole-mounted femoral arteries demonstrated no significant difference in endothelial regeneration between WT and IGFBP-1 mice. (Re-endothelialised area 61.65+/-3.87% vs. 56.16+/-3.08% at day 5 following injury, N=7, p=0.29) (Figure 4.4A).

4.4.1.2 IRKO v IRKO*IGFBP-1

In order to investigate the effects of IGFBP-1 over-expression on endothelial repair in insulin resistant animals, IRKO and IRKO*IGFBP-1 mice underwent femoral artery denuding wire injury. In keeping with our previous experience, endothelial regeneration was significantly impaired in IRKO mice compared to WT mice (re-endothelialised area 47.38+/-2.56% vs. 61.19+/-3.43% at day 5 following injury, p=0.03) (Figure 4.4B). Evans blue staining of whole-mounted femoral arteries demonstrated significantly enhanced endothelial regeneration in IRKO*IGFBP-1 mice compared to IRKO. (re-endothelialised area 54.42+/-1.81% vs. 47.38+/-2.56% at day 5 following injury, p=0.03) (Figure 4.4B).

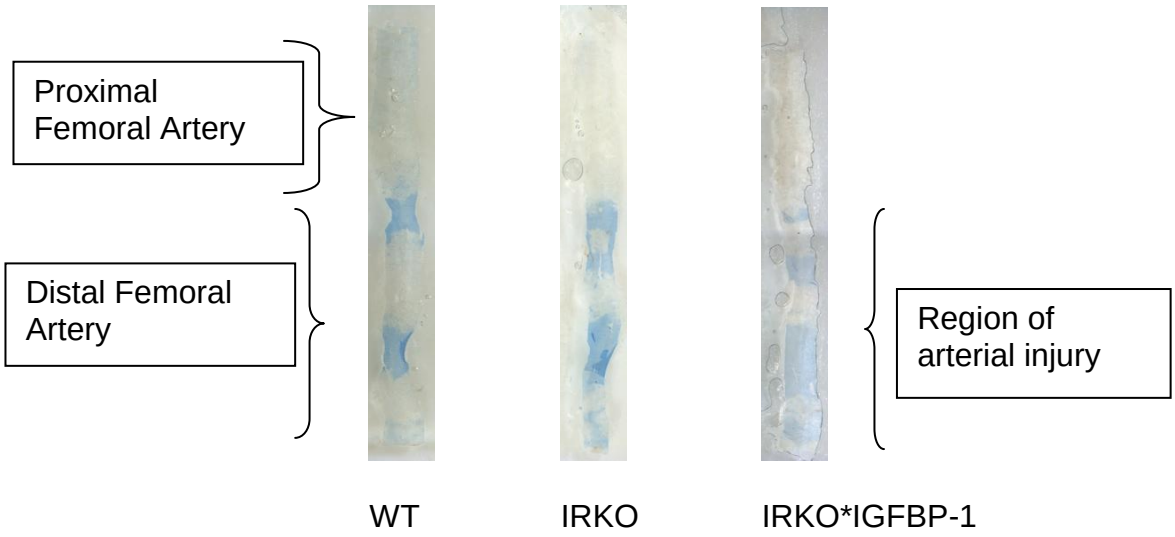
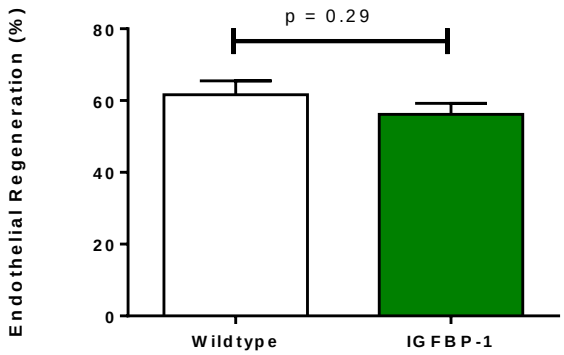


Figure 4-3 Endothelial regeneration following wire-injury of the femoral artery.
Representative *in situ* Evans blue staining in vessels 5 days post vascular injury (blue staining indicates denuded endothelium) in WT, IRKO and IRKO*IGFBP-1 mice (magnification 20x).

A



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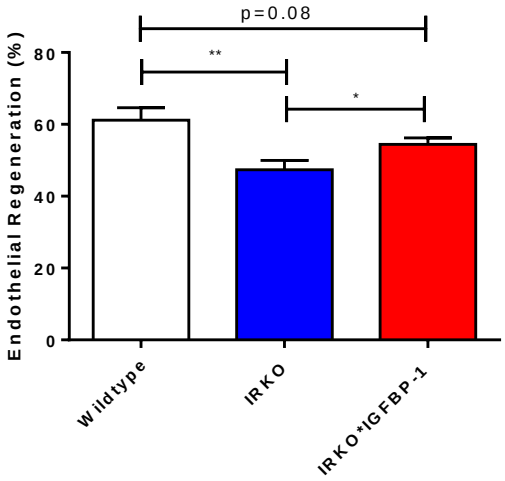


Figure 4-4 Endothelial regeneration from femoral artery denuding injury.
(A) WT v. IGFBP-1 over-expression, ns=non-significant, unpaired t-test (two-tailed), n=7. (B) WT (n=5) v. IRKO (n=7) v. IRKO*IGFBP-1 (n=10), **p<0.01, *p<0.05, one-way ANOVA with Newman-Keuls post hoc test.

4.4.2 Aortic ring angiogenesis assay

In order to investigate whether IGFBP-1 affects angiogenesis in the absence of circulating endothelial progenitor cells, an aortic ring angiogenesis assay was performed. This *in-vitro* assay is considered to come closest to simulating *in-vivo* angiogenesis, because it includes the surrounding non-endothelial cells and also the endothelial cells have not been preselected by *ex-vivo* expansion[274]. The results found no significant difference in the length of aortic micro-vessel sprouting between aortic explants from WT mice that were exposed to control or IGFBP-1 (500ng/mL). In conclusion, IGFBP-1 did not modulate the angiogenic potential of native mature endothelial cells in the vasculature of WT mice (Figure 4.5).

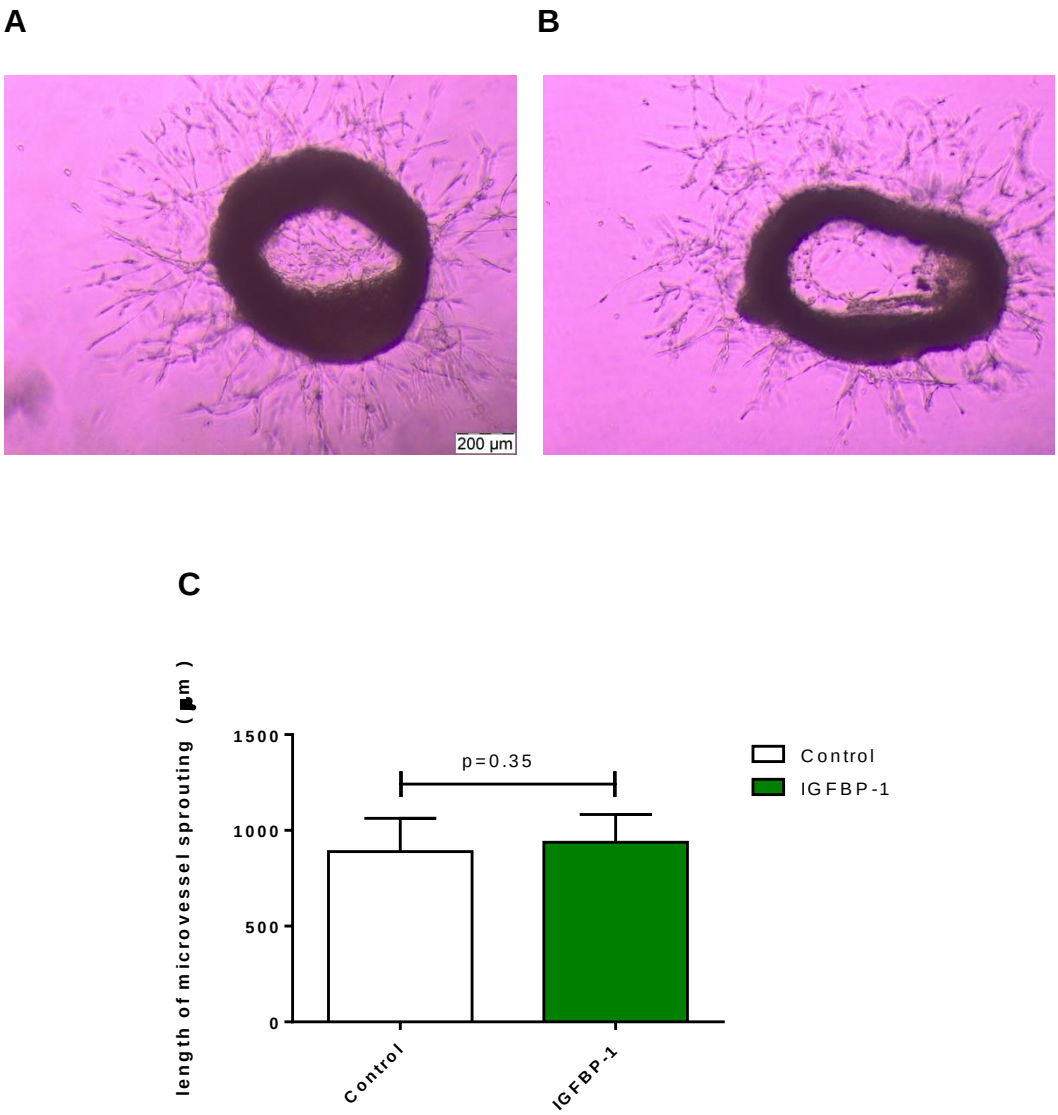


Figure 4-5 Aortic ring angiogenesis assay.
A (WT) & B (IGFBP-1): Representative image demonstrating sprouting microvessels from an aortic ring cultured in Matrigel (magnification 40x).
C: Quantification of microvessels sprouting from aortic rings from WT mice treated either with control or IGFBP-1 (500ng/ml), ns=non-significant, paired t-test, n=4 per group.

4.5 Discussion

Defective endothelial repair mechanisms in the setting of IR are important factors in the delayed and impaired endothelial regeneration following vascular injury[206], which leads to bypass graft failure[104] following surgery and restenosis[105] and stent thrombosis[106] following angioplasty in the clinical setting. IGFBP-1 overexpression has been shown to rescue endothelial function and reduce CV risk factors in murine models of both obese and non-obese IR. Over-expression of human IGFBP-1 in atherosclerosis-prone mice protects against the development of atherosclerotic plaques, raising the possibility that IGFBP-1 could constitute a novel treatment in the prevention of cardiovascular disease[267].

The present chapter provides new insights into the effect of over-expression of human IGFBP-1 on endothelial regeneration in an insulin-sensitive (healthy) and insulin resistant setting. Following endothelium denuding arterial injury, over-expression of human IGFBP-1 had no effect on endothelial regeneration in insulin-sensitive mice. In contrast, over-expression of human IGFBP-1 restored endothelial regeneration to WT levels in the insulin resistant setting (Figure 4.4B). These data represent the first time IGFBP-1 has been investigated in its role in endothelial regeneration and clearly support an *in vivo* modulatory role for IGFBP-1 in endothelial repair in IR. The favourable effects of IGFBP-1 may be specific to endothelial repair rather than angiogenesis, as no difference in angiogenic sprouting was observed following acute *ex vivo* exposure of aortic segments to IGFBP-1.

Surprisingly, no difference was observed between WT mice and mice over-expressing IGFBP-1 in endothelial regeneration after denuding injury. A possible explanation for this is that in ‘healthy’ animals the endothelial reparative measures may already be sufficient and there may be no capacity for IGFBP-1 to improve endothelial regeneration further. In support of this argument, a study looking at over-expression of IGF-1 in healthy mice found that IGF-1 did not enhance revascularisation and regeneration of skeletal

muscle grafts[275]. However, in insulin resistant IRKO mice, expression of human IGFBP-1 clearly improved endothelial regeneration. It is not possible to comment on whether the favourable effects of IGFBP-1 are truly specific for insulin resistance, as it is possible that IGFBP-1 could enhance repair in other settings in which endothelial regeneration is suboptimal. Similarly, whether the favourable effects are recapitulated in other models of IR was not investigated. IR is characterised by multiple biochemical and signalling abnormalities, which impact on endothelial regeneration and could potentially be reversed by IGFBP-1. Of these, the cellular effects of IGFBP-1 in a biochemical milieu characterised by increased production of pro-inflammatory cytokines will be explored further *in vitro* in Chapter 6.

Limitations of these data should be taken into account. During the vascular injury experiments, endothelial regeneration was recorded at one time-point only (5 days). Therefore possible effects of IGFBP-1 on endothelial repair beyond 5 days cannot be excluded. Provisional experiments were performed looking at 7 and 14 days, however due to time and resource restraints, these were discontinued. Only one method of assessing endothelial regeneration was used through Evan's blue staining. Other methods that could have been used include scanning electron microscopy, or assessing endothelial function by assessing the production of NO. However, Evans blue staining is a well-validated model of regeneration but not function[276].

This project's main focus is to investigate endothelial regeneration rather than angiogenesis, (defined as the formation of new blood vessels from existing vascular networks). Even though both processes have similarities involving endothelial cell migration and proliferation, angiogenesis and endothelial regeneration are distinct processes and may be differentially regulated. Therefore, the fact that no effect was observed with acute *ex vivo* IGFBP-1 treatment in the aortic ring angiogenesis assay does not necessarily detract from a favourable role for IGFBP-1 in vascular regeneration. The aortic ring angiogenesis assay has many advantages over other *ex vivo* angiogenesis assays such as proliferation or migration

assays as the assay is seen to stimulate *in vivo* conditions as surrounding non-endothelial cells (pericytes and smooth muscle cells) are present, which are involved in the key steps of angiogenesis. Additionally, ECs are quiescent and have not been repeatedly passaged[277]. Limitations include the fact that that this model does not represent the microvascular environment, as angiogenesis occurs typically in microvessels and not major arteries such as the aorta which was used in the assay. Also variation can occur within one experiment due to inadequate dissection of surrounding adventitia, and this in turn can influence microvessel growth. The presence of endothelial cells in the angiogenic sprouts was not confirmed with an immunofluorescence staining marker such as BS1 lectin[273]. One could have used tissue from IRKO mice rather than WT mice and therefore assessed the effects of IGFBP-1 on IRKO endothelial cells; in addition one also could have performed a concentration dependent experiment using IGFBP-1.

In summary, overexpression of IGFBP-1 enhanced endothelial regeneration following arterial denuding injury *in vivo*. These effects were restricted to the insulin resistant setting and were not observed in metabolically healthy, insulin-sensitive mice. The findings suggest a favourable, pro-regenerative effect of IGFBP-1 on endothelial repair, which may be specific to insulin resistance. Endothelial regeneration at sites of damage is attributed to two broad paradigms; native endothelial cells migrating and proliferating towards sites of injury[181] and angiogenic progenitor cells migrating to sites of injury and either releasing pro-angiogenic molecules which stimulate EC proliferation or differentiating into mature ECs that integrate into damaged vessels[166,172,174,278]. The effects of IGFBP-1 on these parameters are investigated in Chapters 5 and 6.

5 The effects of IGFBP-1 on Angiogenic Progenitor Cell abundance and function

5.1 Introduction

APCs are thought to exert their function by two main methods: release of pro-angiogenic molecules which stimulate proliferation of ECs or other APCs or differentiation into mature endothelial cells that integrate into the damaged vessels. For these processes to occur, APCs must first "home" to sites of endothelial damage and adhere to damaged/activated ECs or to the extracellular matrix, therefore contributing to the endothelial repair process[167]. Animal studies have shown that APCs contribute to endothelial regeneration following endothelium-denuding injury[172], bypass grafting[173] and hyperlipidaemia[174]. Also, APC infusion reduces the development of endothelial dysfunction[163] and neointima[166]. Taken together, the evidence suggests a role for APCs in maintaining endothelial homeostasis and preventing atherosclerosis and restenosis.

Human studies have shown that lower numbers of circulating APCs are present in diabetic subjects compared to healthy subjects. In diabetes, APCs exhibit functional defects which lead to impaired endothelial regenerative capacity[203,206,265,279,280]. Although hyperglycaemia may impair APC function in diabetes, several abnormalities associated with IR, such as reduced NO bioavailability, increased ROS production and down-regulation of PI3-kinase/AKt signalling pathways, have the potential to disrupt APC numbers and function[281]. Previous research carried out in our laboratory clearly showed impaired endothelial regeneration in IRKO mice, indicating that IR per se is sufficient to jeopardise endothelial repair[206]. The defective repair resulting from IR was attributed to a combination of impaired NO-dependent APC mobilisation, reduced APC abundance, and dysfunctional APCs in this study[206].

IGFBP-1 increases NO production through eNOS stimulation via the PI-3 kinase pathway in ECs[267]. As NO bioavailability plays a critical role in APC mobilisation and function, we hypothesised that IGFBP-1 over-expression would lead to an increase in APC abundance and function in IRKO mice.

Investigating the effects of IGFBP-1 on APC abundance and function forms the focus of this chapter.

5.2 Aims

The aims of the investigations described in this chapter were as follows:

1. To investigate, using flow cytometric enumeration of circulating APCs, whether IGFBP-1 overexpression rescues APC abundance in IRKO mice.
2. To investigate, through *in vitro* culture and expansion of APCs derived from peripheral blood monocytes and storage depots, whether IGFBP-1 overexpression rescues APC abundance and dysfunction in IRKO mice.

5.3 Methods

5.3.1 Breeding colonies and genotype

A breeding colony of IRKO*IGFBP-1 mice was established by mating male IGFBP-1 mice with IRKO females (as described in section 3.3.4). Female offspring were culled at weaning. The genotype of male offspring was determined by polymerase chain reaction of ear-notch tissue digests (as described in section 3.6.2). Animals were housed in a conventional animal facility with a 12-hour light/dark cycle and received standard laboratory chow. Male IRKO*IGFBP-1 mice aged 12-16 weeks were compared with age- and sex-matched wild-type, IRKO and IGFBP-1 littermate controls.

5.3.2 Flow cytometry (FACS)

100µL of peripheral blood was taken from the saphenous vein of each non-fasted mouse aged between 12-16 weeks (as described in section 3.4.2) and collected into capillary blood collection tubes containing 150µL Heparin (CP Pharmaceuticals Ltd, Wrexham UK). This was immediately added to 3mL of red blood cell lysis solution 'PharmLyse' (BD Biosciences) (diluted 1:10 with sterile water) and incubated for 10 minutes at room temperature. The samples were then centrifuged for 10 minutes at 300g and the mononuclear cells were resuspended in 1mL of FACS buffer (stock: 500mL PBS; 2.5mL FCS; 0.25g BSA), divided into 2 microcentrifuge tubes per sample and centrifuged once again. After pouring off the supernatant and equilibrating to 100µL with FACS buffer, the samples were incubated with 10µL FcR blocker (BD Biosciences) for 10 minutes at 4^0C to prevent non-specific antibody binding. Appropriate volumes of the following antibodies, or their respective isotype controls, were then added for 10 minutes at 4^0C (in darkness): FITC-anti mouse Sca-1 (BD Biosciences); PE-anti mouse Flk-1 (BD Biosciences). EPCs were enumerated using flow cytometry (Becton Dickinson BD-LSRFortessa) to quantify dual stained Sca-1/Flk-1 cells in accordance with other studies[282]. Isotype control specimens were used to define the threshold for antigen presence and to correct for non-specific

fluorescence. The cytometer was set to acquire 100,000 events within the lymphocyte gate, defined by typical light scatter properties.

5.3.3 APC isolation and culture

Cell culture experiments were conducted using aseptic techniques. For cell number quantification before cell culture, isolated mononuclear cells from peripheral blood, spleen or bone marrow were mixed with an equal volume of trypan blue. This cell suspension was then loaded onto a haemocytometer and viable cells (i.e. did not stain with trypan blue) were counted. A fixed number of peripheral blood- (1 million), bone marrow- (1 million) and spleen- (8 million) derived MNCs were seeded into 6-well plates (spleen-derived MNCs) and 24-well plates (blood- and bone marrow derived-MNCs). Investigations were restricted to 'early outgrowth' APCs due to difficulties in culturing late outgrowth endothelial colony cells from the blood of mice with insulin resistance[191].

5.3.3.1 Peripheral blood

To prevent blood clotting, 50µL of Heparin was aspirated into a 1mL insulin syringe (BD Micro-fine, BD Medical). From this syringe, 1mL of blood was aspirated from the inferior vena cava under terminal anaesthesia (as described in section 3.4.2) and immediately mixed with 3mL sterile PBS (stored at 4°C). This mixed solution was then expelled into a 50mL falcon tube via a 70µm cell strainer (BD Falcon) to separate out any cell clumping or clots and kept at 4°C. Blood samples were carefully layered on top of 5mL of Histopaque-1083 (Sigma) into 15mL centrifuge tubes. Density gradient centrifugation at 400g for 30 minutes then took place as per manufacturer's instructions. After density gradient centrifugation, blood mononuclear cells were aspirated from the buffy layer and re-suspended in PBS before a re-centrifugation washing step at 400g for 10 minutes. After removal of supernatant, the cells were re-suspended in 2mL of lysis buffer (1:10) and 8 mL of PBS. A further re-centrifugation washing step at 400g for 10 minutes took place. Following purification, mononuclear cells were re-

suspended in EGM-2 medium supplemented with EGM-2 Bullet kit (Lonza) in addition to 20% FCS. Mononuclear cells were seeded on fibronectin coated 24-well plates at a seeding density of $1x10^6$ cells/well (suspended in 0.5mL medium).

5.3.3.2 Spleen

Spleens obtained from mice via a left lateral incision under terminal anaesthesia were immediately placed into 5mL of PBS in a 15mL centrifuge tube and placed on ice. Spleen samples were mechanically minced through a 70µm cell strainer (BD Falcon) into a sterile petri dish containing 3mL PBS using a syringe plunger. The suspension was then expelled into a 50mL centrifuge tube through the cell strainer. The petri dish was then washed with 2mL of PBS. The wash was then aspirated and added to the 50mL centrifuge tube via the cell strainer (total of 5mL Spleen + PBS) to ensure any cellular debris was removed.

Spleen samples were carefully layered on top of 5mL of Histopaque-1083 (Sigma) into 15mL centrifuge tubes and underwent the same centrifugation, aspiration and washing step as in the peripheral blood samples as mentioned in the previous section. The spleen samples did not undergo a lysis buffer step and had 2 further washing steps with PBS and mononuclear cells were re-suspended in EGM-2 medium supplemented with EGM-2 Bullet kit (Lonza) in addition to 20% FCS. Mononuclear cells were seeded on fibronectin coated 24-well plates at a seeding density of $8x10^6$ cells/well (suspended in 2 mL medium).

5.3.3.3 Bone marrow

Tibias and femurs were obtained from mice under terminal anaesthesia. To obtain the bone marrow, the tibias and femurs were flushed 3x with 3mL PBS using a 26G needle, and placed into a 15mL centrifuge tube and placed on ice. The bone marrow suspension was mechanically minced through a 70µm cell strainer (BD Falcon) into a petri dish using a syringe plunger. The suspension was then expelled into a 50mL centrifuge tube through the cell strainer. The petri dish was then washed with 2mL of PBS. The wash was then aspirated and added to the 50mL centrifuge tube via the cell strainer to ensure any cellular debris was removed. Marrow samples were carefully layered on top of 5mL of Histopaque-1083 (Sigma) into 15mL centrifuge tubes. Density gradient centrifugation, aspiration from the buffy layer and washing steps as for Spleen samples took place for the bone marrow samples and re-suspended in the same medium used. Mononuclear cells were seeded on fibronectin coated 24-well plates at a seeding density of $1x10^6$ cells/well (suspended in 0.5 mL medium).

5.3.4 APC characterisation

Following incubation for 4 days at 37°C in 5% CO_2, non-adherent cells were discarded by gentle washing with PBS and fresh medium was added to the well containing the adherent cells. At day 7, the phenotype of APCs was confirmed during an enumeration assay in which APCs were defined as adherent cells co-staining for 1,1'-dioctadecy-3,3,3',3'-tetramethyllindocarbocyanine-labelled acetylated low-density lipoprotein (DiI-Ac-LDL) (Life technologies, Paisley, UK) and lectin from *Ulex europaeus* FITC conjugate (Sigma-Aldrich, Gillingham, UK). APCs from peripheral blood, bone marrow and spleen were incubated with DiI-Ac-LDL (Molecular Probes) diluted to 10µg/mL in supplemented EGM-2, at 37°C for 3 hours prior to fixation in 4% paraformaldehyde for 10 minutes. Cells were next washed with PBS twice and incubated with lectin from *Ulex europaeus* FITC conjugate (Sigma), diluted to 10µg/mL in PBS, for a further hour. Cells were finally washed thoroughly (3 times with PBS), prior to collecting multiple high

power (100x) phase contrast images, along with corresponding red (DiI) and green (FITC) fluorescent images using an Olympus CKX-41 fluorescence microscope. Cells appearing yellow on overlaid fluorescence images (using imageJ) were defined as EPCs in view of their co-staining for DiI-Ac-LDL and lectin, as previously reported[283]. Cell counts were expressed as APCs per high power field (Figure 5.1).

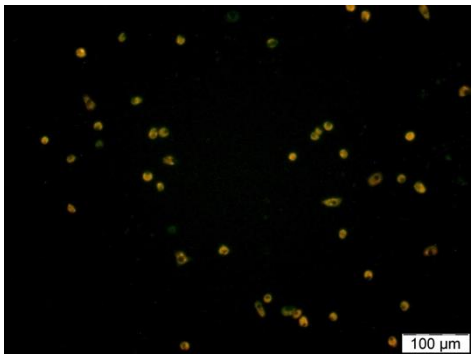


Figure 5-1 Representative image of APCs following cell culture from spleen.
(100x magnification).

5.3.5 *In vitro* APC functional assays

To assess functional capacity of APCs, spleen-derived APCs were cultured as previously described in section 5.3.3.2 and harvested from the plate using enzymatic digestion. Firstly, cells were washed with warm PBS (37°C) twice to ensure removal of latent trypsinases present within FCS. Pre-warmed trypsin/EDTA solution was added at a volume of 40µL/cm^2. Following incubation at 37°C for 2 minutes culture plates were forcefully agitated to assist cellular detachment, then EGM-2 culture medium containing FCS was added to deactivate trypsin. The resultant cell suspension was then centrifuged at 400g for 10 minutes, washed with PBS and re-centrifuged. The cell pellet was resuspended in culture medium according to the functional assay being performed. For the adhesion assay APCs were used at day 7.

5.3.5.1 APC Adhesion

For evaluation of APC adhesion, 50000 spleen-derived APCs were re-suspended in EGM-2 medium, plated onto fibronectin 24-well plates and incubated for 1 hour at 37°C. After washing 3 times with PBS, attached cells were counted after collecting 5 high power (100x) phase contrast images using an Olympus CKX-41 fluorescence microscope. Adhesion was then evaluated as the mean number of attached cells per High Power Field (100x).

5.4 Results

5.4.1 Quantification of circulating APCs using flow cytometry

Cell identification by flow cytometry in terms of lymphocyte, monocyte and granulocyte populations were defined on the basis of forward and side scatter. This was also used to exclude necrotic cells and debris. Investigation of cell structure and function was done through fluorescent labelling. APCs were defined as cells that dual stained for phycoerythrin labelled Flk-1 and fluorescein labelled Sca-1. Significantly reduced numbers of Sca-1/Flk-1 positive APCs were observed in the IRKO mice compared to WT littermate controls (Figure 5.2). Over-expression of human IGFBP-1 in IRKO mice did not alter the number of APCs (Figure 5.2).

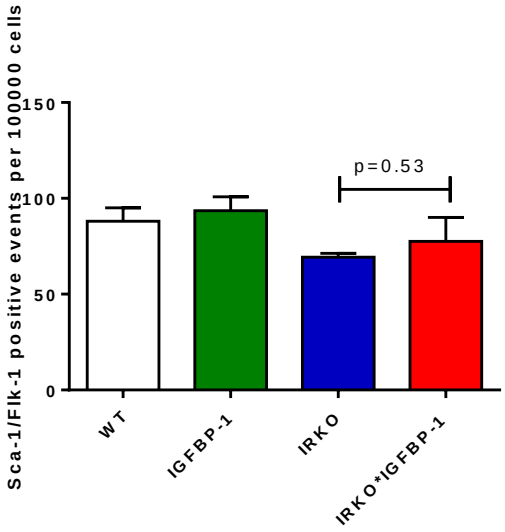


Figure 5-2 Enumeration of APCs in blood using flow cytometry.
Numbers of Sca-1/Flk-1-positive cells in WT, IRKO, IGFBP-1 over-expressing mice and IRKO mice with IGFBP-1 over-expression. (one-way ANOVA with Newman-Keuls post hoc test, *P<0.05, n=6 per group).

5.4.2 *In vitro* APC culture – phenotypic analysis

DiI-ac-LDL/lectin positive early-outgrowth APCs were quantified in cells expanded from peripheral blood-, spleen- and bone-marrow-derived mononuclear cells. On day 7, cultivated cells were incubated with DiI-ac-LDL and lectin. Cells staining positive for both lectin and DiI-ac-LDL were judged to be APCs and quantified in 10 randomly selected HPFs (100x) (Figure 5.3). Significantly fewer APCs were cultured from peripheral blood of IRKO mice compared to WT controls. There was no difference in APC yield from blood of IRKO and IRKO*IGFBP-1 mice (Figure 5.4A). There were no significant differences in the numbers of spleen or bone marrow derived-APCs cultured from all four groups of mice (Figure 5.4B and 5.4C).

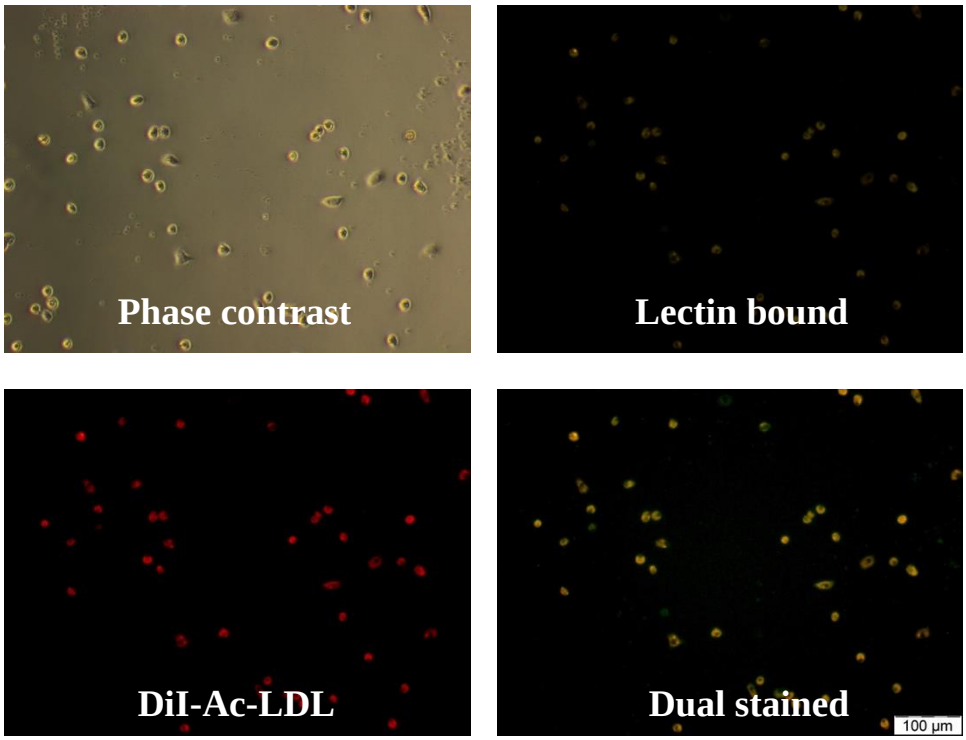
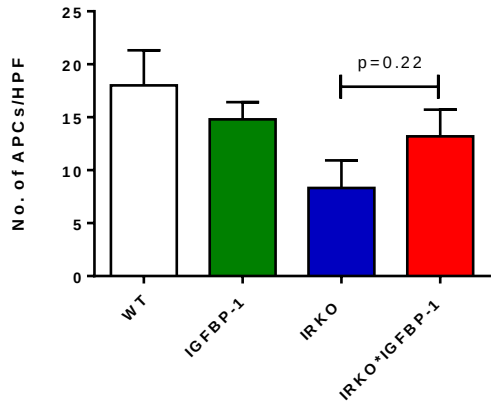


Figure 5-3 Enumeration of APCs derived from blood, spleen and bone marrow by cell culture.
Fluorescence microscopy revealing the spindle shaped APCs (Phase contrast image), lectin bound (green), DiI-ac-LDL uptake (red) and dual stained APCs (yellow), (100x magnification).

A



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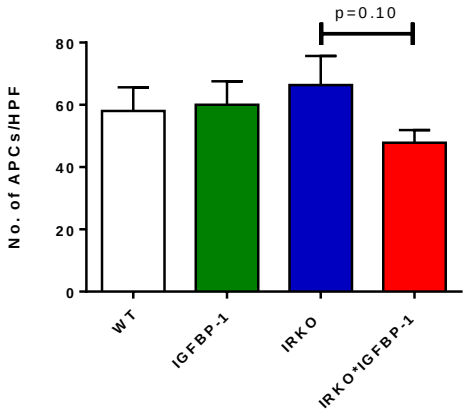

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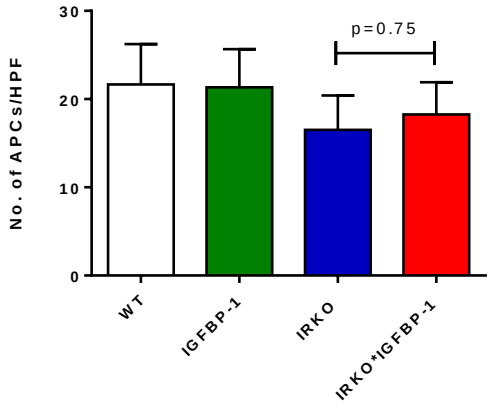
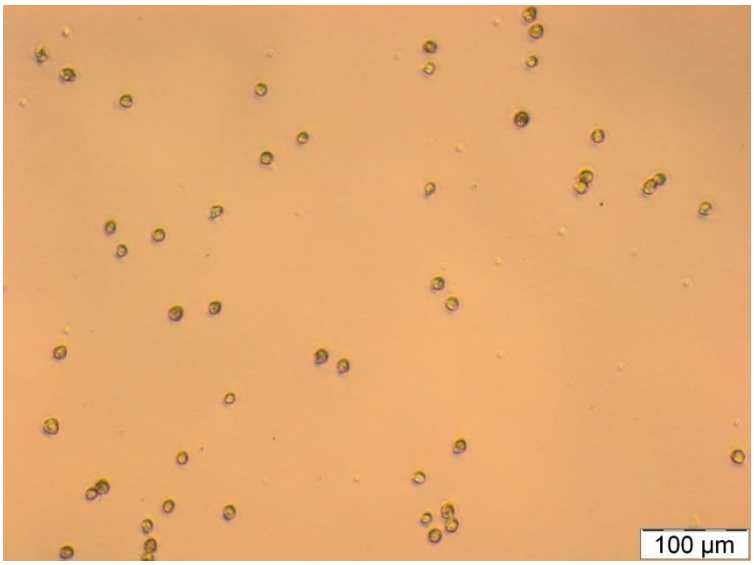


Figure 5-4 Enumeration of APCs derived from cell culture.
Blood (A), spleen (B), and bone marrow (C) by cell culture after 7 days in WT, IGFBP-1 over-expressing mice, IRKO and IRKO mice with IGFBP-1 over-expression. Numbers of peripheral blood (n=5-6), spleen (n=6) and bone marrow (n=6-9) derived cultured APCs (one-way ANOVA with Newman-Keuls post hoc test *P<0.05, n=6 mice per group). Data are presented as mean and SEM.

5.4.3 APC function: Adhesion capacity

To determine whether IGFBP-1 affects the adhesion capacity of APCs, an APC adhesion assay was performed. After 7 days of culture, 50000 spleen-derived early APCs from WT, IGFBP-1, IRKO and IRKO*IGFBP-1 mice were re-suspended in EGM-2 medium and plated onto 24-well plates coated with fibronectin (an important extracellular matrix component involved in the initial steps of angiogenesis). Over-expression of human IGFBP-1 did not modulate adhesive capacity to fibronectin in insulin-sensitive or insulin-resistant mice (Figure 5.5).

A



B

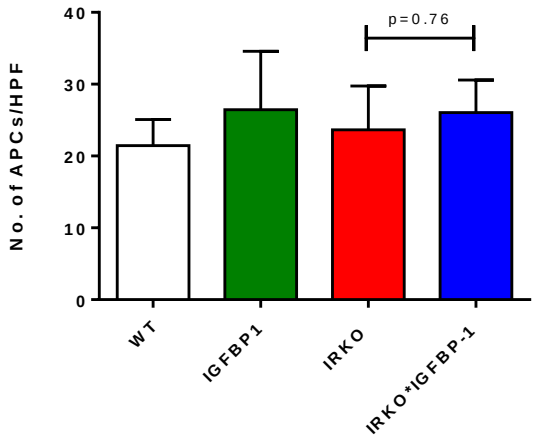


Figure 5-5 APC functional assay.
APCs were spleen-derived and cultured for 7 days. (A) Representative image of APCs (10x magnification). (B). Adhesion capacity of spleen-derived APCs cultured from WT, IGFBP-1, IRKO and IRKO*IGFBP-1 mice expressed as number of cells adhering to fibronectin-coated plates (n=5-6 mice per group). Data are presented as mean and SEM. One way ANOVA with Newman-Keuls post-hoc test.

5.5 Discussion

The principal findings of this chapter may be summarised as follows: IRKO mice had fewer circulating APCs compared to WT littermate controls in keeping with a detrimental effect of IR on endothelial repair. Surprisingly, similar circulating APC abundance was seen in WT and IGFBP-1 mice, and in IRKO and IRKO*IGFBP-1 mice, indicating that the enhanced endothelial regeneration observed in Chapter 4 is not explained by altered abundance of APCs. Finally, no difference was seen between WT, IRKO, IGFBP-1, IRKO*IGFBP-1 mice in APC adhesive capacity, suggesting that IGFBP-1 does not modulate APC reparative functions.

An era of APC-focussed research was born when Asahara's landmark study[180] identified and isolated APCs from peripheral blood. Other findings included CD34+ mononuclear blood cells acquiring endothelial-like qualities, and homing into angiogenic sites when intravenously transfused into animals with hind-limb ischaemia[180]. However there is no clear consensus in the scientific community over the identification and biological properties of APCs. Two broad methods have been used to identify and characterise APCs, namely flow cytometry assays and cell culture assays. With respect to flow cytometry, there is no perfect marker to identify and quantify circulating APCs, hence making it difficult to compare APC quantification in different studies[181]. APCs in mice are commonly classified as co-expressing the stem cell marker Sca-1 and the endothelial marker KDR[172], and these were the markers used for flow cytometry studies in this thesis. However other markers, such as c-kit$^+$ have been used by some groups[284].

This chapter shows evidence (although non-significant) of reduced APC numbers in the peripheral blood of IRKO mice – a finding which is in line with published data from our group[206]. However there was no difference in the number of spleen or bone-marrow derived APCs, suggesting an APC mobilisation defect secondary to impaired NO bioavailability[206]. Surprisingly, IGFBP-1 over-expression did not affect APC numbers in the insulin-sensitive or insulin resistant settings nor did it affect APC function as assessed

through adhesion. Overall, this suggests that over-expression of IGFBP-1 did not rescue the mobilisation defect seen in peripheral blood APCs observed in IRKO mice.

IGFBP-1 is known to increase NO production[267] in ECs and NO is believed to play an important role in EPC mobilisation. Therefore, contrary to the observations reported in this chapter, one would assume over-expression of IGFBP-1 would reverse the APC mobilisation defect seen in IRKO mice. Kahn *et al*[206] observed that eNOS expression was not detected in bone marrow of IRKO but was present in WT mice. Because IGFBP-1 enhances NO bioavailability by phosphorylating eNOS, it is likely that IGFBP-1 overexpression was unable to enhance bone marrow NO bioavailability in IRKO mice in the absence of detectable eNOS. To investigate this possibility, one could assess eNOS expression in the bone marrow in IRKO*IGFBP-1 mice. Other important factors facilitating APC mobilisation and homing to sites of injury include stromal cell-derived factor-1-alpha (SDF-1α). In fact, reduced SDF-1α expression is observed in diabetic wounds compared to non-diabetic wounds and exogenous administration of SDF-1α increased APC numbers and improved wound repair[285]. Whether IGFBP-1 influences expression of SDF-1α is worthy of investigation.

Results presented in chapter 4 indicate that over-expression of IGFBP-1 rescued endothelial repair in the insulin resistant setting *in vivo*. From the results presented in this chapter, one can speculate that the effects of IGFBP-1 on vascular endothelial repair are not mediated by alterations in APCs but may be occurring at the level of native endothelial cells, perhaps through enhancing cell migration and proliferation. It is interesting to note that the trend of endothelial regeneration research may be shifting away from APCs back towards the traditional paradigm of native endothelial cell migration and proliferation. Recent research has suggested that endothelial regeneration following vascular injury did not involve circulating APCs and was mediated solely by EC migration from adjacent healthy endothelium[286].

Limitations of the data presented in this chapter should be acknowledged. There is inconsistency over the identification and characterisation of APCs in the published literature with limitations to the two common methods used for isolation and enumeration of APCs. Flow cytometry cell counting is based upon immunolabelling cells with antibodies directed against intracellular or surface antigens[182]. There is no universally accepted antigenic phenotype to identify APCs, because surface marker expression overlaps with other cell lineages. In addition, the rarity of circulating APCs in peripheral blood restricts detection to a limited number of surface antigens, which renders it a difficult task to identify a pure cell population. Despite these limitations, flow cytometry is considered the gold standard to obtain quantitative data on APCs[182,281].

APCs were also identified as DiI-Ac-LDL and lectin positive cells in culture expanded mononuclear cells. These 'early outgrowth' APCs are not identical to flow cytometrically defined APCs. However, using these two techniques enables us to glean different biological information, even if they have shown consistent variation in many studies[182], *In-vitro* culture of APCs requires cell expansion from their natural environment and is recognised to change the phenotype of cells. Therefore, the results obtained *in vitro* may not necessarily reflect closely endothelial repair mechanisms occurring *in vivo*. Caution is therefore required when interpreting the functional assay described in this chapter into APC function at the cellular level[287].

Perhaps more importantly, during culture of APCs, there was no cell exposure to IGFBP-1 *in vitro*. Cultured primary cells often retain a 'metabolic memory' of the environment to which they were exposed *in vivo*. However, experiments investigating the effects of acute and prolonged exposure of APCs to IGFBP-1 *in vitro* would be required to fully exclude a modulatory effect of IGFBP-1 on APC-mediated repair.

The investigations described involved cell culture of 'early outgrowth' APCs, these cells form within 3 days and have an elongated and spindle shape and die within 4 weeks. It is suggested that 'early outgrowth' APCs display

features of myeloid cells which secrete angiogenic cytokines, rather than integrating into neoendothelium to stimulate endothelial regeneration and do not differentiate to form mature endothelial cells[288]. It is possible that IGFBP-1 in an insulin resistant setting can affect 'late outgrowth' APCs which possess typical endothelial cell morphology, form a confluent cobblestone layer and have a higher proliferative potential[182,289], this would require further study.

In summary, the data presented in this chapter suggest that the enhanced endothelial regeneration in IRKO mice over-expressing human IGFBP-1 is not explained by modulation of APCs by IGFBP-1. Another possible explanation for increased endothelial regeneration is a local effect of IGFBP-1 on endothelial cell function. This will be examined in the next chapter.

6 The effects of IGFBP-1 on endothelial cell adhesion, migration and proliferation

6.1 Introduction

Multiple studies of arterial injury have shown the central mechanism in endothelial regeneration is through local endothelial cell migration and proliferation from adjacent non-injured endothelium[110,116–118]. Another paradigm suggests APCs contribute to repair and could be exploited therapeutically to enhance endothelial regeneration[166,175–179]. However, despite the large number of reports implicating APCs in endothelial repair there is no clear evidence that APCs actually develop into ECs during re-endothelialisation. Tsuzuki *et al* failed to demonstrate any bone-marrow derived ECs in re-endothelialised areas following vascular injury in animals[290]. A recent study by Hagensen *et al*[286] tested whether circulating APCs contributed to endothelial repair and found that, contrary to current theories, migration of arterial ECs from adjacent intact endothelium was the only source of cells involved in endothelial regeneration after endovascular injury in mice. Results presented in Chapter 5 of this thesis indicate that over-expression of IGFBP-1 does not modulate the abundance of APCs or their function. Therefore this chapter investigated potential effects of IGFBP-1 on native endothelial cell function – in particular cell adhesion, migration and proliferation.

Adhesion, migration and proliferation are the key attributes of ECs required to mediate endothelial repair. IGFBP-1 has been shown to promote migration in various cell types[225,242] but has not until now been studied in ECs. As described in this chapter, an *ex-vivo* model of EC adhesion was developed, using denuded human saphenous vein to assess the ability of IGFBP-1 to modulate the number of human ECs adhering to the saphenous vein matrix. Two different methods of analysing EC migration were employed; linear wound assay and modified Boyden Chamber. Finally, the role of IGFBP-1 on EC proliferation was investigated. In healthy blood vessels, the basal rate of EC proliferation is low as mature ECs are terminally differentiated, however certain regions in the endothelium display a higher rate of EC proliferation, for example, regions where there is disturbed blood flow[111]. In addition, cardiovascular risk factors and age lead

to increased EC proliferation[112]. Following arterial injury, the denuded vessel is exposed to many circulating factors which can impede EC proliferation and therefore regeneration, hence it is vital to investigate agents to promote EC proliferation.

6.2 Aims

The aims of the investigation described in this chapter were as follows:

To assess the effects of IGFBP-1 on native human endothelial cell adhesion, migration and proliferation.

6.3 Methods

6.3.1 Adhesion Assay

The potential for IGFBP-1 to modulate EC adhesion was investigated in adhesion assays performed with HCAECs. EC suspensions (100,000 cells/mL) were seeded onto sterile cover slips which were placed on the surface of a culture well plate a well, 6 in total of a 12 well culture plate were used. Vehicle-treated wells contained 1% FCS and treatment wells contained 500ng/mL of IGFBP-1 in 1% FCS. One vehicle-treated well and one corresponding IGFBP-1 well were fixed after 2 hours, 4 hours and 6 hours of incubation at 37°C in 4% paraformaldehyde and stained with Haematoxylin & Eosin for 1 minute. Finally, the cover slips were mounted onto microscope slides using glycerol gelatin and 10 random fields containing the adherent cells were counted at 400x magnification.

6.3.2 *Ex-vivo* endothelial regeneration

To assess the effects of IGFBP-1 on EC adhesion and regeneration in human vessels *ex-vivo*, I set-up and optimised an experiment in which endothelial cell adhesion to the lumen of a human saphenous vein matrix was quantified. This was to try and mimic an environment akin to that found *in vivo*. Pilot studies looked at the feasibility of ECs adhering on the saphenous vein assessed through confocal imaging. One of the first problems encountered was that staining of ECs with CellTracker CM-DiI (Lot:983910, Invitrogen, Oregon, USA) was not effectively staining cells. The manufacturer's protocol advised 1 hour of incubation with CellTracker, however this was advised for non-endothelial cells. I then proceeded to incubate the EC with CellTracker overnight, which proved to be successful.

Excess lengths of EC denuded human saphenous vein segments were kindly provided by Mr. David O'Regan (Consultant Cardiac Surgeon at Leeds General Infirmary) from patients who were undergoing coronary artery bypass graft surgery. Segments of human saphenous vein were placed and

each corner of the segment pinned on to an organ culture plate, followed by 250µL (50,000 cells) of EC suspension (20% FCS ECGM) being placed onto the segment. Following incubation at 37°C/5% CO_2 for indicated times, the organ dish (Figure 6.1) was washed 3 times with PBS and full (20% FCS) ECGM was added to cover the whole dish and segment. The saphenous vein segments were imaged on a coverslip plate using confocal microscopy (Zeiss LSM S10 META Upright Confocal). Once the technique was optimised, the time course of EC adhesion was evaluated to determine the most appropriate time point at which to investigate a potential effect of IGFBP-1. 5 minutes of EC exposure to the vein matrix was felt to be ideal to detect a difference using IGFBP-1.

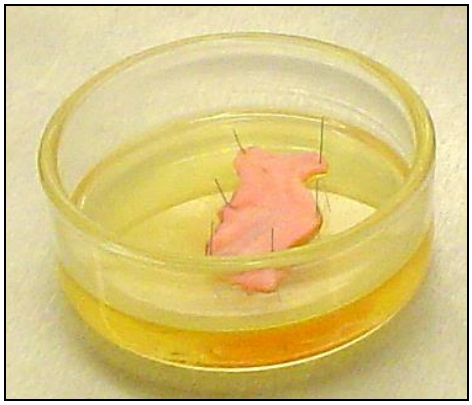


Figure 6-1 Sample of human saphenous vein pinned to an organ culture plate.

HCAECs stained with CellTracker CM-Dil (Lot:983910, Invitrogen, Oregon, USA) were incubated at 37°C/5% CO_2 for 1 hour at a fixed density (250,000 cells/mL) in full (20%) ECGM suspension with either vehicle-treated or IGFBP-1 (500ng/mL). 50,000 cells from each treatment group were seeded onto a denuded segment of human saphenous vein, incubated at 37°C/5% CO_2 for 5 minutes and the cell suspension was gently washed with PBS, Hoecht stain (to identify cell nuclei) were added to the saphenous vein segment and finally re-suspended in full (20%) ECGM. Images were obtained by confocal microscopy (as mentioned) of the vein segments, assessing the number of cells adhering to the saphenous vein matrix per high powered field (100x magnification).

6.3.3 Linear wound healing assay

Before plating ECs, the underside of the plates were marked with linear reference points using a blade not more than 1mm apart (Figure 6.2). This was used as a reference point for the linear wounds and imaging. ECs were plated at 100,000 cells per well (12-well plate) in full ECGM. Once confluent, they were serum depleted (1% FCS) overnight and the following morning the cell layer was "scratched" using a sterile 10mL Gilson pipette tip. The tip was used to scrape off the ECs in a straight line. The cells were then washed with PBS, placed into appropriate media with treatment for "time zero" images and incubated at 37°C/5% CO_2 with images taken at 24 hours and 48 hours. Observations were quantified by counting the number of cells that had migrated more than 200 microns past the wounded edge at the end of 48 hours. 4 fields per well were imaged and analysed[291].

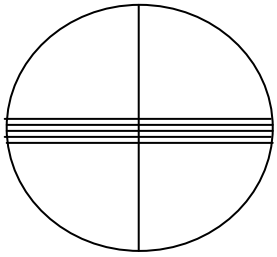


Figure 6-2 Example of underside of culture well marked with reference points for linear wound assay.

Preliminary studies were conducted to ascertain the optimum concentration of FCS to use in subsequent experiments investigating the effects of IGFBP-1 on migration. After selecting 5% and 10% serum growth media concentrations as the baseline growth media concentration, comparisons were made with vehicle-treated versus IGFBP-1 (500ng/mL) and finally comparisons were made with co-incubation with TNF-α (Peprotech Incorporated, Rocky Hill, New Jersey, USA) (10ng/mL).

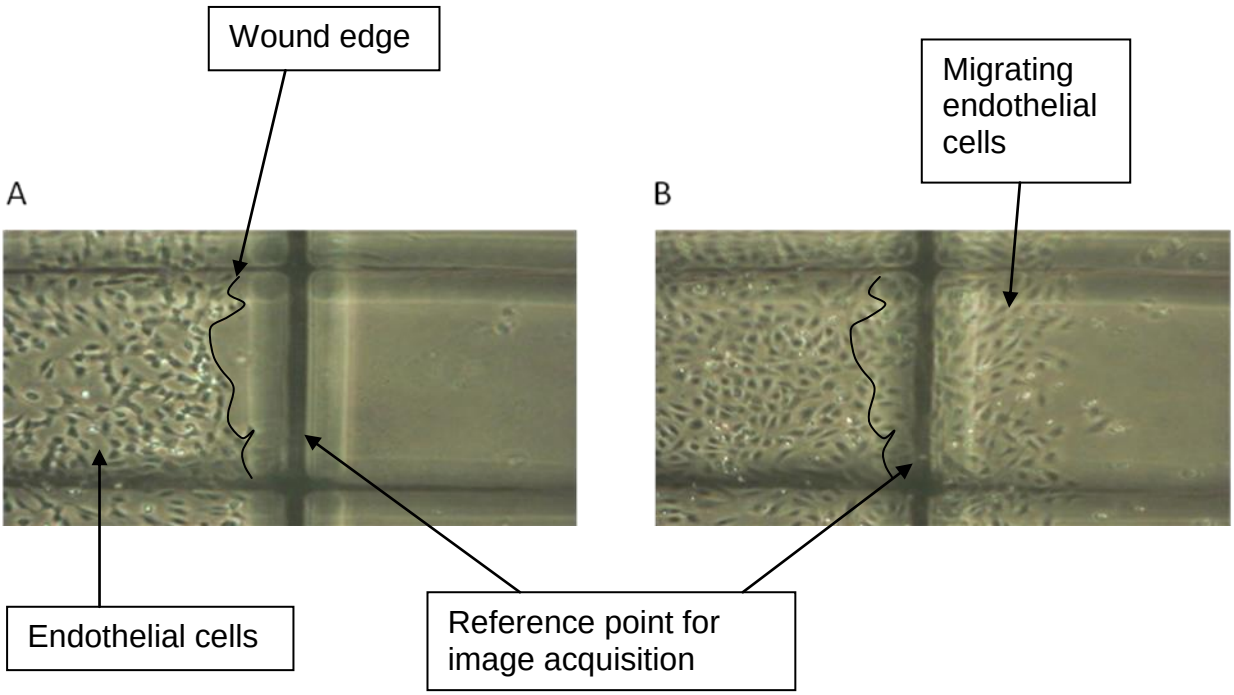


Figure 6-3 Representative image of linear wound assay.
(A) a linear wound is made initially, then a 0 hour time point image is acquired. (B) Endothelial cells are seen migrating past the wound edge after 48 hours.

6.3.4 Boyden Chamber

The modified Boyden chamber assay consists of two chambers, an upper and a lower chamber that are separated by a porous membrane (pore size 8 μm). ECs were seeded in the upper chamber and a chemotactic agent was added to the lower chamber. The cells sense and migrate along the chemotactic gradient. After six hours, cells that have crossed the membrane are stained and counted.

Confluent 25cm^3 tissue culture flasks of EC were serum deprived in 1% FCS for 12 hours. 750μL of chemoattractants was added to each of the lower chambers and all migration assays were set up in triplicate. The chemoattractants used were serum-containing growth media in different concentrations, IGFBP-1 (500ng/mL), and Vascular Endothelial Growth Factor (VEGF) (50ng/mL). Cells were prepared at 200,000 cells/mL in 1% FCS and 500μL of cell solution were added to each of the upper Boyden chambers. The cells were left to migrate for 6 hours in an incubator at 37°C/5% CO_2 then fixed overnight at -20°Celsius in 70% ethanol.

Each Boyden chamber was washed with tap water and using a cotton bud non-migrated cells were removed from the upper surface of the membrane. The chambers were Haematoxylin & Eosin stained for 30 seconds per stain and the membrane of the Boyden chambers was then cut out using a small surgical blade and mounted using glycerol gelatin on a microscope slide. 10 high-powered microscope fields were randomly selected and the migrated cell nuclei were counted at 400x magnification. Migration was quantified as the average number of cells per high-powered field for each condition.

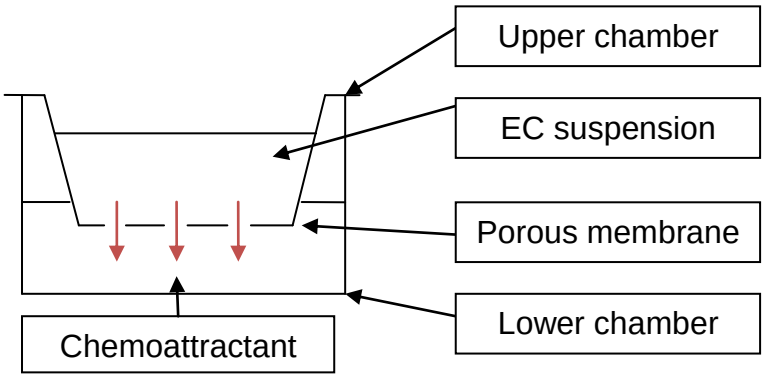


Figure 6-4 Modified Boyden Chamber.
Modified Boyden chamber assay used to study EC migration. ECs are loaded into the upper chamber and actively migrate towards a chemotactic stimulus through the porous membrane.

6.3.5 Cell Proliferation Assay

HUVECs and HCAECs were seeded at 20,000 cells/well on a 24-well plate and left to attach to the fibronectin coated surface for 24 hours. Cells were then serum deprived overnight (16 hours) using 1% FCS to quiesce the cells. The following morning (designated day 0), medium was removed from the wells, cells were washed with PBS and were then treated accordingly in triplicates. Day 0 counts of cells were also performed using a haemocytometer. Following treatment, cells were incubated at 37ºC and 5% CO_2 and treatments were replaced every 2 days. On day 5, cells were washed with PBS then 200µL of trypsin added, once cells were detached 800 µL of full ECGM were added to each well. The cell suspensions were then centrifuged at 600g for 6 minutes in 1.5mL microcentrifuge tubes. The supernatant was then aspirated off and the pellet was re-suspended in 50µL of full ECGM. 50µL trypan blue were added and mixed to the suspension, ready to be counted.

Initially, different FCS concentrations were compared to assess their effects on cell proliferation and in order to find an optimal concentration whereby a potential benefit of IGFBP-1 could be observed. After selecting 2.5% serum

growth media concentration, proliferation was quantified in response to IGFBP-1 (500ng/mL). Insulin (100ng/mL) was used as a positive control.

Finally, the ability of IGFBP-1 to rescue TNF-α (10ng/mL)-induced inhibition of cell proliferation was investigated in (20% FCS) ECGM. 20% FCS was chosen as lower concentrations of FCS would not have been conducive to cell survival in the presence of TNF-α. Initial studies found 10ng/mL of TNF-α led to marked inhibition of cell proliferation where no effects of co-incubation of IGFBP-1 could be observed. A dose response assay was subsequently performed comparing the inhibitory effects of 0.01ng/mL, 0.1ng/mL, 1ng/mL and 10ng/mL TNF-α on cell proliferation. From this set of studies 0.1ng/mL of TNF-α was selected to investigate the effects of IGFBP-1 on cell proliferation.

6.4 Results

6.4.1 Adhesion on to a plastic surface

HCAECs were incubated with IGFBP-1 and the time course of adhesion of ECs onto a plastic sterile cover slip was investigated. IGFBP-1 did not alter adhesive capacity over the time course studied (Figure 6.5).

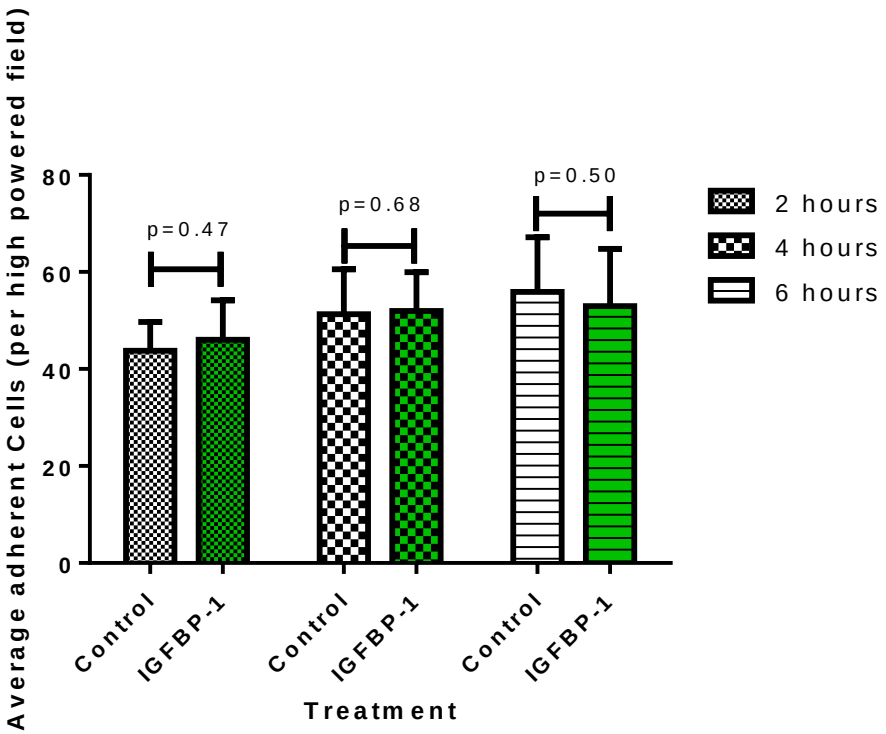


Figure 6-5 Time course Adhesion Assay – Control v. IGFBP-1 in HCAECs.
No significant difference (paired t-test) was seen between control and IGFBP-1 (500ng/ml) at 2, 4 and 6 hours (n=3).

6.4.2 *Ex-vivo* endothelial regeneration

6.4.2.1 Pilot studies

Pilot studies were performed to assess the feasibility of the experiment in terms of labelling endothelial cells and ascertaining whether cells would adhere to the saphenous vein matrix. Representative images of exposed smooth muscle cells on the surface of endothelium-denuded human saphenous vein (Figure 6.6A) and of endothelial cells labelled with CellTracker adhering onto the saphenous vein matrix (Figure 6.6B) are provided.

6.4.2.2 Time course experiments

Initial experiments investigated the effect of exposing the saphenous vein matrix to HCAECs for 2 hours, then washing away the unattached cells in the cell suspension and re-suspending the vein sample in full ECGM. The vessel was imaged at the following time-points: 0 hours, 24 hours, 48 hours and 72 hours. At 24 hours there was a peak in the number of cells adhering and spreading towards each other but after this time point the EC tended to detach (Figure 6.7A).

Subsequent studies investigated shorter time points of exposure and peak cell exposure was found to occur at 30 minutes. Reproducible and sub-maximal cell adhesion was observed after 5 minutes of exposure, which was therefore selected to examine any potential effects of IGFBP-1 on cell adhesion (Figure 6.7B).

6.4.2.3 IGFBP-1 enhances EC adhesion *ex vivo*

CellTracker-labelled HCAECs were pre-treated for 1 hour with IGFBP-1 (500ng/mL) at 37°C in 5% CO_2. These cells were then placed onto denuded saphenous vein for 5 minutes after which the cell suspension was washed away and the vein was immediately imaged. There was a significant

increase in adhesion to saphenous vein of HCAECs co-incubated with IGFBP-1 in comparison to untreated cells (Figure 6.8C).

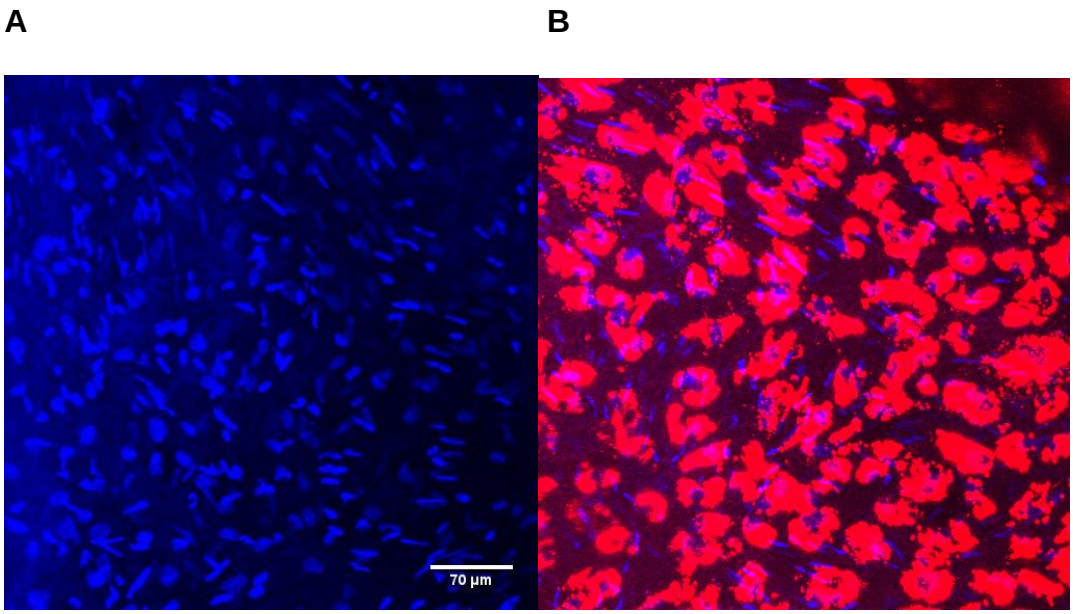
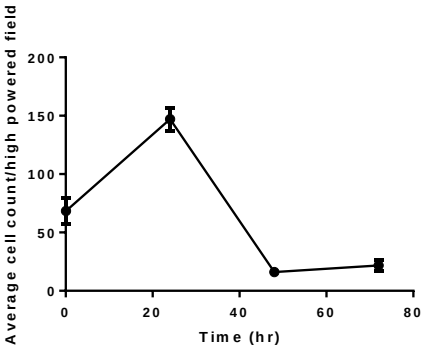


Figure 6-6 Representative images (200x magnification) of human saphenous vein under confocal microscopy
(A) Nuclei of smooth muscle cells of endothelium-denuded human saphenous vein (B) CellTracker-labelled endothelial cells adhering onto the human saphenous vein matrix.

(A) Time course of HCAEC retention to human saphenous vein following 2 hour exposure



(B) Acute exposure of HCAEC attachment to human saphenous vein over a time course of 5-30 minutes

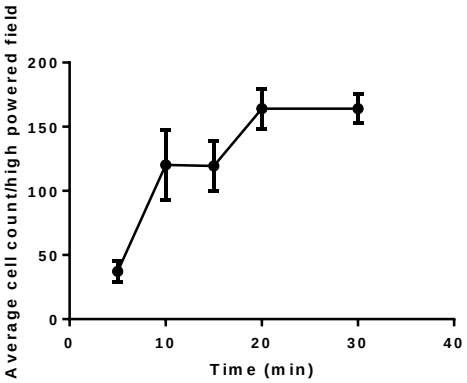


Figure 6-7 Time course of re-endothelialisation of human saphenous vein matrix by HCAECs.
(A) Time course imaging experiment where denuded saphenous vein segments were imaged serially after 2 hours of incubation with endothelial cell suspension. Images were taken immediately, then at 24, 48 and 72 hours after incubation with HCAECs. Data represent mean numbers of adherent CellTracker +ve cells per high power field (n=5-6). (B) Re-endothelialisation of saphenous vein segments following short term incubation with HCAECs. Images were acquired immediately after incubation of denuded saphenous vein with HCAECs for 5, 10, 15, 20 or 30 minutes. Data represent mean numbers of adherent CellTracker +ve cells per high power field, n=5-10.

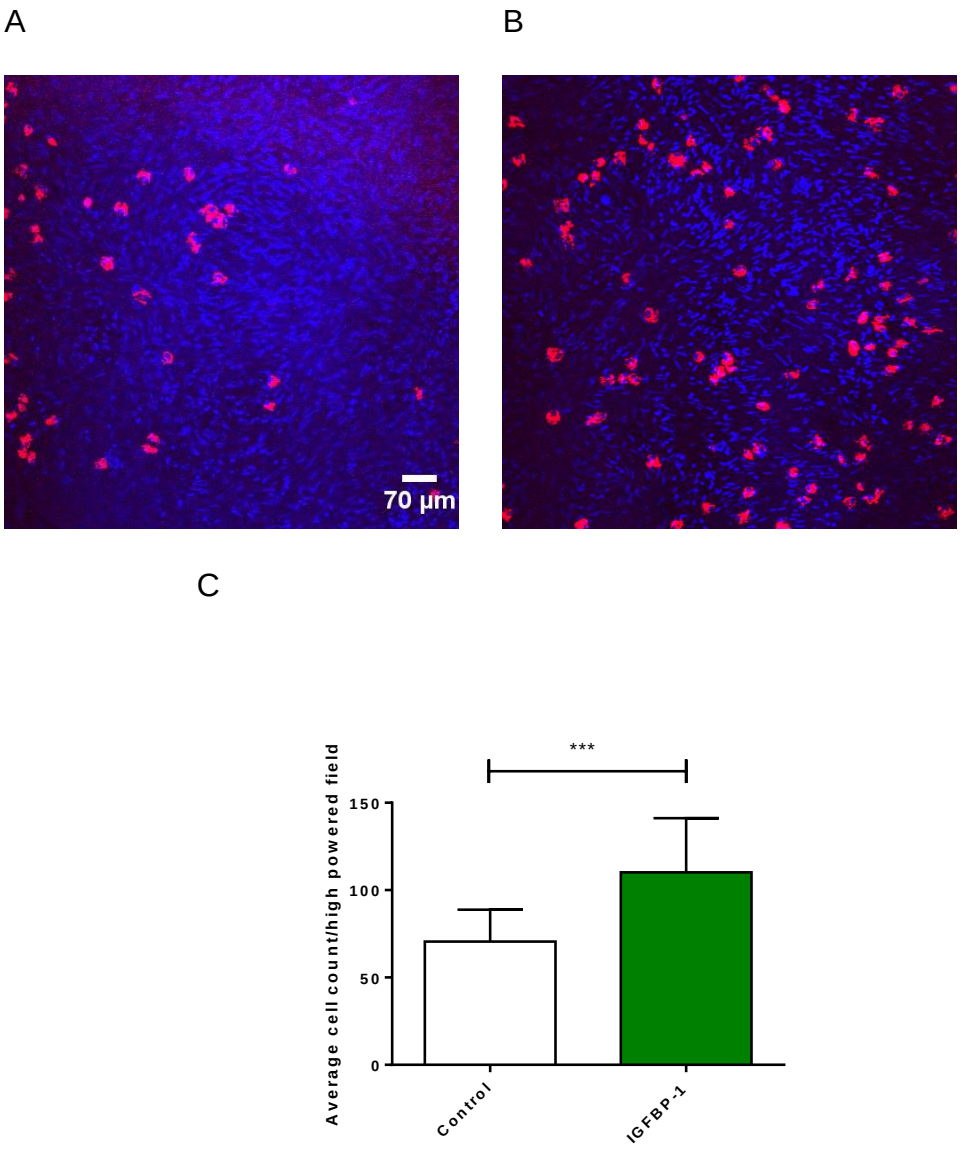


Figure 6-8 Effects of IGFBP-1 on adhesion of HCAECs to human saphenous vein.
Endothelium-denuded segments of human saphenous vein were imaged after incubation with CellTracker-labelled HCAECs for 5 minutes. Representative confocal microscopy images (100x magnification) of human saphenous vein following incubation with vehicle treated HCAECs (A) or IGFBP-1 (500ng/ml) treated HCAECs (B). Mean data are presented in (C) ***p<0.001, paired t-test; n=5.

6.4.3 Linear Wound Assay

6.4.3.1 Concentration dependent effect of foetal calf serum

Initial experiments were performed to identify the optimal concentration of FCS to use for studies of cell migration. In both HCAECs and HUVECs, a significant concentration-dependent increase in cell migration was observed at increasing FCS concentrations at 24 hours and 48 hours (Figure 6.9).

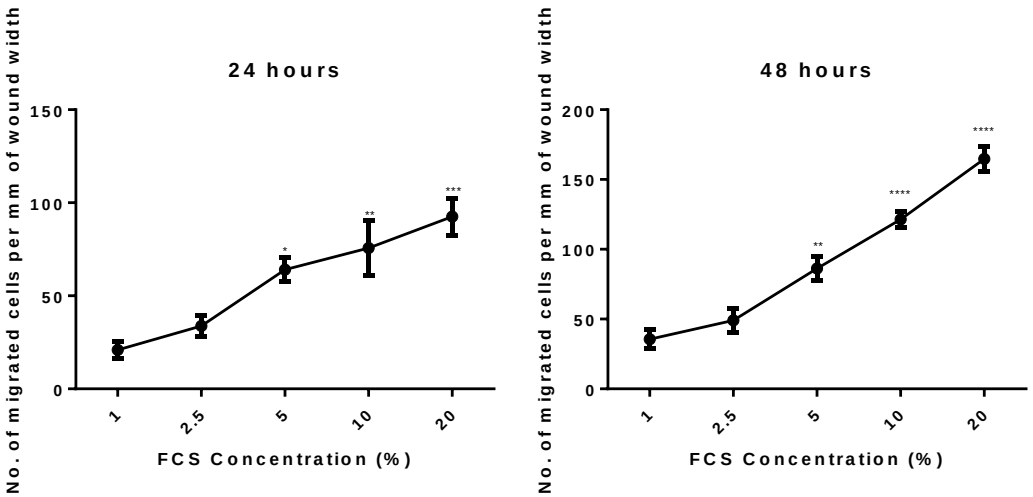
6.4.3.2 IGFBP-1 does not modulate EC migration

From the above experiments, 5% and 10% concentrations of ECGM were selected for use in subsequent experiments to investigate whether IGFBP-1 has a pro-migratory effect on EC. We considered that higher concentrations of serum growth media may mask any IGFBP-1 response. Incubation of HUVEC or HCAEC with IGFBP-1 did not modulate migration in the linear wound assay. (Figure 6.10)

6.4.3.3 In a pro-inflammatory setting IGFBP-1 reverses impaired EC migration

Having observed no effect of IGFBP-1 on migration of 'healthy' ECs, we speculated that any modulatory effect of IGFBP-1 of EC migration may be restricted to an insulin resistant setting. This would parallel the augmentation of endothelial regeneration by IGFBP-1 *in vivo*, described in Chapter 4, which was only observed in insulin resistant mice. In order to simulate insulin resistance *in vitro*, we incubated ECs with TNF-α, a pro-inflammatory cytokine which is upregulated in insulin resistant states. In HUVECs, incubation with TNF-α significantly inhibited cell migration (Figure 6.11A). The addition of IGFBP-1 to TNF-α rescued cell migration to vehicle-treated levels (Figure 6.11A). In HCAECs, a trend to impaired migration was observed in cells incubated with TNF-α (Figure 6.11B). Co-incubation of TNF-α-treated HCAECs with IGFBP-1 significantly enhanced migration (Figure 6.11B). It is interesting to note that overall fewer HCAECs migrated compared to HUVECs.

(A) HCAECs



(B) HUVECs

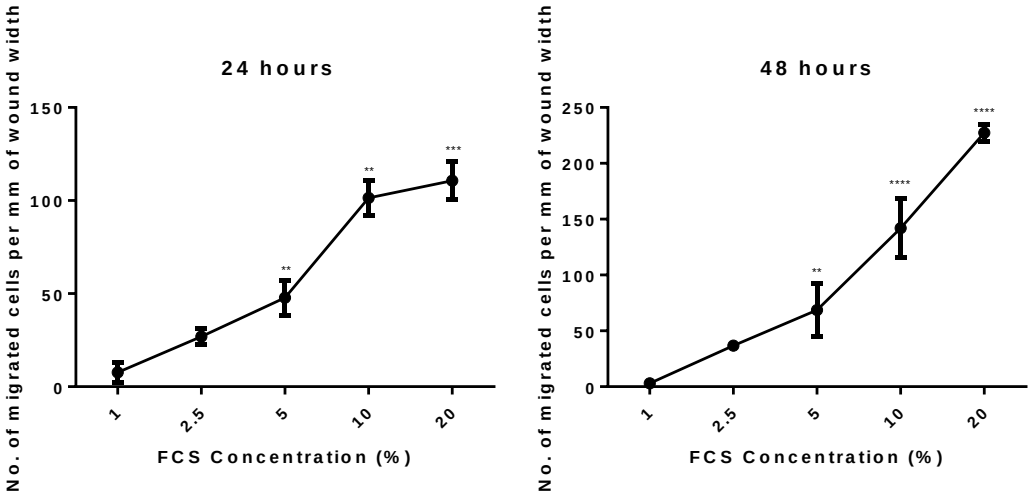


Figure 6-9 Concentration dependent effect of Foetal calf serum (FCS) on cell migration (linear wound assay).
(A) Concentration dependent effect of FCS on cell migration in HCAECs. **P<0.01, ***P<0.001, ****P<0.0001, one-way ANOVA with Newman-Keuls post hoc test. Comparisons are made with 1% ECGM. n=4. (B) Concentration dependent effect of FCS on cell migration in HUVECs. **P<0.01, ***P<0.001, ****P<0.0001, one-way ANOVA with Newman-Keuls post hoc test. Comparisons are made with 1% ECGM, n=3.

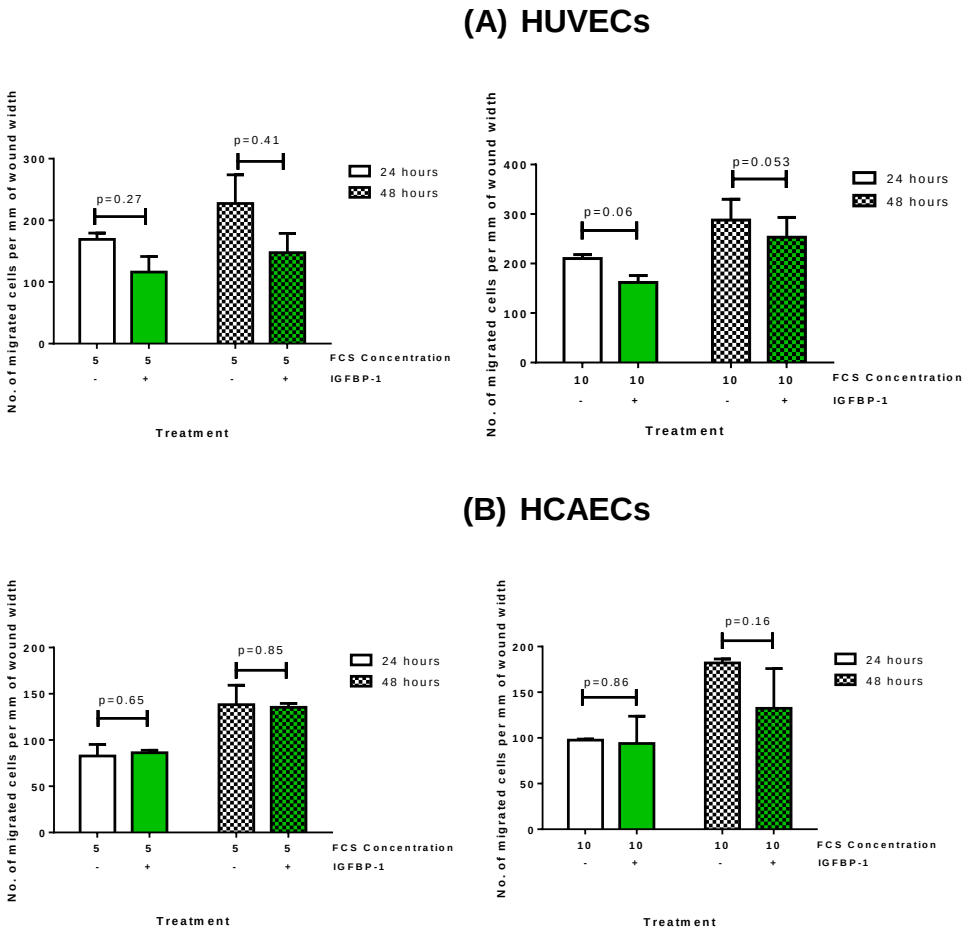


Figure 6-10 Effect of IGFBP-1 on EC migration in a linear wound assay

(A) Effect of incubation with IGFBP-1 (500ng/ml) on cell migration in HUVECs at 48 hours, paired t-test, n=3. (B) Effect of incubation with IGFBP-1 on cell migration in HCAECs at 48 hours, paired t-test, n=3.

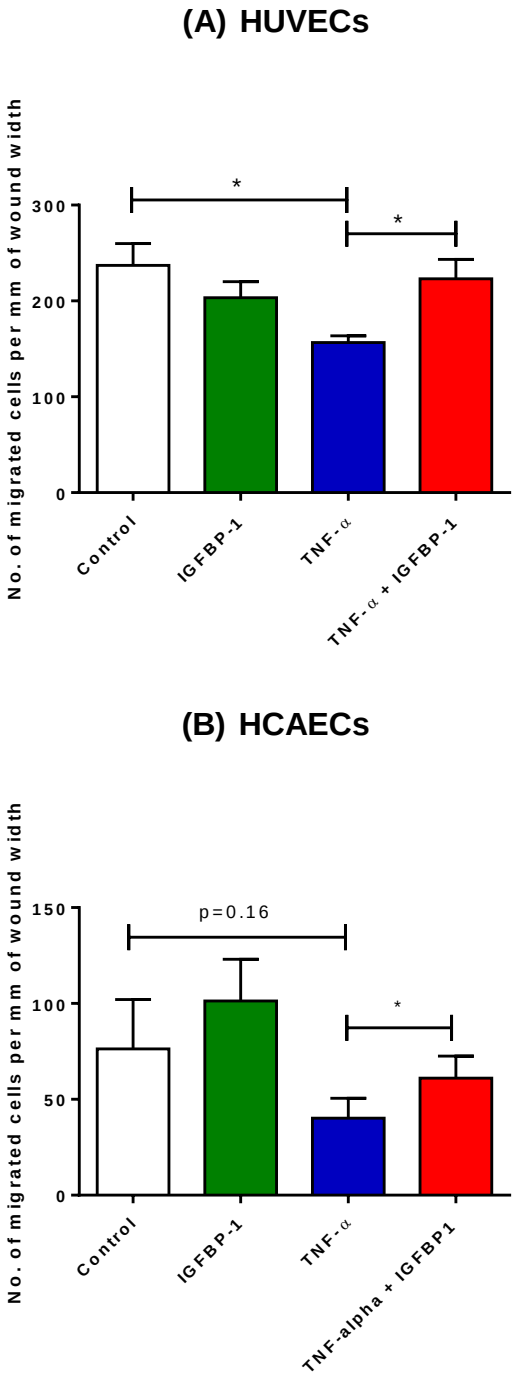


Figure 6-11 Effects of IGFBP-1 on migration of TNF-α-treated ECs in a linear wound assay.
(A) Effect of TNF-α (10ng/ml) and IGFBP-1 (500ng/ml) on migration of HUVECs at 48 hours, **p<0.01, *p<0.05; one-way ANOVA with Newman-Keuls post hoc test (n=9). (B) Effect of TNF-α (10ng/ml) and IGFBP-1 (500ng/ml) on migration of HCAECs at 48 hours *p<0.05; one-way ANOVA with Newman-Keuls post hoc test) (n=6).

6.4.4 Boyden chamber Assay

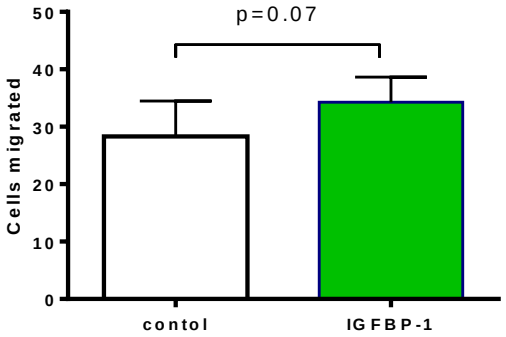
6.4.4.1 Effects of IGFBP-1 on EC chemotaxis

Having shown that IGFBP-1 modulated migration in a scratch wound assay, here whether IGFBP-1 acted as a chemotactic stimulus in a Boyden chamber assay was investigated. When IGFBP-1 (500ng/mL) was present in the lower chamber, a trend towards increased migration was seen in HCAECs (P=0.07) but not in HUVECs (Figure 6.12A and B).

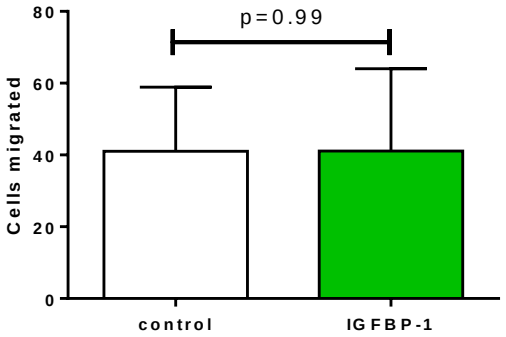
6.4.4.2 Effects of VEGF and IGFBP-1 on chemotaxis

Next, we investigated whether incubation with IGFBP-1 modulated the chemotactic response of ECs to other agents. VEGF is a potent chemo-attractant for endothelial cells and plays an essential role in angiogenesis. When VEGF (50ng/mL) was in the lower chamber, a significant increase in migration was observed (Figure 6.12C). When VEGF and IGFBP-1 were placed in the lower chamber, no additional effect on migration was observed (Figure 6.12C).

A



B



C

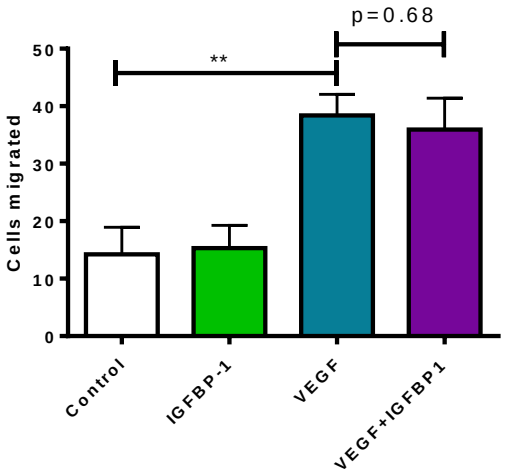


Figure 6-12 Effects of IGFBP-1 and VEGF on cell migration in HCAECs & HUVECs (Boyden Chamber).

(A) Effects of IGFBP-1 (500ng/ml) on cell migration in HCAECs P value =0.07 paired t-test, n=5, (B) Effects of IGFBP-1 (500ng/ml) on cell migration in HUVECs, P=0.99, paired t-test, n=3, (C) Effects of IGFBP-1 (500ng/ml) and VEGF (50ng/ml) on cell migration in HCAECs, **P<0.01, One way ANOVA with Newman-Keuls post hoc test, n=6.

6.4.5 Cell Proliferation Assay

6.4.5.1 Concentration dependent effect of foetal calf serum

Initial experiments were performed to define a sub-maximal EC proliferative response so either an increase or decrease could be detected when IGFBP-1 was applied in subsequent experiments. In HUVECs, an increase was observed in cell proliferation at increasing FCS concentrations at day 2 and day 5 (Figure 6.13). 2.5% FCS concentration was selected for subsequent experiments with insulin and IGFBP-1,

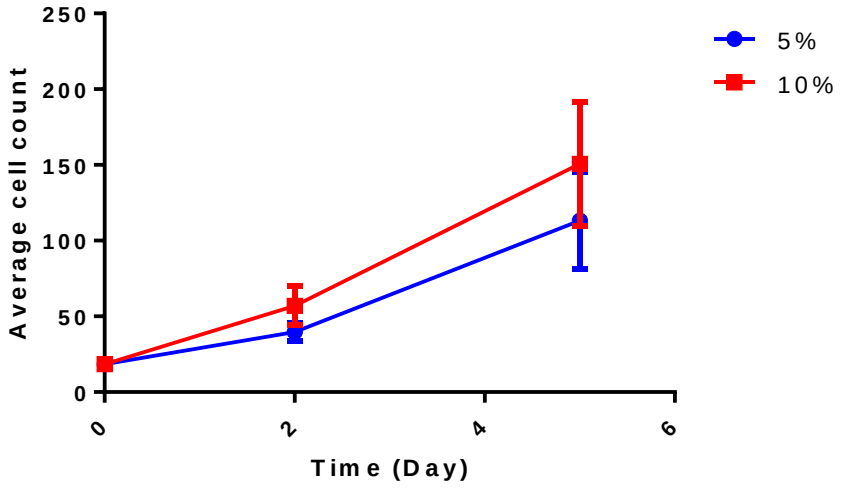
6.4.5.2 Effects of IGFBP-1 on EC proliferation

A strong trend was seen toward IGFBP-1 promoting proliferation of both HUVECs (P=0.076) and HCAECs (P=0.01). (Figure 6.14 and 6.15A)

6.4.5.3 In a pro-inflammatory setting IGFBP-1 reverses impaired EC proliferation

As in the linear wound assay, TNF-α incubation was again used to assess whether the modulatory effect of IGFBP-1 on cell proliferation was exaggerated in a pro-inflammatory setting. In HCAECs incubated in full (20%) ECGM, TNF-α (10ng/mL) significantly inhibited cell proliferation but appeared to induce apoptosis (n=3). The addition of IGFBP-1 to TNF-α at this concentration (10ng/mL) did not rescue cell proliferation (Figure 6.13A). A concentration-response experiment was then performed to select the optimum concentration of TNF-α at which to examine modulatory effects of IGFBP-1 (n=3) (Figure 6.15B). A sub-maximal anti-proliferative concentration of TNF-α (0.1ng/mL) was selected. At this lower concentration of TNF-α, IGFBP-1 significantly improved cell proliferation on par with vehicle-treated levels (n=6) (Figure 6.15C).

A



B

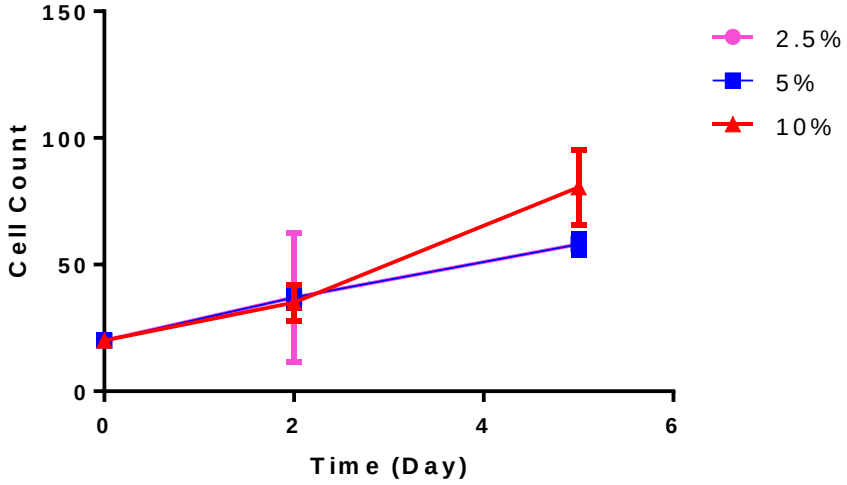


Figure 6-13 Concentration dependent effect of Foetal calf serum (FCS) on cell proliferation.
(A) Concentration dependent effect (5% vs 10% FCS) on cell proliferation in HUVECs, n=4 (B) Concentration dependent effect (2.5% vs 5% vs 10%) on cell proliferation on HUVECs, n=2.

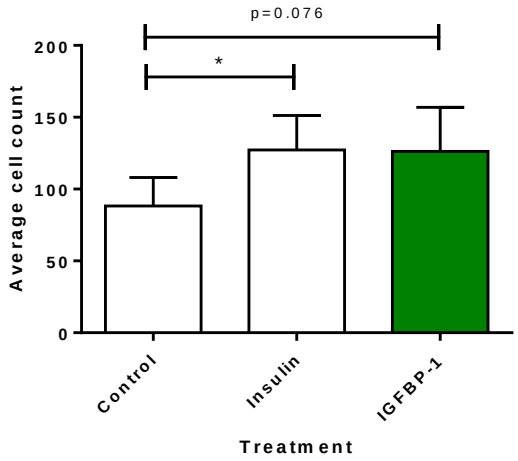
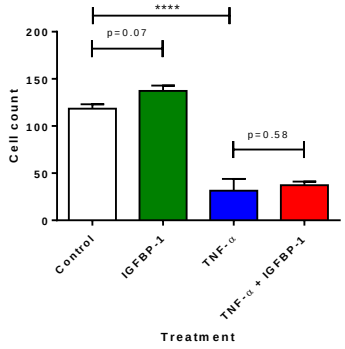
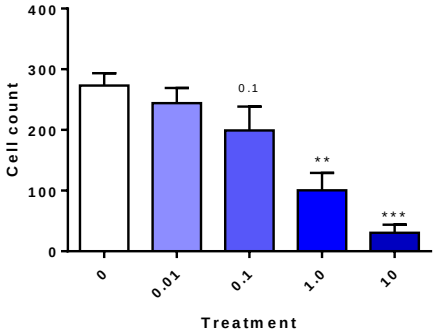


Figure 6-14 Effect of IGFBP-1 on cell proliferation in HUVECs.
Effect of IGFBP-1 (500ng/ml) on cell proliferation in HUVECs in (2.5% FCS) ECGM, insulin was used as a positive control. *p<0.05, one-way ANOVA with Newmans-Keul post hoc test, n=4.

(A) Effect of TNF-α (10ng/mL) on cell proliferation with and without IGFBP-1 in HCAECs



(B) Concentration-dependent effect of TNF-α on cell proliferation in HCAECs



(C) Effects of TNF-α (0.1ng/mL) on cell proliferation with and without IGFBP-1 in HCAECs

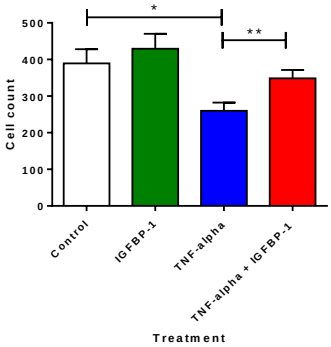


Figure 6-15 Effects of IGFBP-1 and TNF-α on cell proliferation in HCAECs.

(A) Initial experiment demonstrating the effect of TNF-α and IGFBP-1 (500ng/ml) on cell proliferation, n=3. (B) Concentration dependent effect of TNF-α on cell proliferation. Cells were incubated with decreasing concentrations of TNF-α, n=3 (except TNF-α = 0.01ng/ml, n=2). (C) Effect of TNF-α and IGFBP-1 (500ng/ml) on cell proliferation, ****p<0.0001, ***p <0.001, **p <0.01, *p <0.05. One-way ANOVA with Newmans-Keul post hoc test, n=6.

6.5 Discussion

The experiments conducted in this chapter provide a number of new insights in to the modulatory effects of IGFBP-1 on the properties of EC pertinent to endothelial regeneration. First, IGFBP-1 was shown to enhance regeneration of human saphenous vein segments by augmenting the adhesion of HCAECs to the denuded vein. Second, IGFBP-1 was shown to rescue the impairment of endothelial cell migration induced by a pro-inflammatory stimulus. Finally, IGFBP-1 was found to rescue the impairment of endothelial cell proliferation induced by a pro-inflammatory setting.

Effective endothelial regeneration of injured vessels is dependent on ECs adhering to the extra-cellular matrix. In this chapter, it was shown that pre-treatment of cells with IGFBP-1 significantly enhanced adhesion onto a native human saphenous vein matrix. The denuded vessel matrix consists of vascular smooth muscle cells and the extra-cellular matrix to which the ECs bind. The saphenous vein model is of particular interest as this correlates with the clinical setting. It mimics the changes which happen following coronary artery bypass graft surgery in humans, in which ECs in the vein are damaged and then repopulated by neighbouring endothelial cells. Identification of a pro-reparative effect of IGFBP-1 in this model supplements the data derived in mice in chapter 3 and suggests the potential for translation in to clinical studies. However, further studies using the saphenous vein model could first be considered. For example, the rate of re-endothelialisation by human 'late-outgrowth' APCs pre-treated with IGFBP-1 could be investigated with this model, as Xu *et al* successfully showed that de-cellularised human internal mammary artery samples could be transplanted into a murine model and are repopulated after 2-4 weeks by cells expressing endothelial cell markers suggestive of angiogenic progenitor cells[292].

In the saphenous vein model, ECs adhere to denuded vascular matrix. Although this is biologically relevant, adhesion to specific matrix components (e.g. fibronectin and collagen) could also be investigated individually.

Adhesion of ECs to extra-cellular matrices is dependent on the action of cell surface integrins. Integrins are heterodimeric transmembrane adhesion receptors comprising α and β subunits, which play an important role not only in adhesion but also in migration[139]. Experiments investigating the effect of IGFBP-1 on cell surface expression of integrins are presented in the next chapter.

The *in vitro* studies assessed endothelial cell migration using a linear wound assay and modified Boyden Chamber assay. HUVECs were used initially as these are most widely studied ECs, are readily available and are relatively simple to culture. However HUVECs are of foetal origin and are venous cells, factors which may limit the translation of experiments conducted in HUVECs to arterial regeneration in adult humans. It is recognised that adult ECs have a different phenotype to HUVECs due to exposure to hormones and cytokines[293]. In later experiments, HCAECs were employed as they are adult cells which are derived from coronary arteries. HCAECs potentially provide a more relevant insight into endothelial cell reparative functions as endothelial dysfunction and atherosclerosis mainly affect arteries. It may be considered good practice, as here, to replicate experiments in ECs derived from more than one primary source, as ECs do differ widely in morphology and function[294]. From the results presented, it is interesting to note that in the linear wound assay the HCAECs migrated at a slower rate compared to HUVECs, possible because HCAECs are mature cells and have less regenerative capacity.

The experiments conducted in this chapter found that IGFBP-1 does not modulate EC migration in healthy cells. In the modified Boyden Chamber assay, IGFBP-1 did not promote EC migration. However, this finding should be interpreted with caution as this assay investigates migration in response to a chemotactic stimulus. One perhaps would not expect there to be a chemotactic effect of IGFBP-1 as *in vivo* there is unlikely to be a natural concentration gradient of IGFBP-1 in injured vessels. However, neither did IGFBP-1 modulate EC migration to the known chemotactic stimulus VEGF. Pre-incubation of IGFBP-1 was also investigated prior to seeding and was

not found to modulate the migratory response of ECs in the Boyden Chamber assay.

The linear 'scratch' wound assay is arguably a more appropriate assay as to a degree the assay mimics cell migration *in vivo.* Here, ECs migrate to areas of denuded endothelium to close the wound. In the linear wound assay, there was no significant effect of IGFBP-1 on migration of 'healthy' cells, perhaps because there was no 'damaging' stimulus similar to interpretation of the *in vivo* studies comparing femoral arterial regeneration in WT and IGFBP-1 mice described in Chapter 4. Another possibility could be that IGFBP-1 binds to IGFs in the growth medium. IGF-1 on its own has been shown to enhance endothelial cell migration[295]. In this case, inhibition of IGF bioavailability by IGFBP-1 could potentially counteract any IGF-independent effects of IGFBP-1 on cell migration. To investigate this possibility further, synergy studies with and without IGF-1 and IGFBP-1 would need to be conducted. Synergy studies using des-IGF-1[296] would be particularly informative. Des-IGF-1 is an IGF-1 variant, which has a very low affinity for binding proteins.

A potential limitation from the in vitro studies are the growth factors being present in the Endothelial Cell Growth Media, especially whether IGF-1 or IGFBP-1 are already present and whether the effects of IGFBP-1 being applied are inhibited or enhanced. It was not known by the manufacturers whether IGF-1 or IGFBP-1 were present in the growth media. One could have performed ELISA studies assessing IGFBP-1 and IGF-1 levels of the growth media used or could have added IGF-1 receptor antibodies to ensure that adding IGFBP-1 would have not bound to IGF-1 and therefore inhibit the actions of IGFBP-1.

Having observed no modulatory effect of IGFBP-1 on migration of 'healthy' or 'insulin-sensitive' EC, the linear wound assay was repeated following incubation of ECs with TNF-α to compromise EC function and to induce a pro-inflammatory stimulus characteristic of IR. As discussed in chapter 1, pro-inflammatory cytokines such as TNF-α, Interleukin-1,-6,-8, are

upregulated in IR and have been implicated in vascular pathology[297–299]. In this chapter, TNF-α was shown to inhibit cell migration significantly in HUVECs with a trend to inhibition of migration observed in HCAEC. In both types of primary cells, incubation with IGFBP-1 was found to rescue cell migration to control levels. These findings suggest that IGFBP-1 exerts a modulatory effect on EC migration which appears to be specific for a pro-inflammatory state characteristic of IR.

Certain limitations from the studies reported in this chapter must be acknowledged. TNF-α concentrations were not measured in IRKO mice in Chapter 4. Such information would be useful to better correlate the *in vitro* data generated in this chapter with the *in vivo* data in Chapter 4. However, it has been shown that raised TNF-α levels are present in other insulin-resistant mice[300].

We selected incubation with TNF-α as this cytokine is increased in individuals with IR, correlates with endothelial dysfunction and cardiovascular events and is known to impair insulin signalling in EC[86,301]. However, other methods of inducing *in vitro* insulin resistance in ECs could have been considered. For example, ECs could be pre-treated with insulin receptor blocking antibodies or incubated with palmitate, a free fatty acid which is increased in insulin resistant states *in vivo*. Other options include culturing cells in high insulin or high glucose concentrations. Although time pressures precluded these experiments being conducted in this thesis, ideally the *in vitro* experiments should be repeated to confirm these effects in other models of IR.

The linear wound assay is an inexpensive method and easy to perform, however there is a limitation presented by the manual nature of the wound formed. Other methods of cell migration are available – for example the stamping assay, in which an EC monolayer is punched to form a reproducible and consistent pattern of injury. Also, an optical method is available in which a laser induces photomechanical effects on the cell layer[302]. This is computer controlled and has the advantages of consistency

reproducibility, automatic analysis of wound healing and the ability to create differing wound shapes[302].

The *in vitro* studies also assessed the effects of IGFBP-1 on cell proliferation in ECs. There was a trend towards enhanced cell proliferation when ECs were incubated with IGFBP-1. Having observed restoration of the inhibitory effect of TNF-α on EC migration with the addition of IGFBP-1, the same conditions were applied in the cell proliferation assay. TNF-α has also been observed to inhibit cell proliferation *in vitro* in studies[303]. Using the same concentration of TNF-α as in the linear wound assay (10ng/mL) provoked toxicity in ECs, probably due to cells requiring continued exposure for 5 days in proliferation assays rather than 48 hours in the linear wound assay. It is likely that any potential benefits of IGFBP-1 on proliferation were masked at this concentration of TNF-α. It was therefore necessary to perform a TNF-α concentration-response experiment to select a concentration at which the anti-proliferative effects of TNF-α were sub-maximal and apoptosis could be avoided. From this set of studies, a TNF-α concentration of 0.1ng/mL was chosen. The cell proliferation assay was then repeated to demonstrate that impaired proliferation induced by TNF-α, was significantly enhanced by co-incubation with IGFBP-1. Other proliferation assays are available, such as a DNA synthesis cell proliferation assay where 5-bromo-2'-deoxyuridine (BrdU) is applied to cells and the BrdU becomes incorporated into newly formed DNA. However, the direct cell counts performed here are superior to DNA synthesis assays because an assumption is made with the DNA assays that cells have divided. In addition, effects of TNF-α may not have been apparent at the shorter time points typically used with the DNA assay.

In summary, the data presented in this chapter demonstrate multiple effects of IGFBP-1 on functional properties of ECs contingent with effective endothelial repair, albeit apparently specific for a pro-inflammatory setting. The potential mechanisms underpinning the effects of IGFBP-1 on endothelial responses such as migration and adhesion will be investigated in the next chapter.

7 Mechanistic pathways and effects of IGFBP-1 on endothelial repair

7.1 Introduction

So far in this project IGFBP-1 has been demonstrated to enhance endothelial regeneration in an insulin resistant setting *in vivo*, enhance endothelial cell migration, proliferation in a pro-inflammatory environment *in vitro* and augment EC adhesion *ex vivo*. The effects of IGFBP-1 on endothelial regeneration are likely to be mediated by modulation of endothelial cells rather than angiogenic progenitor cells. It is therefore important to determine the underlying mechanistic basis by which IGFBP-1 improves endothelial repair processes such as adhesion, migration and proliferation.

Cell migration is a coordinated process that involves rapid changes in actin filament dynamics, together with the formation and disassembly of cell adhesion sites[141]. External stimuli that affect cell migration are transduced into intracellular biochemical signals through the interaction of transmembrane integrins which bind to the extra-cellular matrix (ECM). Integrin activation allows the functional connection between focal adhesions and actin cytoskeleton that is needed for cell migration[304].

Remodelling of actin, the major cytoskeletal component, is seen as the central factor involved in EC migration. Actin is composed of 43-kDa monomeric subunits (G-actin) that polymerize into filaments (F-actin). The actin cytoskeleton constantly remodels into filopodia, lamellipodia and stress fibres, which are essential for cell migration. The Rho family GTPases RhoA, Rac and Cdc42 are major regulators of the actin cytoskeleton and cell migration[140] and are the most studied members[157]. These members are molecular switches cycling between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state to activate the GTPase[158], RhoA induces the formation of actin stress fibres and focal adhesions and stimulates cell contraction through downstream effectors ROCK and mDia. Rho-kinase (ROCK) is an effector of GTPase RhoA[159], whilst Rac and Cdc42 regulate the polymerisation of actin to form peripheral lamellipodial and filopodial protrusions[158].

Integrins are a family of cell adhesion molecules, which interact with the ECM or with adhesion molecules on other cells[135]. In all, 24 integrins have been identified and each is a heterodimer of an α and a β subunit[135]. Integrins exhibit both inside-out/outside-in (bidirectional) signalling, i.e. signals within the cell cause integrins to change which leads to integrin activation and therefore an increased affinity for extracellular ligands. In the opposite direction, integrins binding to their ligands can initiate change to their cytoplasmic domains and activate multiple signalling pathways[136]. An ECM protein such as fibronectin can engage several integrins, the most commonly expressed fibronectin-binding integrins are $\alpha_V\beta_3$ and $\alpha_5\beta_1$, both bind to the RGD sequence within fibronectin[134].

Focal adhesion kinase (FAK) was identified in 1992 in normal cells as a highly tyrosine-phosphorylated protein that localised to integrin-enriched cell adhesion sites known as focal adhesions[141]. Focal adhesions are formed at ECM-integrin junctions and bring together cytoskeletal and signalling proteins during the processes of cell migration and adhesion. Early studies showed that FAK could be activated by growth factors and phosphorylation of FAK is a rapid event associated with focal adhesion formation[143]. FAK regulates cell migration through a number of pathways but in particular through affecting the Rho subfamily of small GTPases and the assembly/disassembly of actin cytoskeleton[148]. It has also been shown that FAK-deficient cells spread slower on ECM, exhibit an increased number of prominent focal adhesions and migrate poorly in response to haptotactic and chemotactic signals[144–147].

Data presented in this thesis show that IGFBP-1 enhances EC adhesion, and promotes EC migration and proliferation in a pro-inflammatory setting. IGFBP-1 has previously been shown to promote cell migration in non-vascular cells independently of IGFs via the Arg-Gly-Asp (RGD) motif present in its C-domain[305]. This RGD motif is an integrin recognition sequence and can bind to integrins such as $\alpha_5\beta_1$ on the cell surface leading to integrin-mediated signalling. Therefore a potential mechanism for the findings reported in this thesis is that IGFBP-1 mediates its effects on

endothelial cells via integrin signalling which in turn phosphorylates FAK. In this chapter, the role of IGFBP-1 in modulating integrin expression, FAK phosphorylation, RhoA activity and cytoskeletal rearrangement will be examined.

7.2 Aims

The aims of this chapter are to explore potential candidates by which IGFBP-1 enhances adhesion, migration and proliferation.

The following will be investigated in detail:

1. The effects of IGFBP-1 on FAK phosphorylation and RhoA activity
2. The role of IGFBP-1 on the actin cytoskeleton in ECs and additionally on integrin expression on the EC surface

7.3 Methods

7.3.1 Western blot analysis of endothelial cells assessing FAK phosphorylation

Western blot analysis, undertaken by Mrs Jessica Smith who had previously optimised the experimental conditions in our laboratory, was used to quantify FAK phosphorylation in HUVECs. After 4 hours of serum-starvation, endothelial cells were treated with IGFBP-1 for 15 minutes. VEGF (10 minutes) served as a positive control. Vehicle treated cells were used as a negative control. Protein was extracted in lysis buffer and quantified using the protein Bicinchoninic Acid (BCA) assay (Sigma-Aldrich, Gillingham, UK). Then, 30µg of protein were separated by electrophoresis through 4–12% SDS-PAGE gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred onto polyvinylidene fluoride membranes. Membranes were probed with primary antibodies (FAK antibody 1:1000 (#3285, Cell Signalling technology, USA and phosphoFAK antibody 1:1000 (#8556S, Cell Signalling technology, USA), and 1:3000 actin antibodies (BD Bioscience), as previously described[211]. The steps are described in detail as follows:

7.3.1.1 Total protein quantification

Equal protein concentration was estimated using the Bicinchoninic Acid assay (Pierce, Rockford, USA). Initially 12.5µL of each lysate sample were diluted 12-fold in distilled water in a centrifuge tube. 25 µL of this solution were then loaded onto a 96-well plate in duplicate. Standards were obtained by serial dilution of 2mg/mL BSA (Sigma-Aldrich, Gillingham, UK) (0, 25,125, 250, 500, 750, 1000, 1500 and 2000µg/mL) and loaded in duplicate. 200µL of 50:1 BCA:4% copper (ii) sulphate were then pipetted into each well. The amount of copper reduced is proportional to the amount of protein present in the solution, reduced copper is chelated by BCA to form a purple coloured complex. After covering the plate with sealing foil, the plate was incubated at 37^0C for 30 minutes. After this time period, absorption spectra were measured by colorimetric assay at 562nm. Protein content was calculated

by reference to the standard curve, with correction for the initial 5-fold dilution. Co-efficient of variation between duplicates was only accepted at <10%, and R^2 value of 0.99 for standards.

7.3.1.2 Protein gel-resolution transfer

Lysate sample volume containing 30µg of protein was calculated as above and boiled for 5 minutes after mixing with appropriate volumes of 4X loading buffer and 10X reducing agent (NuPage, Invitrogen, CA). Reduced samples were chilled on ice, vortexed and then pulse-centrifuged, and finally loaded onto 4-12% SDS-PAGE gels (Invitrogen). One well for reference was loaded with a protein standard ladder. Samples were resolved through electrophoresis at 160V for 60 minutes using a gel running system (Invitrogen) bathed in running buffer (NuPAGE, Invitrogen). Next, the gel with resolved protein was extracted from the gel-case and placed over a PVDF membrane (Millipore, MA) soaked in methanol (15 seconds), distilled water (2 minutes) and transfer buffer (2 minutes; see appendix)**.** The gel / membrane were then sandwiched between 3mm-thick filter papers with sufficient pressure to ensure firm contact, and placed in a transfer-cassette orientated to ensure correct direction of transfer. The cassette was bathed in chilled transfer buffer conducting 90V for 90 minutes.

7.3.1.3 Western immunoprobe

Membranes were re-activated by immersing in methanol for 15 seconds and then placing in water for 2 minutes and then TBS/Tween-20 (5 minutes; see appendix). The membrane was then blocked for non-specific proteins by washing in 10mL of 5% non-fat milk / TBS / Tween-20 solution for 1 hour, using a container on a roller at room temperature. Blocked membranes were then transferred to a new container with 5mL 5% milk / TBS / Tween-20 solution containing the primary antibody of interest (1:1000 FAK antibody, 1:1000 phospho-FAK antibody and 1:5000 actin antibodies). After being probed overnight at 4^0C by washing on a roller, the membrane was washed in TBS / Tween solution on a rocking device for 3 cycles of 10 minutes each,

with fresh solution at each cycle. Next, the membrane was probed by a secondary rabbit anti-mouse antibody conjugated to HRP (1:5000) (Dako) in fresh 5mL 5% milk / TBS / Tween solution for 1 hour at room temperature. TBS / Tween washes were performed and repeated three times to remove excess antibody. The membrane was then superficially covered with chemiluminescent substrate solution (Millipore) for 5 minutes in darkness, after which excess solution was allowed to run off and the membrane sandwiched between 2 translucent acetate films. Fluorescence was then detected by exposure of the sandwiched membrane to a Kodak Image Station 2000R. The blot densities were analysed with Image J (version 1.47) software (NIH, USA). Band densities were quantified and background signal subtracted. Proteins of interest (FAK and phospho-FAK**)** were normalised to same-sample actin density as a control.

7.3.2 RhoA Activity Assay

The RhoA activity assay was performed using a Rho G-LISA kit (Cytoskeleton Inc., Denver, Colorado, USA) which contains a Rho-GTP-binding protein linked to the wells of a 96-well plate.

Initially, HCAECs were seeded at 100,000 cells/well into a 6 well fibronectin-coated plates. Once they achieved 80% confluence cells were serum starved overnight and then treated with IGFBP-1 for different time points (0 minutes, 10 minutes, 20 minutes and 40 minutes). After treatment, the medium was aspirated and washed thrice with ice-cold PBS being especially careful to remove all residual PBS. Cells were then lysed with 120 µL of ice-cold lysis buffer (1:100 of Protease inhibitor:lysis buffer), harvested and transferred into microcentrifuge tubes on ice. The samples were centrifuged at 10,000g, 4 °C for 2 minutes. 20µL of lysate were taken off and stored at 4 °C for protein quantification and the remainder used for the RhoA activity assay by the RhoA ELISA (Cytoskeleton, Inc., Denver, Colorado, USA).

7.3.2.1 Total protein quantification

It is important to measure the total protein concentrations of the cell lysates to ensure standardisation of results. Using a 96-well plate, 10µL of lysis buffer were added into the top 2 wells, and then 10µl of each sample place in duplicates. 300 µL of Precision RedTM Advanced Protein Assay Reagent were added into each well and left to incubate for 1 minute at room temperature. After this time period, absorbance was then measured at 595nm.

7.3.2.2 Running G-LISA Assay

The next step was to set up a standard curve, this consisted of a blank sample (60µL lysis buffer and 60µL Binding buffer = lysis buffer solution), sample 0.25 (15µL of a pre-made mix {24 µL Rho Control Protein + 96µL lysis buffer} + 45 µL lysis buffer solution), sample 0.5 (30 µL pre-made mix + 30 µL lysis buffer solution) and sample 1 (60 µL of pre-made mix). Next, 60 µL of Binding buffer were added to each sample. Using the assay strips, the powder in the wells was dissolved with 100µL of milliQ water, whilst the plate with the strips was kept on ice.

Using the treated cell lysate samples, 60 µL were placed into a fresh microcentrifuge tube and then 60 µL of binding buffer were added to each sample. With the 96-well plate, water was completely removed by a series of vigorous pats onto a paper towel. This is an important step as complete removal of solution from the wells is required between steps of the G-LISA as this avoids high background readings in the buffer only wells. 50 µL of each sample in duplicate were added including the standard curve samples onto each well and the plate placed on a cold orbital microplate reader (200rpm) at 4°C for 30 minutes.

After 30 minutes, the solution was removed and washed twice with 200 µL of wash buffer with vigorous removal after each wash. 200 µL of Antigen Presenting Buffer were pipetted into each well and incubated at room temperature for exactly 2 minutes, followed by vigorous removal and washed

thrice with 200 µL of wash buffer. 50 µL of diluted anti-RhoA primary antibody (1/250 ratio, 2 µL of anti-RhoA primary antibody to every 500 µL Antibody Dilution buffer) were added to each well and the plate was placed on the orbital microplate shaker (200rpm at room temperature) for 45 minutes.

Following this, the solution was removed vigorously, washed thrice with 200 µL of Wash Buffer and 50 µL of diluted secondary antibody (1/62.5 ratio, 8 µL of secondary HRP labelled antibody to every 500 µL Antibody Dilution buffer) were added to each well and the plate was placed on an orbital microplate shaker (200rpm at room temperature) for 45 minutes. Following this, the secondary antibody solution was removed vigorously and the wells washed thrice with 200µL of Wash Buffer. 50µL of HRP detection reagent were added to each well and incubated at 37°C for 15 minutes. Then 50µL of HRP Stop buffer were added to each well. The signal was immediately read by measuring the absorbance at 490nm using a microplate spectrophotometer.

7.3.3 Visualisation of cytoskeletal re-arrangement

HCAECs were seeded at 3000 cells/well into 1% gelatin-coated chamber slides in full (20% FCS) ECGM for 48 hours. After gentle washing with PBS, cells were treated in 10% FCS ECGM with IGFBP-1 (500ng/mL) or TNF-α (10ng/mL). After treatment for 24 hours, HCAECs were washed with PBS twice before fixation in 4% paraformaldehyde for 10 minutes and washed with PBS twice again. The cells were permeabilised and blocked in PBS containing 10% goat serum and 0.05% Triton X-100 (this allows the rhodamine phalloidin to enter the cells but prevents non-specific binding). After washing twice with PBS, fixed cells were treated with a fluorescent marker to stain the F-actin cytoskeleton (rhodamine phalloidin, 1:40) in PBS for 20 minutes in the dark. After 20 minutes the solution was removed and the slide was then mounted on to a cover slip using ProLong Gold. The slide was then left overnight in the dark and incubated at room temperature to allow the ProLong Gold to set and therefore be able to obtain images.

Stained cells were visualised using 20× objective on a LSM510 Axiovert 200M high throughput fluorescence microscope the following day. Interpretation was assessed subjectively through observing cytoskeletal arrangement.

7.3.4 Integrin-mediated cell adhesion assay

Effects of IGFBP-1 on cell surface expression of subunit or heterodimer integrins were quantified using an integrin-mediated cell adhesion array kit (ECM532, Millipore, Billerica, Massachusetts, USA). In the assay, mouse monoclonal antibodies directed against specific human subunit (α_1, α_2, α_3, α_4, α_5, α_V, β_1, β_2, β_3, β_4, β_6) or heterodimer ($\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_5\beta_1$) integrins were immobilised to the surface of the wells using goat anti-mouse antibody. Negative wells were coated with the goat anti-mouse antibody only. HCAECs were grown to confluence in 75cm^3 flasks and harvested using Gibco® Cell Dissociation Buffer. HCAECs were co-incubated with or without IGFBP-1 for 1 hour before 100,000 cells were added to the integrin antibody-coated and control wells and incubated for 2 hours at 37°C. Unbound cells were then washed off and the adherent cells stained. The optical density of nuclear stain extracts was measured at 540nm (OD540nm) on a MRX TC 2 microplate reader (DYNEX Technologies, U.K.).

Prior to starting the assay, the strips from the kit were rehydrated with 200 µL of PBS per well for 10 minutes at room temperature. The PBS was removed from the strip by turning over the plate and gently tapping out the PBS. HCAECs were harvested using Gibco® Cell Dissociation Buffer and rinsed twice with Gibco® Cell Dissociation Buffer. After re-suspension, cells were re-suspended in Assay Buffer and aliquoted into 1,000,000 cells/mL and were treated and incubated with vehicle or IGFBP-1 (500ng/mL) for 1 hour. After 1 hour, the cell suspension was gently mixed and 100 µL applied to each well of the mouse anti-α or anti-β integrin capture and control wells in duplicate. The plate was then incubated for 2 hours at 37°C in a CO_2 incubator, followed by gentle aspiration and washed 3 times with 200 µL of Assay Buffer. 100 µL of cell stain solution were added to each well and allowed to incubate for 5 minutes at room temperature, followed by gentle aspiration and washed 5 times with deionized water, aspirated, allowed to dry and 100 µL of Extraction Buffer added to each well. The plate was incubated on an orbital shaker at room temperature until the cell-bound stain

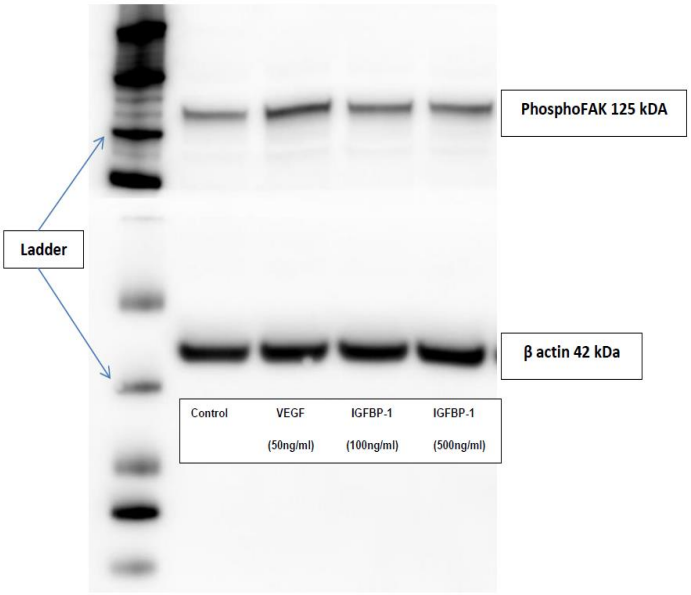
was completely solubilised, which approximately took 5-10 minutes. The absorbance was determined at 540-570 nm on a microplate reader.

7.4 Results

7.4.1 The role of IGFBP-1 in focal adhesion kinase phosphorylation

Because phosphorylation of FAK is critically implicated in integrin signalling and EC migration, western blotting was employed to assess whether acute exposure to IGFBP-1 induces FAK phosphorylation. There was a strong trend towards IGFBP-1 induced FAK phosphorylation in HUVECs (Figure 7.1) suggesting that integrin-FAK signalling may contribute to the functional effects on EC migration observed in Chapter 6.

A



B

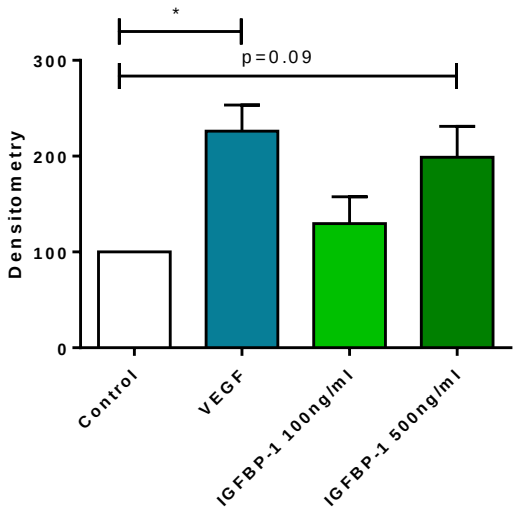


Figure 7-1 Evaluation of FAK phosphorylation in HUVECs.

IGFBP-1 (100ng/ml and 500ng/ml) concentration-dependently stimulated FAK phosphorylation in HUVECs, VEGF was used as a positive control. (A) representative Western Blot showing FAK phosphorylation in HUVECs in tyrosine 397 residue. Beta actin is presented as a loading control. (B) mean data of FAK phosphorylation (values normalised to beta actin), *p<0.05**;** one-way ANOVA with Newman-Keuls post hoc test; n=3.

7.4.2 IGFBP-1 activates RhoA

RhoA is a small GTP-binding protein which regulates actin cytoskeleton in the formation of stress fibres and plays a role in cellular motility. HCAECs were serum-starved overnight and exposed to IGFBP-1 for different time intervals. A significant rapid yet transient activation of RhoA in HCAECs (n=5) following exposure to IGFBP-1 (Figure 7.2) was observed, which returned to baseline levels within 40 minutes. RhoA was activated within 10 minutes of exposure to IGFBP-1 and was maintained at 20 minutes.

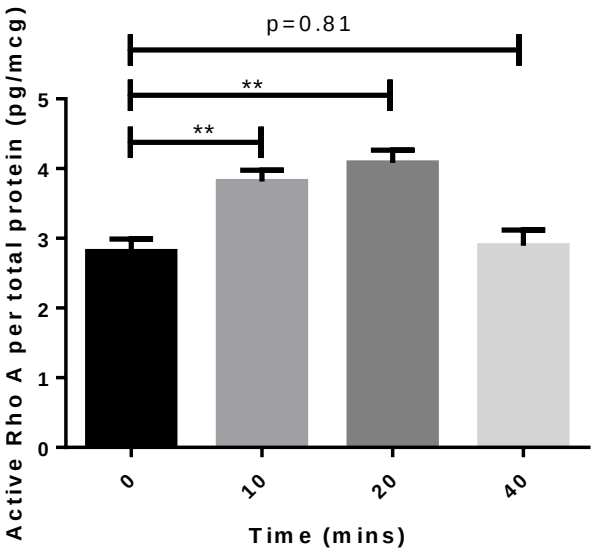


Figure 7-2 RhoA activation of HCAECs in response to IGFBP-1.
HCAECs were exposed to IGFBP-1 (500ng/ml) at different time points and RhoA activity was measured at the times indicated (0 = serum starved cells with no IGFBP-1 treatment). **p<0.01 vs unstimulated cells; one-way ANOVA with Newman-Keuls post hoc test, n=5.

7.4.3 Rhodamine Phalloidin staining of endothelial cells

Having shown that IGFBP-1 improves EC migration, proliferation and adhesion, cytoskeletal remodelling in response to IGFBP-1 was investigated next. The clear effect on cell migration suggests a potential role for IGFBP-1 in modulating cyto-skeletal rearrangement. To investigate this, ECs were stained with rhodamine phalloidin to visualise the F-actin cytoskeleton. Pilot studies were performed investigating the correct density of cells to seed initially as if the wells were too confluent it may not prove possible to visualise individual cells completely. 5000 cells/well were used initially, this led to a confluent well and therefore difficulty in visualising individual cells. Cell density was then reduced to 3000 cells/well. Initial studies investigated whether TNF-α disrupted the actin cytoskeleton of HCAECs after 24 hours of incubation. TNF-α (10ng/mL, 24 hours) was qualitatively noted to induce substantial disruption of the cytoskeleton (Figure 7.3B and 7.3C).

IGFBP-1 had no discernable effect on the cytoskeleton of quiescent cells (Figure 7.4B). However, IGFBP-1 (500ng/mL, 24 hours) appeared to almost completely ameliorate the cytoskeletal disruption induced by TNF-α (Figure 7.4D).

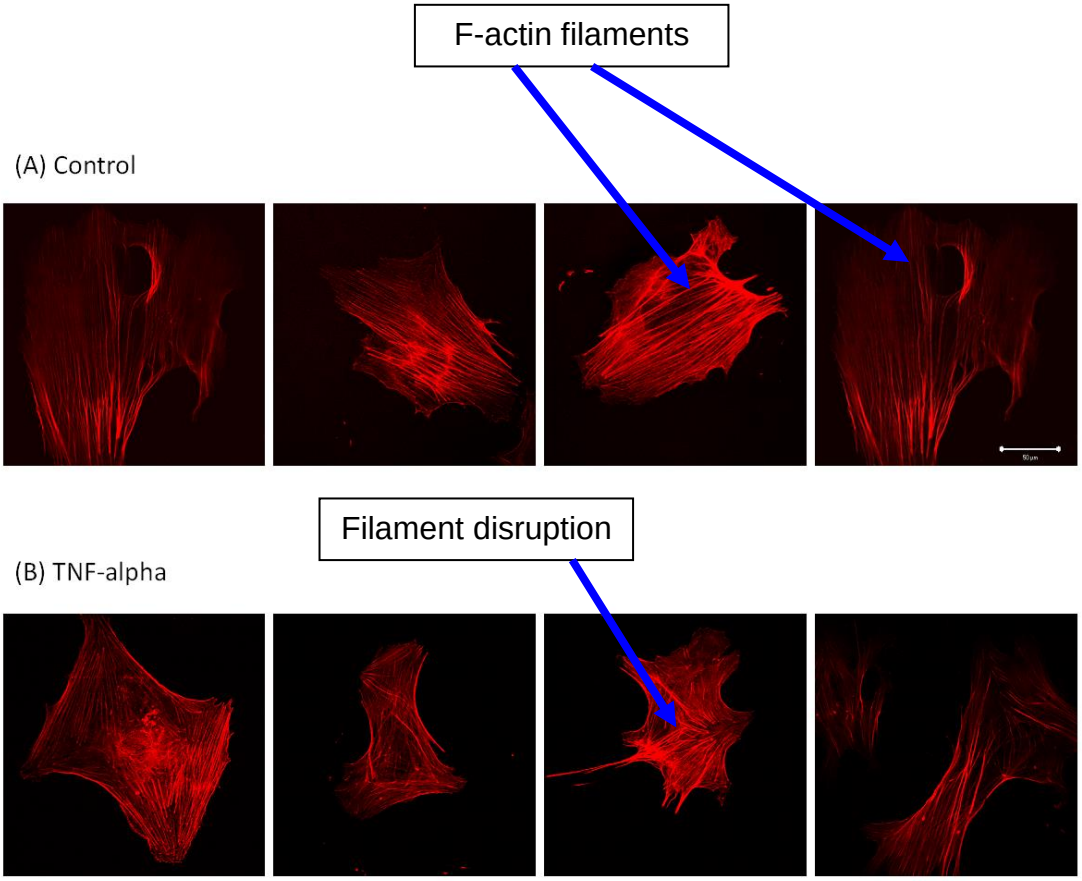


Figure 7-3 Rhodamine Phalloidin staining of HCAECs with TNF-α. Representative images (200x magnification) showing the actin cytoskeleton of control HCAECs (A) and those exposed to TNF-α (10ng/ml) for 24 hours (B). Possible TNF-α induced cytoskeletal disruption after 24 hours, n=3.

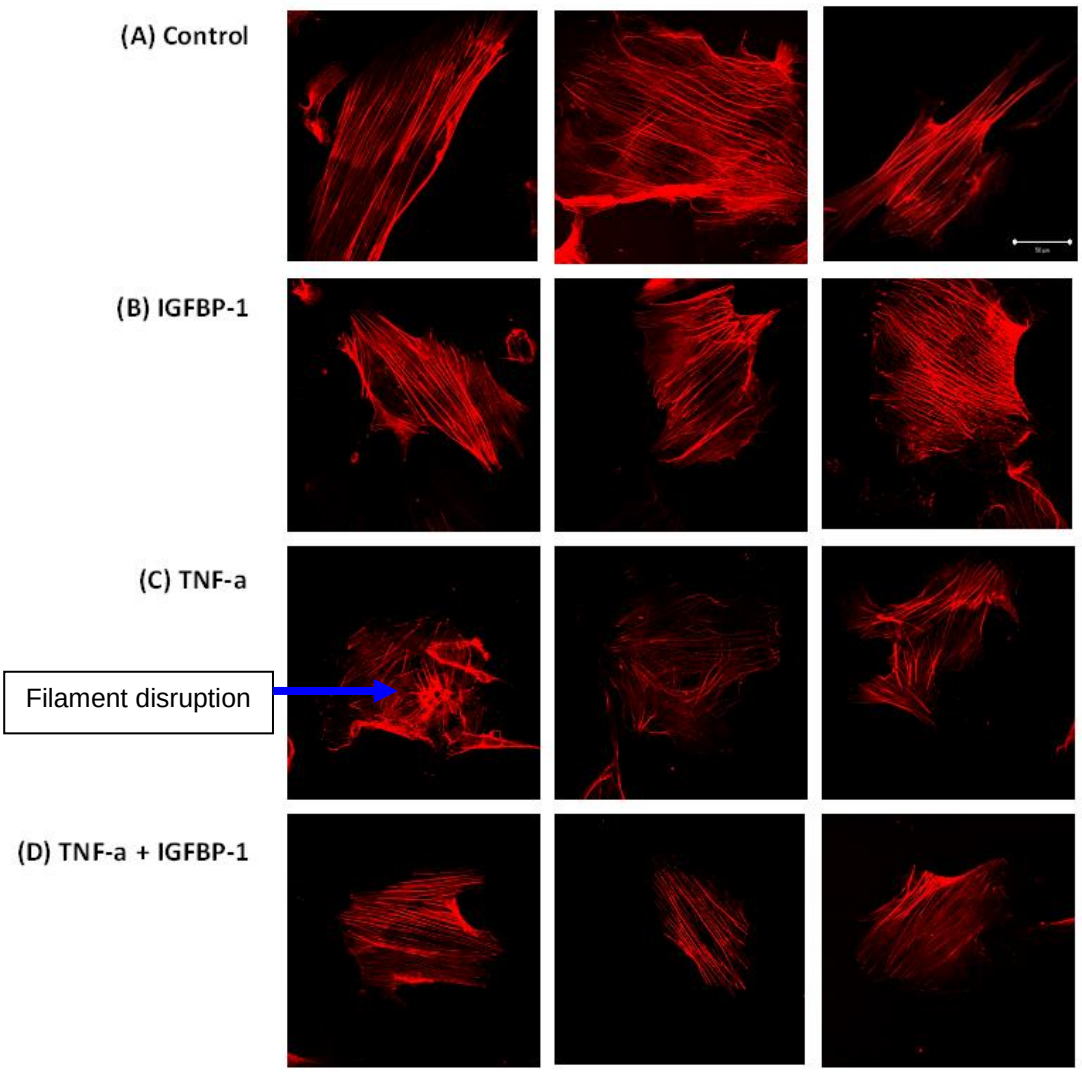
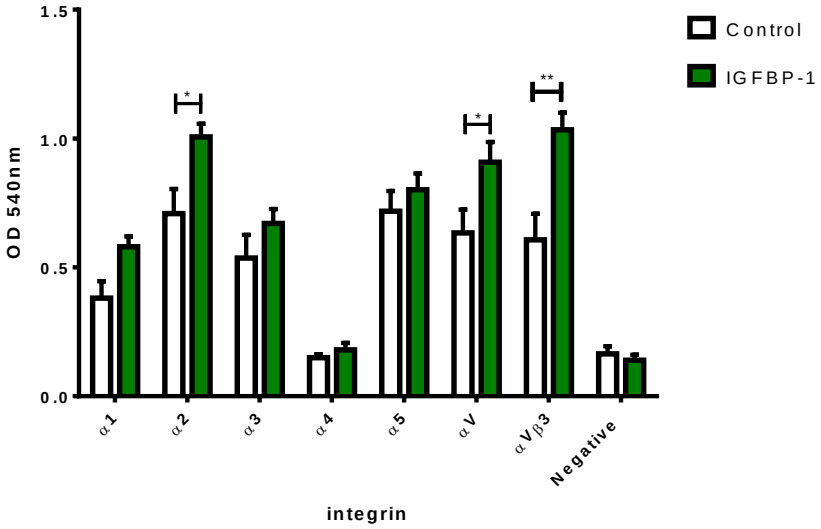


Figure 7-4 Rhodamine Phalloidin staining of HCAECs with TNF-α and IGFBP-1.
Representative images (200x magnification) showing the actin cytoskeleton of (A) control HCAECs and those exposed to (B) IGFBP-1 (500ng/ml) (C) TNF-α and (D) TNF-α + IGFBP-1 (500ng/ml) for 24 hours. IGFBP-1 (500ng/ml) alone seemed to have no effect on cytoskeletal rearrangement. TNF-α appears to have caused disruption of the cytoskeleton which then appeared to be partially restored with co-incubation of IGFBP-1 (500ng/ml), n=4.

7.4.4 IGFBP-1 increases cell-surface expression of $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins

In the previous chapter, IGFBP-1 was shown to increase endothelial cell adhesion. This raises the possibility that IGFBP-1 may increase cell-surface availability of integrins capable of binding extra-cellular matrix. Here, incubation of HCAEC with IGFBP-1 (500ng/mL) for 1 hour significantly increased cell-surface expression of $\alpha_5\beta_1$, α_2, α_V (Figure 7.5A) and $\alpha_V\beta_3$ integrins (Figure 7.5B) (n=6).

A



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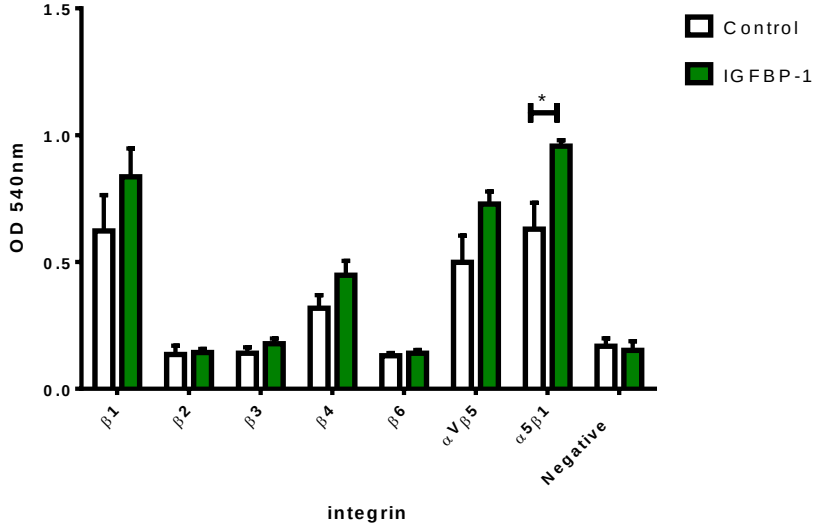

Figure 7-5 Effects of IGFBP-1 on cell surface integrin expression in HCAECs.

HCAECs were incubated with IGFBP-1 (500ng/mL) for 1 hour before quantifying cell-surface expression of a panel of integrins (A) Effects of IGFBP-1 on cell surface α integrin expression in HCAECs (B) Effects of IGFBP-1 on cell surface β integrin expression in HCAECs, *p<0.05 and **p<0.01; Paired t-test; n=6.

7.5 Discussion

This chapter extends the functional insights reported in the preceding chapters by providing a number of key mechanistic insights into the influence of IGFBP-1 on key signalling nodes and cytoskeletal changes in ECs. First, IGFBP-1 was shown to potentially phosphorylate the signalling node FAK which plays a critical role in cell proliferation and migration. Second, IGFBP-1 was found to activate RhoA which mediates cytoskeletal rearrangements necessary for EC motility. Third, IGFBP-1 was seen to potentially restore partial disruption of actin remodelling induced by the pro-inflammatory cytokine TNF-α. Finally, IGFBP-1 was noted to increase cell surface expression of integrins recognised to mediate adhesion to extracellular matrices.

Since the 1960s and 1970s it has been long thought that endothelial regeneration in response to arterial injury is a local process involving cell migration and proliferation of EC adjacent to the site of injury. In the present chapter the mechanisms by which IGFBP-1 exerts its effects on EC adhesion, migration and proliferation were elucidated. IGFBP-1 was found to modulate multiple processes involved in EC responses, including actin cytoskeletal remodelling and the upregulation of cell surface integrins.

The findings of this chapter lead to a proposed mechanism by which IGFBP-1 promotes functional responses in ECs (Figure 7.6). First, the RGD-domain of IGFBP-1 binds to integrins such as $\alpha_5\beta_1$ and $\alpha_V\beta_3$ on the cell surface of ECs. This in turn induces FAK phosphorylation, which leads to assembly and disassembly of focal adhesions permitting increased cell adhesion and proliferation. A separate pathway is transduced by FAK phosphorylation, where the Rho-Family GTPases are activated and interact with downstream targets. In particular, RhoA is activated, targeting ROCK, which in turn activates actin:myosin filament assembly, ultimately leading to increased cell contraction and migration.

This is the first demonstration of these effects of IGFBP-1 in ECs and is supportive of a modulatory role of IGFBP-1 in vascular regeneration. The findings are consistent with previously reported data on IGFBP-1 actions in non-vascular cells, reflecting the recognised roles of IGFBP-1 in other organ systems – for example the central nervous system and reproductive system. IGFBP-1 migration in oligodendrocytes by activating integrin-mediated intra-cellular signalling[306]. IGFBP-1 interaction with $\alpha_5\beta_1$ integrin has been shown to be responsible for enhancing migration in Chinese hamster ovary cells[305], smooth muscle cells[307] and extra-villous trophoblast cells[308]. These actions of IGFBP-1 proved to be independent of IGF-1. It has also been demonstrated that members of the RHO GTPase family, such as RHO kinase, RHOC and RAC1, are essential for IGFBP-1 induced migration in extravillous trophoblasts[309]. In schwannoma cells, enhanced adhesion and proliferation were shown to be elicited by IGFBP-1-induced FAK phosphorylation[310].

The increased cell surface expression of certain integrins in cells incubated with IGFBP-1 is intriguing, as it is assumed that interaction of IGFBP-1 itself with $\alpha_5\beta_1$ integrin is the mechanism by which IGFBP-1 can signal independently of IGF-1. The short duration of IGFBP-1 incubation used in these experiments is unlikely to be sufficient for integrins to be transcriptionally and translationally upregulated. Instead, it is likely that IGFBP-1 incubation promotes recycling of integrins from intra-cellular stores and increase their availability for binding extra-cellular matrices at the cell surface[311,312]. Elucidation of the mechanism by which IGFBP-1 facilitates integrin expression requires further study.

Although the finding of FAK phosphorylation in ECs is likely, in keeping with other studies, to have been mediated through interaction of IGFBP-1 with integrins, it must be acknowledged that IGFBP-1-integrin interactions were not specifically investigated in this thesis. One could demonstrate that enhancement of cell migration/proliferation/adhesion by IGFBP-1 is integrin-mediated by pre-incubating ECs with anti-$\alpha_5\beta_1$ or $\alpha_V\beta_3$ integrin-blocking antibodies and repeating the endothelial cell functional assays with IGFBP-1.

Another potential limitation is that the effects of IGFBP-1 on cytoskeletal rearrangement were seen only after TNF-α incubation, whereas the RhoA activity assay was performed in quiescent ECs unstimulated by TNF-α. The benefit of investigating IGFBP-1 in isolation, allowed us to elucidate the role of IGFBP-1 without confounding factors such as TNF-α. Nevertheless, the data confirming that IGFBP-1 activates RhoA are supportive of these mechanisms being implicated in the modulatory effects of IGFBP-1 on cell migration.

In summary, the studies reported in this chapter have uncovered important mechanisms by which IGFBP-1 stimulates cell proliferation, adhesion and migration. Understanding the mechanistic basis of the actions of IGFBP-1 in ECs is an essential step for IGFBP-1 to be considered as a potential therapeutic to enhance endothelial repair in an insulin resistant setting.

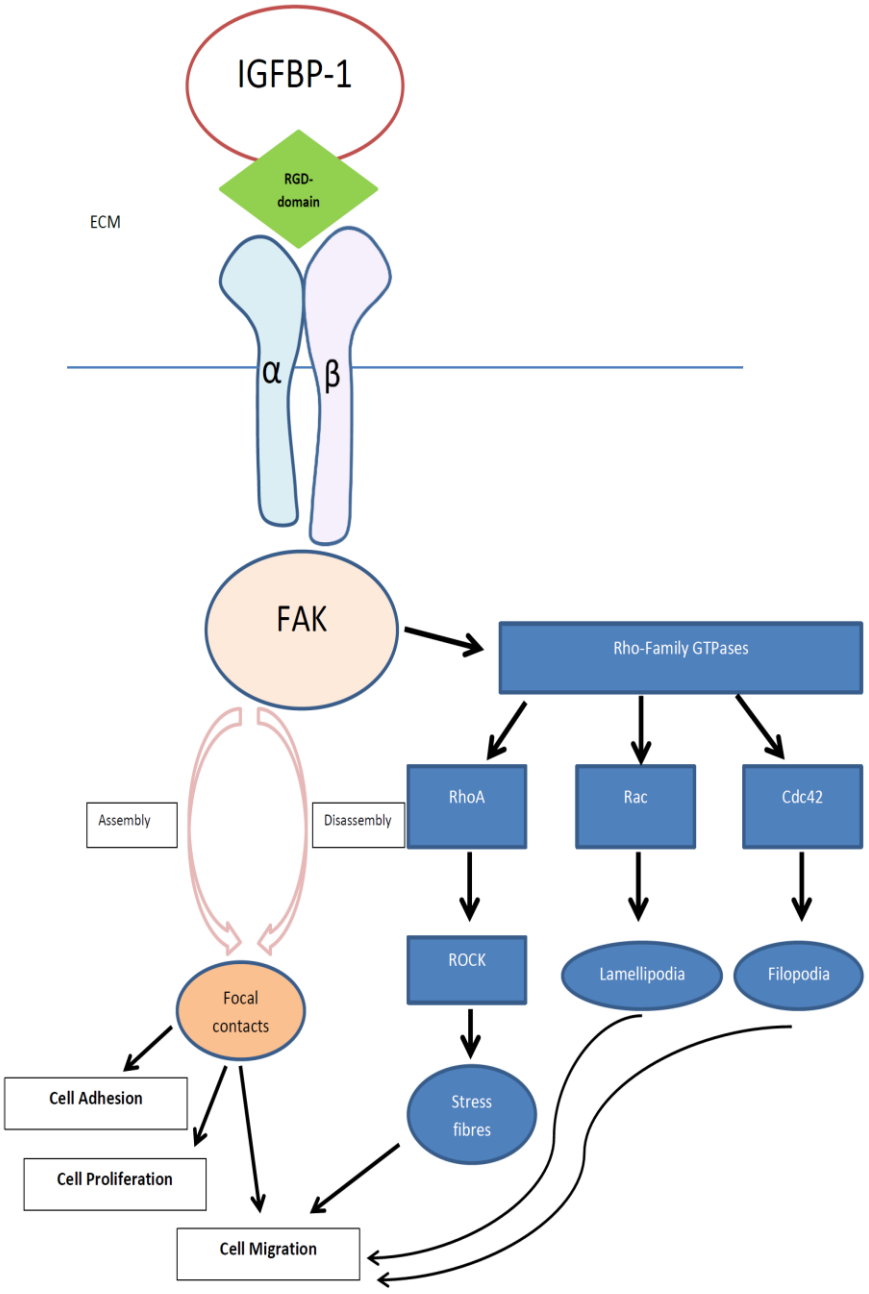

Figure 7-6 Proposed mechanistic pathway by which IGFBP-1 modulates adhesion, migration and proliferation in ECs.
This schematic summarises the proposed signalling pathways by which IGFBP-1 increases proliferation, cell-matrix adhesion and migration through interaction of its RGD domain and $\alpha_5\beta_1/\alpha_V\beta_3$ integrins on endothelial cells. IGFBP-1 binds to integrins on the cell membrane, which activates focal adhesion kinase (FAK) by promoting its phosphorylation. The effects of FAK on formation of focal adhesions leads to enhanced cell adhesion, increased cell migration and cell proliferation. FAK activates the Rho GTPase family (RhoA, Rac, Cdc42) which regulates cytoskeletal arrangement (actin organisation) and thus cell migration.

8 General Discussion

8.1 Summary of key findings

The investigations into the vascular role of IGFBP-1 and in particular endothelial regeneration described in this thesis were prompted by a considerable body of epidemiological data inversely associating IGFBP-1 with IR and CVD. Our laboratory has shown previously that IGFBP-1 directly upregulates NO generation in ECs, independently of IGF-1, via PI-3 kinase activation and eNOS activation. Also, in an insulin resistant murine model, overexpression of human IGFBP-1 rescued endothelial function and endothelial insulin sensitivity[267].

During the course of this research project, *in vivo* studies were first carried out investigating the effects of over-expression of human IGFBP-1 on endothelial regeneration following arterial injury in insulin sensitive mice and in a murine model of IR. IGFBP-1 was found to enhance endothelial regeneration, although this effect was specific for the insulin resistant setting.

Secondly, *in vitro* studies examined the effect of IGFBP-1 on the functional properties of APCs and human ECs implicated in endothelial repair. Impaired APC abundance and mobilisation, as a consequence of reduced NO bioavailability, are known to be responsible for the delayed endothelial regeneration resulting from IR in IRKO mice[206], IGFBP-1 has been shown to enhance NO bioavailability and therefore might have been expected to improve APC abundance and function in IR mice. However, surprisingly flow cytometry and cell culture analyses did not show an improvement in APC abundance in IRKO mice when IGFBP-1 was over-expressed. Furthermore, no improvement in APC function was elicited by IGFBP-1 over-expression. EC functional studies investigated the effects of IGFBP-1 on EC adhesion, migration and proliferation. IGFBP-1 enhanced endothelial cell adhesion on a human saphenous vein matrix, although no direct effect of IGFBP-1 was observed on migration or proliferation in ‘metabolically healthy’ ECs. However, in ECs exposed to the pro-inflammatory cytokine TNF-α

(mimicking IR), co-incubation of IGFBP-1 significantly improved migration and proliferation.

Finally, studies probed the effects of IGFBP-1 on intracellular signalling pathways and cytoskeletal rearrangements necessary for endothelial repair. Mechanistically, IGFBP-1 was shown to increase expression of $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins on the surface of ECs, phosphorylate FAK, activate RhoA and improve cytoskeletal arrangement in a compromised setting. A proposed mechanism is that IGFBP-1 through its RGD domain binds to $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins on the EC surface, activating 'outside-in' integrin signalling, and phosphorylating FAK. FAK phosphorylation leads to increased EC adhesion, migration and proliferation. In addition to this direct effect, the Rho GTPase family is activated which regulates cytoskeletal arrangement and therefore cell migration.

Endothelial regeneration is vital in a clinical setting, e.g. post-angioplasty and stent insertion to prevent neointima formation and thrombosis and to restore vascular homeostasis expediently[125,313]. Through this project, IGFBP-1 has been identified as an endogenous protein which enhances endothelial regeneration in an insulin resistant setting and exhibited multiple favourable effects on EC function *in vitro*. These results have been demonstrated for the first time and show that not only is IGFBP-1 a biomarker of CVD, but plays an important role in endothelial repair especially in a diseased setting.

8.2 Differential effects of IGFBP-1 on endothelial cells and angiogenic progenitor cells

There has been a considerable shift in focus of vascular biology research over the last twenty years. Asahara's[180] landmark study identifying angiogenic progenitor cells stimulated intense interest in investigating APCs and their involvement in endogenous regenerative mechanisms and maintenance of endothelial integrity. However, more recent reports draw

into question the contribution of APCs to endothelial regeneration[286,290] and the tide may now be turning back towards recognition that native ECs hold the key towards endothelial repair.

Data presented in this thesis show that over-expression of IGFBP-1 has no effect on the abundance of APCs or their function. In contrast, the results from this thesis suggest that IGFBP-1 mediates its effects *in vivo* through modulating the reparative function of endothelial cells rather than through APCs which is consistent with Hagensen's findings[286].

8.3 Selective effects of IGFBP-1 on endothelial regeneration in the insulin resistant setting

Another intriguing finding was that in a 'healthy' or a normal metabolic setting, whether *in vivo* or *in vitro*¸ IGFBP-1 had no effect on endothelial regeneration or EC function. However, in an insulin resistant setting, IGFBP-1 enhanced endothelial regeneration, and in a pro-inflammatory setting, migration and proliferation were enhanced. The observation of a more pronounced effect of IGFBP-1 in IR is not unexpected, given the epidemiological links between IR, CVD and low circulating concentrations of IGFBP-1[254,256,259,314,315]. Low IGFBP-1 concentrations in individuals with IR may be permissive for the development of CVD. Based on the findings of this thesis, one could argue that increasing IGFBP-1 concentrations could have a prominent effect in these individuals.

Finding that favourable effects of a protein under investigation are specific for IR is not exclusive to IGFBP-1. A study in the literature, for example, investigated the effects of interferon tau (an anti-inflammatory agent) on IR in an obesity-induced mouse model, reversal of IR (decreased hyperglycaemia and decreased blood insulin levels) was observed with lower levels of pro-inflammatory cytokines such as TNF-α, however no effect of interferon tau was found in insulin sensitive mice[316].

TNF-α was chosen in this thesis to induce a pro-inflammatory state *in vitro* characteristic of the altered biochemical milieu in individuals with IR. TNF-α is known to induce impaired insulin signalling in ECs[86,301] however, this was not confirmed in experiments reported here. TNF-α is also known to induce endothelial dysfunction through other mechanisms including increased ROS formation[317] and to have a direct effect on the p38 MAP kinase pathway[301]. Further studies could look into whether IGFBP-1 has a direct effect on ROS production through inhibition of the p38 MAP kinase pathway.

8.4 Future studies

8.4.1 Therapeutic strategy

The *in vivo* studies reported in Chapter 4 demonstrate a favourable effect of IGFBP-1 on endothelial regeneration following arterial injury in insulin resistant mice. Although these data were corroborated by the experiments conducted in primary ECs and reported in chapters 6-7, potential limitations of data generated from gene-modified mice should be acknowledged. IGFBP-1 was expressed using conventional transgenic techniques under the control of its native promoter. Whilst this permits regulation of IGFBP-1 in response to physiological cues, animals will have been exposed to increased IGFBP-1 concentrations throughout their development. It cannot, therefore, necessarily be inferred that the enhanced endothelial regeneration observed would be translated to a favourable effect if IGFBP-1 concentrations were to be increased acutely at the time of injury. This is an important question if IGFBP-1 is to be exploited as a therapeutic target. To address this issue, a gene-modified mouse in which IGFBP-1 is expressed conditionally would be preferable. A number of approaches are available to regulate transgene expression in a temporal fashion in mice – e.g. tamoxifen-inducible cre-lox technology[318]; or tetracycline-inducible systems[319]. Alternatively, IGFBP-1 could be expressed transiently in mice using adenoviral transduction.

A perhaps more attractive approach from the translational/therapeutic perspective would be to investigate short term administration of IGFBP-1 to mice following arterial injury. This would necessitate parenteral administration and the short half-life of IGFBP-1 in the circulation may present challenges in achieving consistently elevated plasma concentrations. Reassuringly, however, other investigators have successfully employed parenteral administration of IGFBP-1 with favourable effects in other organ systems[320,321].

8.4.2 Integrin signalling as a therapeutic target

The findings reported in chapter 7 suggest that the functional effects of IGFBP-1 in EC may be mediated through outside-in integrin signalling. Further studies are now required to confirm that interaction of the RGD domain of IGFBP-1 with integrins on the EC surface is required for IGFBP-1 effects. This could be achieved by repeating the signalling studies using antibodies to block integrin binding or following knock-down of integrin sub-units in ECs. Additionally, a recombinant IGFBP-1 protein in which the RGD motif has been mutated has recently been generated in our laboratory. Unfortunately this only became available after the experimental work reported here had been completed. This would be ideal to determine whether an intact RGD domain is required to mediate the functional effects of IGFBP-1 on ECs.

Pending the findings of these further studies, $\alpha_5\beta_1$ mediated signalling may be confirmed as a potential target to increase endothelial regeneration. However, there will then be the question of whether the entire IGFBP-1 protein is required to mediate modulatory effects on ECs, or whether these could be reproduced by shorter peptides able to activate integrin signalling. Small hexapeptides such as Gly-Arg-Gly-Asp-Thr-Pro which activate integrins are already available and could be used to investigate their effects on cell migration and proliferation *in vitro*, and on endothelial regeneration *in vivo*.

8.4.3 IGF-independent effects of IGFBP-1

Although it is likely that the effects of IGFBP-1 on EC signalling and functional properties reported in this thesis were IGF-independent, a contribution to the findings through modulation of IGF bioavailability by IGFBP-1 cannot be fully excluded. IGF-1 was not added in any of the *in-vitro* experiments, however IGF-1 will have been present *in vivo* and small amounts of IGF-1 are produced by cultured cells. Synergistic studies could be performed with IGF-1, des-IGF-1 and IGFBP-1 to evaluate the relative contributions of IGF-1 and IGFBP-1 to EC responses *in vitro*. A perhaps more attractive approach is to repeat the *in vitro* experiments with a recombinant form of IGFBP-1 in which the IGF-binding domain of the protein has been mutated. This has recently become available in our laboratory but not whilst this project was being undertaken.

Determining whether the favourable effects of IGFBP-1 are independent of IGF-1 binding is also highly relevant to exploiting IGFBP-1 as a potential therapeutic target. If IGF-1 interaction is not required, a mutated IGFBP-1 protein able to interact with cell surface integrins but unable to alter IGF-1 bioavailability may offer the attraction of favourable vascular effects without off-target effects.

8.4.4 APC studies

Although the results of chapter 5 suggest that a modulatory effect of IGFBP-1 on APCs is unlikely, this possibility has not been fully discounted. Further studies could investigate whether addition of IGFBP-1 to cells directly modulates the phenotype or expansion of early outgrowth APCs *in vitro*. Future studies could also look at whether IGFBP-1 modulates the properties of human ‘late outgrowth’ APCs. Such experiments may be productive, as the phenotype of late outgrowth APCs is close to that of native ECs.

Finally the findings of this project require human translation, the rationale for phase one human trials of IGFBP-1 are strengthened by the findings of this project, especially as IGFBP-1 is an endogenously expressed protein.

8.4.5 Mechanistic studies

Chapter 7 provided evidence of some of the molecular mechanisms by which IGFBP-1 modulates EC responses. For example, IGFBP-1 was shown to activate RhoA in EC. It would be interesting to further investigate this link by repeating functional endothelial cell assays such as cell migration by using the Rho kinase inhibitor Y-27632. Further western blot analyses could be conducted investigating downstream Rho-kinase signalling, probing ROCK1 and ROCK2 using available primary antibodies. Finally, investigating RhoA activity in an insulin resistant setting in animal and cellular models would help deepen our understanding of the apparent specificity of the functional responses to IGFBP-1 to insulin resistant or pro-inflammatory environments.

8.5 Conclusions

The experiments reported in this thesis have yielded intriguing and novel results which could play a key role in the development of potential new therapies to augment vascular repair. IGFBP-1 has been shown in this project to enhance endothelial regeneration in an insulin resistant setting. These reparative effects are mediated through multi-factorial influences of IGFBP-1 on EC function associated with enhanced FAK-RhoA signalling and cytoskeletal modulation. IGFBP-1 was thus shown to be a vasculo-protective agent and relative IGFBP-1 deficiency has already been implicated in the increased CV risk in individuals with low plasma IGFBP-1. Further investigations are now required to corroborate understanding of the mechanistic basis for IGFBP-1 effects, before considering therapeutic studies looking at the translational effects of IGFBP-1. IGFBP-1 or EC FAK signalling could be targeted therapeutically to enhance repair in insulin resistance.

Appendix

Endothelial Cell (20% FCS) Growth Medium (for cell culture):

380 mL Basal Medium M199 (Sigma)
100 mL FCS 20%
10 mL 1M HEPES
5 mL Antibiotic Anti-mycotic solution (Penicillin 10,000 units/mL, Streptomycin 10,000 µg/mL, Fungizone 25 µg/mL)
2.5 mL ECGS (3 mg/mL)
1 mL Sodium Pyruvate (55 mg/mL)
2.5 mL Heparin (1,000 units/mL)

Serum Free Media:
500 mL M199
10 mL 1M HEPES
5 mL Antibiotic Anti-mycotic solution (Penicillin 10,000 units/mL, Streptomycin 10,000 µg/mL, Fungizone 25 µg/mL)
2.5 mL ECGS (3 mg/mL)
1 mL Sodium Pyruvate (55 mg/mL)
2.5 mL Heparin (1,000 units/mL)

Endothelial Cell Basal Medium-2 plus Clonetics EGM-2 Bulletkit CC-3162 (for APC culture): Commerical kit, medium was made according to manufacturer's instructions
500 mL Endothelial Cell Basal Medium-2
50 mL FCS 20%
Hydrocortisone 0.2mL
hFGF-B 2mL
VEGF 0.5mL
R3-IGF-1 0.5mL
Ascorbic acid 0.5mL
hEGF 0.5mL
GA-1000 0.5mL
Heparin 0.5mL

Transfer Buffer (Western Blot)
70.5g Glycine (Sigma)
15g Tris-Base (Sigma)
1000mL methanol
Then made up to 5L with ddH_20

10x TBS/Tween
12.1g Tris-base (Sigma)
87.65g NaCl (vWR)
Made up to 1L with ddH_20, pH to 7.4
20µL Tween added to 1L of 1 x TBS

Publication list

Publications arising from this project:

1. **Aziz A**, Wheatcroft S.
   Insulin resistance in Type 2 diabetes and obesity: implications for endothelial function.
   Expert Rev Cardiovasc Ther. 2011 Apr;9(4):403-7. doi: 10.1586/erc.11.20.

Abstracts arising from this project:

1. **Aziz A**, Yuldasheva N, Smith J, Haywood N, Cordell P, Cubbon R, Porter KE, Kearney MT, Wheatcroft SB.
   Insulin-like Growth Factor Binding Protein (IGFBP)-1 enhances vascular endothelial regeneration in the setting of insulin resistance
   American Diabetes Association, June 2014, San Francisco, USA

2. **Aziz A**, Yuldasheva N, Smith J, Riches KE, Gage M, Mughal R, Mercer BN, Sengupta A, Ali N, Cordell P, Haywood N, Cubbon RM, Kearney MT, Porter KE, Wheatcroft SB.
   Insulin-like Growth Factor Binding Protein (IGFBP)-1 enhances vascular endothelial regeneration in the setting of insulin resistance
   British Cardiac Society, June 2014, Manchester, UK

3. **Aziz A**, Yuldasheva N, Smith J, Haywood N, Cordell P, Cubbon R, Porter KE, Kearney MT, Wheatcroft SB.
   The effects of Insulin-like Growth Factor Binding Protein-1 on endothelial repair in the insulin resistant setting.
   International Congress of Physiological Sciences. July, 2013 Birmingham, UK. – **Winner ESM/EVBO Poster award**

4. **Aziz A**, Yuldasheva N, Smith J, Haywood N, Cordell P, Cubbon R, Porter KE, Kearney MT, Wheatcroft SB.
   Enhancing vascular endothelial repair in the setting of insulin resistance: effects of insulin-like growth factor binding protein-1.
   British Cardiac Society. June 2013 London, UK.

Other publications arising during this project:

1. Yuldasheva NY, Rashid ST, Haywood NJ, Cordell P, Mughal R, Viswambharan H, Imrie H, Sukumar P, Cubbon RM, **Aziz A**, Gage M, Mbonye KA, Smith J, Galloway S, Skromna A, Scott DJ, Kearney MT, Wheatcroft SB.
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