Examining the Actions of Insulin-Like Growth Factor Binding Protein 2 in Vascular Biology

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I formally confirm that the work carried out and submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

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Acknowledgements

The research included in this thesis is my own, although I have had assistance from other contributors throughout my studies and this contribution is explicitly indicated in the text.

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I would like to dedicate this thesis to my younger sister Sophie, whose sudden death as I was starting my PhD cast a very large shadow over the first year of my fellowship. My

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Abstract

The formation of new blood vessels; physiological function of quiescent vasculature; and remodelling of vasculature after injury, is regulated by a wide variety of complex and overlapping signalling pathways. The endothelial cell monolayer, vascular smooth muscle cells and adventitial tissue which constitute blood vessels are influenced by multiple circulating and locally secreted factors, with each tissue type exerting effects on the other; especially in the context of angiogenesis and in the pathophysiological response to vascular injury. Insulin-like growth factor binding protein-2 (IGFBP-2) has been considered an important factor in tumour angiogenesis and cellular proliferation, but little is known about its effects on vascular function, vascular remodelling, and its effects on the individual cell types within the vascular environment.

In this work, we describe the effects of IGFBP-2 on vascular physiological and pathophysiological processes. We achieved this predominantly through the use of two transgenic murine models: of conditional endothelial specific IGFBP-2 overexpression, and conditional global IGFBP-2 overexpression. We demonstrate that endothelial specific IGFBP-2 overexpression has no effect on *ex vivo* aortic vasomotor function, whilst global overexpression of IGFBP-2 causes significant aortic hypocontractility, but without any change in endothelium-dependent vasodilatation, and without altering nitric oxide bioavailability. No change for *in vivo* blood pressure was evident, with increased aortic distensibility seen with global overexpression of IGFBP-2. We also demonstrate that global overexpression of IGFBP-2 leads to reduced re-endothelialization after vascular injury, but does not affect neointimal quantity, although possible changes in neointima composition were observed. Finally, increased exposure to IGFBP-2 caused an increase in sprouting angiogenesis across multiple endothelial cell types.

In conclusion, increased expression of IGFBP-2 may exert multiple effects on murine vasculature in the setting of vascular vasomotor function, vascular injury and angiogenesis. More work is required to further understand the effects IGFBP-2 exerts in vascular biology, and the signalling mechanisms through which these occur.

Table of Contents

Table of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Abbreviations

AAA	Abdominal aortic aneurysm
Ach	Acetylcholine
AGE	Advanced glycation end-products
ALS	Acid labile subunit
ANGPT1	Angiopoietin-1
ANGPT2	Angiopoietin-2
ApoE	Apolipoprotein E-deficient
ASMA	Alpha smooth muscle actin
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CAD	Coronary artery disease
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CAV	Cardiac allograft vasculopathy
Cdh5	VE-Cadherin
CEB	Cell extraction lysis buffer
CFA	Common femoral artery
cGMP	Cyclic guanosine monophosphate
CMV	Cytomegalovirus
DMEM	Dulbecco Modified Eagle Medium

ECFC	Endothelial Colony Forming Cells
ECGM	Endothelial Cell Growth Medium
ECM	Extracellular matrix
EDHF	Endothelium derived hyper-polarising factor
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptors
EGM-2	Endothelial Cell Growth Medium 2
EKV	ECG gated Kilohertz visualisation
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
Fn-EDA	Fibronectin–splice variant containing extra domain A
GFP	Green fluorescent protein
GH	Growth hormone
HBD	Heparin binding domain
HBSS	Hanks’ Balanced Salt Solution
HGF	Hepatocyte growth factor

HIF-1α	Hypoxia Inducible Factor 1 Subunit Alpha
hIGFBP-2	Human IGFBP-2
hPGK	Human phosphoglycerate kinase
HPSC	Human pluripotent stem cells
HSC	Haematopoietic stem cell
HSP90	Heat shock protein 90
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGFBP-2	Insulin-like growth factor binding protein-2
IL-1	Interleukin-1
IL-6	Interleukin-6
ILK	Integrin-linked kinase
IMA	Internal mammary artery
IP3	Inositol triphosphate
IRS	Insulin receptor substrates
KCl	Potassium chloride
LAM	Lymphangioleiomyomatosis
LCC	Left common carotid
L-NMMA	NG-Methyl-L-arginine
LTCC	L-type Ca^{2+} channels
MAC	Myeloid Angiogenic Cells
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase

MFAP4	Microfibrillar-associated protein 4
MLC	Myosin light chains
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteases
MOI	Multiplicity of Infection
MPA	Megapascal
MYH11	Smooth muscle myosin heavy chain
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localisation sequence
NO	Nitric oxide
NOX	NADPH oxidase
PAD	Peripheral arterial disease
PAPP-A	Pregnancy Associated Plasma Protein-A
PBMC	Peripheral blood derived monocytes
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFRα	PDGF receptor-alpha
PDGFRβ	PDGF receptor-beta
PE	Phenylephrine
PEC	Pulmonary endothelial cells
PGI_2	Prostacyclin
PI3K/Akt	Phosphatidylinositol 3′-kinase/protein kinase B

PTEN	Phosphatase and tensin homolog
PVAT	Perivascular adipose tissue
RCT	Randomised control trial
RGD	Integrin binding domain
ROS	Reactive oxygen species
RPTPβ	Receptor protein tyrosine phosphatase β
RT-qPCR	Real-time quantitative polymerase chain reaction
SFA	Superficial femoral artery
SNP	Sodium nitroprusside
SV	Saphenous vein
TAA	Thoracic aortic aneurysms
TBS-T	Tris-buffered saline-tween
TLR4	Toll like receptor 4
TNF-α	Tissue necrosis factor-alpha
TXA_2	Thromboxane
USS	Ultrasound scanning
VEGF	Vascular endothelial growth factors
VESC	Vascular endothelial stem cells
WT	Wild type

Publication list

Publications arising from this project:

These review articles have been referenced but the text has not been used for any significant part of this thesis, and these publications do not contain any data presented in the experimental chapters of this thesis.

Slater T, Haywood NJ, Matthews C, Cheema H, Wheatcroft SB. Insulin-like growth factor binding proteins and angiogenesis: from cancer to cardiovascular disease. Cytokine Growth Factor Rev. 2019 Apr;46:28-35. doi: 10.1016/j.cytogfr.2019.03.005. PMID: 30954375.

Haywood NJ, **Slater TA**, Matthews CJ, Wheatcroft SB. The insulin like growth factor and binding protein family: Novel therapeutic targets in obesity & diabetes. Mol Metab. 2019 Jan;19:86-96. doi: 10.1016/j.molmet.2018.10.008. PMID: 30392760

Other publications arising during this project:

Haywood NJ, **Slater TA** *et al*. IGFBP-1 in Cardiometabolic Pathophysiology-Insights From Loss-of-Function and Gain-of-Function Studies in Male Mice. J Endocr Soc. 2019 Nov 4;4(1):bvz006. doi: 10.1210/jendso/bvz006. PMID: 32190801

Abstracts arising from this project:

American Heart Association Scientific Sessions November 2019: Abstract 13483: Insulin-like growth factor binding protein 2 overexpression leads to aortic

hypocontractility through vascular smooth muscle phenotypic switching. Circulation. 2019;140:A13483

British Cardiovascular Society June 2019: BS52 The effects of IGFBP2 over-expression on vascular function and smooth muscle. Heart 2019;105:A173.

British Cardiovascular Society June 2018: 117 The divergent effects of IGFBP1 and IGFBP2 on vascular endothelial function. Heart 2018;104:A90.

Chapter 1 Introduction

1.1 The vasculature

1.1.1 Vascular structure

The human vasculature can be defined as the arrangement of all blood vessels – arteries, veins and capillaries – throughout the body. The vasculature, along with the heart and lymphatic system, constitutes the circulatory system, first recognised by the Ancient Egyptians and first described accurately five thousand years later by William Harvey in 1628.(1) The circulatory system has multiple roles, allowing the movement of oxygen, nutrients, hormones and waste products, as well as thermoregulation and facilitating the immune response to pathogens.(2)(3)

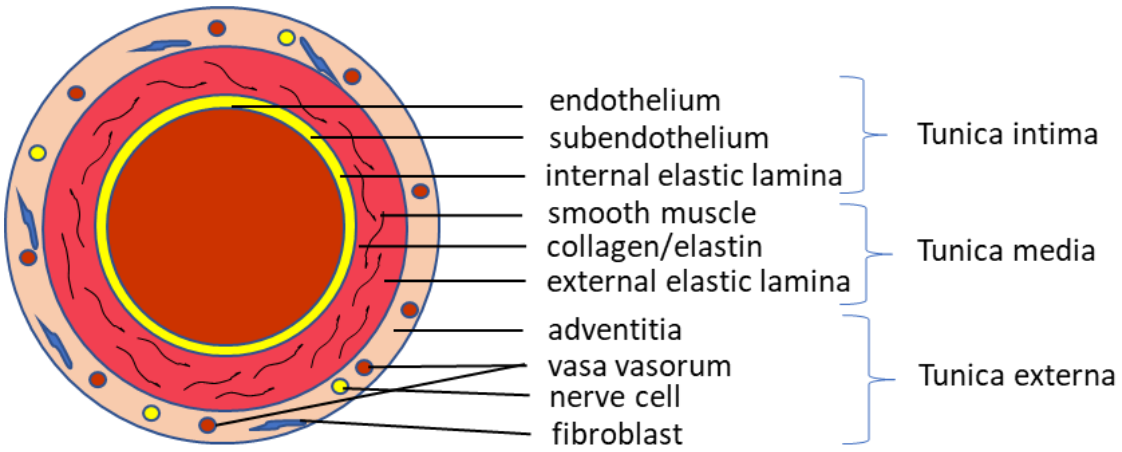


Figure 1-1. Cross section of blood vessel. The tunica intima consists of an endothelium, subendothelium and internal elastic lamina. The tunica media consists of differing proportions of smooth muscle and extracellular matrix, and an external elastic lamina. The tunica externa consists of adventitial tissue, nerve cells and microvasculature.

With the exception of the capillary network, which consists of just an endothelial cell monolayer, basement membrane of varying porosity and pericytes enveloping the basement membrane,(4)(5) all blood vessels within the human body consist of the same

three layers: the tunica externa, tunica media and tunica intima. The tunica externa consists of adventitial tissue, a heterogenous collection of cells including fibroblasts and immune cells anchored by an extracellular matrix of connective tissue, as well as nerve cells and, in larger vessels, a microvascular network called the vasa vasorum. The constitution of the tunica media depends on the type of blood vessel, with arterial media predominantly consisting of vascular smooth muscle cells (VSMC) and elastic tissue in differing arrangements and proportions depending on the size of the artery.(4) In veins the tunica media predominantly consists of connective tissue, with less VSMC and elastic tissue evident. Finally, the tunica intima consists of a monolayer of endothelial cells, a thin subendothelial layer of connective tissue and an internal elastic lamina allowing communication with the tunica media (Figure 1-1).(4)(6)

1.1.2 The endothelium

Despite only being a cellular monolayer, the endothelium plays a vital role in a multitude of functions, and its dysfunction lies at the core of vascular disease.(7) The endothelium acts as both a barrier and communication between circulating factors, the tunica media and the perfused tissue. Beyond this structural role, it plays a key autocrine and paracrine role in cell adhesion, inflammation, thrombosis, angiogenesis and the regulation of vascular tone.(8)(9)(10)

Endothelial homeostasis requires the careful balance of multiple expressed factors, which act to maintain vascular quiescence. If this balance is disrupted, the endothelium switches to an ‘activated’ phenotype, characterised by reduced nitric oxide (NO) bioavailability and upregulation of vasoconstrictive factors. This leads to a cascade of pro-inflammatory, pro-adhesion, proliferative actions and reactive oxygen species (ROS) production.(11)

1.1.3 Vascular smooth muscle cells

Vascular smooth muscle cells are found in the tunica media of all vasculature except capillaries. The primary function of VSMC in normal physiology is to control blood flow through vessels via constriction or relaxation. Through these mechanisms, VSMC play a central role in systemic vascular resistance homeostasis, controlling blood pressure and organ perfusion.(12)

VSMC can be separated into two classically described phenotypes: a ‘contractile’ phenotype and a ‘synthetic’ phenotype. The contractile phenotype predominates in healthy vasculature, whilst the synthetic phenotype contributes significantly to vascular pathology such as atherosclerosis and neointimal hyperplasia. This will be discussed in more detail in section 1.3.2.

1.1.4 Pericytes

Pericytes are perivascular cells present throughout the microvasculature. They are embedded within the basement membrane, helping to provide structural integrity for the microvasculature. Crosstalk between pericytes and endothelial cells is a key component of angiogenesis and the formation of a mature, functioning blood vessel network. Pericyte loss and dysfunction have been associated with diabetes mellitus and occur as a consequence of persistent hyperglycaemia. This alteration of pericyte function contributes to the development of diabetes associated microvascular complications, such diabetic retinopathy and nephropathy, and may also contribute to diabetes associated macrovascular complications.(13)

1.1.5 The adventitia

The adventitia consists of a heterogeneous collection of cell types including fibroblasts, progenitor cells, nerve cells and immune cells such as T cells, B cells, mast cells,

dendritic cells and resident macrophages. In recent years it has been increasingly recognised that these cells play key roles in maintaining normal vascular development and physiology through communication with both endothelial cells and vascular smooth muscle cells. The contained immune cells also play an important role in immune surveillance and detection of foreign antigens.(14)

Pathological activation of the immune cells within the adventitia contribute to local cytokine production, collagen deposition and arterial stiffening, with accumulation of adventitial inflammatory cells seen in atherosclerotic plaque.(15) Additionally, marked proliferation of adventitial fibroblasts has been demonstrated to be a key component of vascular remodelling in response to vascular injury and hypertension, and the relationship between fibroblasts and the extracellular matrix plays an important role in the development of these pathological states.(15)

Although not part of the vasculature *per se*, perivascular adipose tissue (PVAT) borders the adventitia and has been demonstrated to play a significant role in both cardiovascular health and disease. PVAT secretes adipocytokines: a variety of hormones, cytokines and chemokines which in normal vascular homeostasis exert predominantly anti-inflammatory, antioxidant and vasodilatory effects. The PVAT secrotome is affected by cardiovascular risk factors such as obesity and diabetes, and in response to these insults can switch to a pro-inflammatory and oxidative phenotype, potentially contributing to vascular dysfunction. However, it has also recently been discovered that PVAT can respond to vascular inflammation by altering adipocyte size, lipid content and increasing local production of the anti-inflammatory adiponectin, thus acting to protect against the development of atherosclerotic plaque.(16)

1.1.6 The extracellular matrix

The extracellular matrix (ECM) provides structural integrity throughout the vascular wall and consists predominantly of collagens and elastin as well as other proteins such as fibronectin, osteopontin and laminin. ECM is present within the basement membrane, tunica media and tunica adventitia, is found in differing proportions in different types of vasculature, and is produced predominantly by smooth muscle cells in the media, and by fibroblasts in the adventitia.(17) ECM has multiple functions beyond structural support and provides a key role in maintaining vascular quiescence through inhibitory regulation of VSMC proliferation and migration, as well as displaying anti-inflammatory and anti-thrombotic properties. However, in a pathological state excessive ECM deposition by fibroblasts and VSMC contributes to arterial stiffness, neointima formation and pathological vascular remodelling, and binding of these cells to the ECM via cell membrane receptors called integrins further contributes to cellular proliferation, adhesion, migration and excess ECM deposition.(17)

1.2 Endothelial cells in vascular biology

As described in section 1.1.2, endothelial cells play a vital role in normal vascular homeostasis through a variety of mechanisms. Additionally, endothelial cell dysfunction has been determined as a key early mechanism in the development of vascular pathophysiology, including atherosclerosis and restenosis after coronary intervention. Therefore, the role of endothelial cells in vascular homeostasis will be briefly expanded upon further in section 1.2.1, followed by a discussion of the mechanisms by which endothelial cell dysfunction contributes to vascular disease, in section 1.2.2.

1.2.1 Physiological endothelial function

As previously described, the endothelial monolayer plays a central role in vascular tone regulation and the maintenance of vascular homeostasis. Regulation of vascular tone is predominantly related to endothelial secretion of several key factors: the

vasoconstrictors endothelin-1 (ET-1) and thromboxane (TXA_2), and the vasodilators prostacyclin (PGI_2), endothelium derived hyper-polarising factor (EDHF), and, crucially, nitric oxide.(9) Amongst other homeostatic roles, these factors act upon VSMC in the tunica media to cause either constriction or dilatation of the vessel.(9)

Endothelial nitric oxide production in normal conditions is mediated by the constitutively expressed enzyme endothelial nitric oxide synthase (eNOS), and the production and utilisation of NO in the endothelium is commonly referred to as NO bioavailability.(18)(19) Inactivation of eNOS occurs through interaction with caveolin-1 within caveolae, an important interaction for maintaining physiological function.(20) Humoral factors such acetylcholine and bradykinin activate eNOS through upregulation of intracellular calcium ions (Ca^{2+}), binding of calcium to calmodulin (CaM), and subsequent activation of eNOS by the Ca^{2+}/CaM complex.(9)(21) Shear stress within the blood vessel also causes eNOS activation through both calcium dependent and independent mechanisms.(9) Calcium independent activation of eNOS occurs through phosphorylation of eNOS by a number of different pathways including the phosphatidylinositol 3′-kinase/protein kinase B (PI3K/Akt) pathway,(21)(22) an important phosphorylation cascade pathway through which insulin also acts to promote NO production.(23) Activated eNOS subsequently synthesizes NO through hydroxylation of l-arginine and then oxidation of N^{ω}-hydroxy-l-arginine to produce NO and the by-product l-citrulline.(24)

Along with its key role in modulating vascular tone, NO has anti-inflammatory, anti-thrombotic and antioxidant functions.(25) Reduced NO expression is therefore central to the development of endothelial dysfunction, an essential precursor to overt atherosclerosis, and reduced NO bioavailability is associated with all established risk factors for cardiovascular disease.(26)(27)(28)

1.2.2 Endothelial dysfunction

The endothelium in normal physiology is a quiescent structure, and NO plays a key role in maintaining this quiescence through inhibition of inflammatory cytokine expression and leukocyte adhesion molecules, as well as maintenance of vascular structure through inhibition of VSMC proliferation and migration, regulation of vascular tone and inhibition of platelet reactivity.(29)(30) Disruption of vascular homeostasis leads to 'activation' of the endothelium, defined by expression of adhesion molecules usually suppressed by NO activity, and subsequent endothelial dysfunction, defined by a reduction in NO bioavailability.(30) Endothelial activation has been found to be induced by proinflammatory cytokines such as tumour necrosis factor-alpha which downregulate eNOS activity,(31) leading to a cycle of increased cytokine expression, leukocyte adhesion, oxidative stress and subsequent reactive oxygen species production, further impairing NO bioavailability. Traditional cardiovascular risk factors – hypercholesterolaemia, smoking, diabetes, hypertension, obesity – as well as chronic inflammatory conditions all lead to endothelial activation through the effects of the inflammatory mechanisms described, hyperglycaemia and the action of turbulent shear stress on the endothelium.(30)

This continued endothelial dysfunction eventually leads to loss of endothelial integrity, initially through increased paracellular permeability, followed by endothelial cell senescence and apoptosis. These changes leave the endothelium prone to injury, allow unchecked proliferation and migration of VSMC and lead to the development of atherosclerosis.(32)

A key role of endothelial cells is to act in response to vascular insult through reparative mechanisms such as re-endothelialization and angiogenesis, which will be discussed further in sections 1.5.1 and 1.6.1.

1.3 Vascular smooth muscle cells in vascular biology

As described in section 1.1.3, vascular smooth muscle cells have a central role in controlling normal vascular tone, and therefore organ perfusion and blood pressure control. Although VSMC are largely quiescent in normal homeostasis, in a disease state VSMC contribute significantly to the development of atherosclerosis, neointimal formation and aneurysm development. Physiological and pathophysiological actions will be expanded upon further in the sections below.

1.3.1 Physiological vascular smooth muscle cell function

The primary function of VSMC in physiological function is regulation of vascular tone, with vasoconstriction or dilation dependent on external stimuli. As with both cardiomyocytes and skeletal muscle, VSMC contain both actin and myosin filaments, and interaction of the two is the primary mediator of VSMC contraction. The intracellular organisation of myosin and actin and their mechanisms of activation in VSMC however differs significantly from both cardiomyocytes and skeletal muscle cells. VSMC lack both troponin, the key mediator of actin:myosin interaction in cardiomyocytes, and sarcomeres, the contractile units present in both skeletal and cardiac muscle which coordinate synchronised cell contraction through tight proximity of actin and myosin filaments.(33)

1.3.1.1 VSMC contraction and calcium signalling

VSMC contraction is mediated through a variety of complex mechanisms; however, all of these mechanisms are dependent upon Ca^{2+} availability, either through extracellular influx into the cell or through intracellular release from the sarcoplasmic reticulum. Extracellular influx occurs primarily through transmembrane voltage gated L-type Ca^{2+} channels (LTCC), with other channels such as T-type Ca^{2+} channels and transient

receptor potential channels playing a lesser but important role in the dynamic regulation of intracellular Ca^{2+}.(34)(35) These channels are activated by a number of mechanisms, including the myogenic response to increased intravascular pressure, the actions of biological agonists, and membrane depolarisation resulting from increased intracellular Ca^{2+} concentration.(36)(37) After influx, Ca^{2+} forms a complex with calmodulin. This Ca^{2+}-CaM complex binds to myosin light chain kinase (MLCK), which in turn increases the phosphorylation of myosin light chains (MLC). This post translational modification allows MLC to cross bridge with smooth muscle actin filaments, facilitating cellular contraction.(38)

As previously described, intracellular Ca^{2+} is also released from the sarcoplasmic reticulum. This can occur in response to changes in intracellular Ca^{2+} concentration or in response to external stimulus by biological agonists such as angiotensin II, vasopressin or endothelin-1.(39) These agonists act through G-protein coupled receptors to activate phospholipase C, which causes the synthesis of the messenger molecule inositol triphosphate (IP3), in turn activating calcium channels on the sarcoplasmic reticulum, releasing Ca^{2+} into the cytoplasm and thus facilitating contraction and vasoconstriction. (40) Activation of G-protein coupled receptors simultaneously upregulates Rho-kinase activity, inhibiting the dephosphorylation of MLC and so enhancing vasoconstriction.(41)

Vascular smooth muscle cells are primarily innervated by the sympathetic nervous system, which plays a key role in vasoconstriction and vasodilation via alpha- and beta-adrenoreceptor activation respectively. Activation of alpha receptors, primarily by noradrenaline, leads to activation of IP3 and the same cascade of events provoked by other G-protein coupled receptors described above, and therefore predominantly has vasoconstrictive action.(42) Other alpha-adrenoreceptor agonists such as phenylephrine can also act in a similar fashion, and this will be described in more detail in later chapters. Conversely, activation of beta-adrenoreceptors leads to vasodilation as described below.

1.3.1.2 VSMC relaxation

Vascular smooth muscle cell relaxation occurs with dephosphorylation of the MLC and therefore uncoupling of the actin and myosin filaments. Nitric oxide released by the endothelium acts to increase intracellular cyclic guanosine monophosphate (cGMP) levels, which in turn upregulates the activity of myosin light chain phosphatase (MLCP).(33) Relaxation can also occur with reduced MLCK activity, mediated either through reduced intracellular Ca^{2+} or directly via increased cyclic adenosine monophosphate (cAMP) concentration, the latter being the main mechanism by which beta-adrenoreceptor activation contributes to vasodilation.(43) There is also evidence that, in VSMC, intracellular release of calcium from the sarcoplasmic reticulum through ryanodine receptors may lead to hyperpolarisation of the plasma membrane and subsequent reduction in LTCC activity, leading to an overall global reduction in intracellular Ca2+ and reduced MLCK activity.(44) This is in contrast to the action of ryanodine receptors in other cells, such as striated muscle and cardiomyocytes, in which ryanodine receptors play a key role in enhancing intracellular Ca^{2+} levels and facilitating contraction.(44)

It is predominantly through the above described mechanisms that the actions of the sympathetic nervous system, and agonists such as angiotensin II, maintain blood pressure and systemic vascular resistance homeostasis, depending on cardiac output requirements.(42)

1.3.1.3 VSMC extracellular matrix deposition

Another physiological function of VSMC is the continual synthesis and remodelling of the extracellular matrix in the tunica media, providing structural and mechanical support to the vasculature. This is especially important during morphogenesis of blood vessels but

is still a continual and vital process in adult vasculature, with careful balance and regulation required to prevent the development of pathological remodelling and associated excess ECM deposition.(17) In turn, interaction between the ECM and VSMC can also help to maintain the quiescent and non-proliferative contractile VSMC phenotype.(45) Elastin and collagens type I and III are the primary ECM proteins within the media, but laminin, fibronectins and proteoglycans also play important roles.(46) Adhesion receptors known as integrins are present on the cell surface of VSMC and act to anchor VSMC to the extracellular matrix, as well as contributing to ECM synthesis, deposition and maintenance of the contractile VSMC phenotype.(47) Integrins also play a major role in VSMC dysfunction and the development of vascular pathology and will be discussed later in more detail.

1.3.1.4 Blood pressure regulation and aortic distensibility

VSMC within the tunica media plays a crucial role in blood pressure homeostasis through vasoconstriction or dilation in response to extracellular stimuli, leading to changes to systemic vascular resistance and therefore blood pressure. Systemic vascular resistance is predominantly affected by alterations in the lumen diameter of the small arteries and arterioles within the peripheral vasculature, known as ‘resistance vessels’.(48) These vessels therefore contain a high ratio of VSMC within the tunica media, as do the medium size arteries proximal to resistance arteries, such as the radial, femoral and coronary arteries, known as ‘muscular or ‘distributing’ arteries.(49) The large arteries such as the aorta are known as ‘elastic’ arteries, with the primary role of regulating the pulsatile blood flow generated by cardiac systole into continuous flow at the level of the resistance vessels.(50) This is achieved primarily through aortic compliance, with little role for vasoconstriction or vasodilation. In these elastic arteries there is therefore a greater ratio of ECM present, primarily elastin, compared to VSMC, to allow for this distensibility.(51) A variety of mechanisms can affect this ratio and affect

the composition of the ECM with the overall effect of increasing arterial stiffness, which will be described in detail later.

1.3.2 Vascular smooth muscle cell dysfunction

As previously described, there are two predominant phenotypes of vascular smooth muscle cells: the contractile phenotype and the synthetic phenotype. Contractile smooth muscle cells are rod shaped, largely quiescent and highly contractile, demonstrating high expression of contractile proteins such as alpha smooth muscle actin, myosin heavy chain and calponin. The ‘synthetic’ phenotype is characterised by a rhomboid shape, increased propensity to cellular proliferation, migration, reduced expression of contractile proteins and therefore a reduced capacity for contraction (Figure 1-2).(52)

In physiological conditions, the synthetic phenotype is important during embryonic blood vessel development but in adult vasculature the contractile phenotype predominates,(52) and is particularly abundant in the arterial vasculature. However, differentiated mature contractile VSMC display considerable plasticity and, in response to stimuli such as increased shear stress or the proinflammatory milieu present in pathological conditions such as atherosclerosis, contractile VSMC can undergo a phenotypic ‘switch’ to the synthetic type (Figure 1-2), promoting cellular proliferation and subsequently contributing to the development of pathological conditions such as atherosclerotic plaque development or aortic aneurysm formation.(53)(54) Multiple pathways have been implicated in VSMC phenotypic switching, and these will be discussed in further detail in subsequent sections.

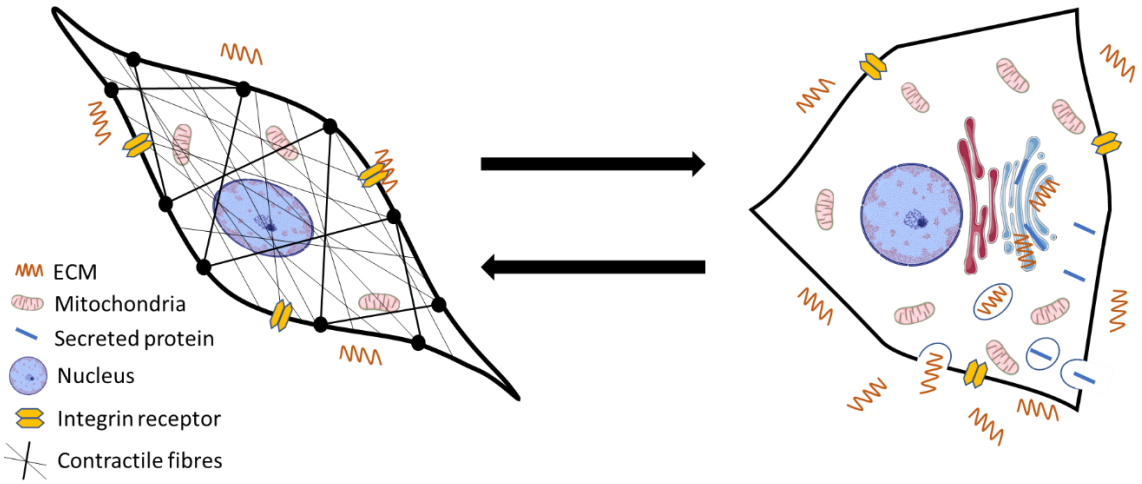


Figure 1-2. VSMC composition. The image on the left represents the spindle shaped contractile phenotype, characterised by multiple contractile filaments and quiescence. The image on the right represents the synthetic phenotype, characterised by proliferative, migratory actions and extracellular matrix production and deposition. External signalling can provoke a phenotypic switch between the two. Created using Servier Medical Art

Both endothelial and VSMC dysfunction are inextricably linked in the development and progression of vascular pathology, through either acute or chronic vascular insult. These will therefore be discussed in tandem in the following section exploring vascular pathology.

1.4 Vascular pathophysiology

1.4.1 Arterial stiffness

Arterial stiffness in the large arteries is a hallmark of vascular aging, and is positively associated with hypertension, coronary atherosclerosis and heart failure (55). Arterial stiffening is characterised as loss of vascular compliance and aortic distensibility and can be the first indication of the development of endothelial and VSMC dysfunction. As previously described, adequate vascular compliance is vital to convert the pulsatile pressure created by ventricular systole into continuous pressure within the microcirculation. Loss of this compliance causes a direct increase in ventricular afterload,

and will also increase systemic vascular resistance as the peripheral arterial circulation becomes less compliant, adding further to ventricular afterload.(56)

Classically it has been thought that arterial stiffening was primarily due to increased ECM deposition and a change in ECM composition. Whilst this does remain a crucial component in arterial stiffening, it is increasingly recognised that other cell types play a major role as well.(56)

Vascular compliance relies on the composition of the tunica media and adventitia, the degree of VSMC present and the composition of the ECM. The large, central arteries such as the aorta contain a smaller proportion of VSMC and therefore have increased distensibility compared to the smaller, muscular arteries and arterioles, in which myogenic tone and vasoconstriction play a crucial role in cardiac output homeostasis.(55) Elastin is the primary ECM constituent that contributes to vascular compliance, whilst collagen provides structural integrity along with the other components of the ECM, glycoproteins such as laminin and fibronectin and proteoglycans.(57) Degradation of elastin and collagen within the ECM is regulated by matrix metalloproteases (MMPs). Dysregulation of these enzymes corresponds to increased elastin degradation and irregular collagen deposition and is therefore central to the development of arterial stiffness. MMPs are upregulated by inflammatory cytokines such as tissue necrosis factor-α (TNF-α) and interleukin-1 (IL-1), as well as ROS, increased shear stress and synthetic VSMC, and additionally are upregulated in the presence of MMPs themselves, leading to a self-perpetuating cycle of dysfunction.(57) Within the pro-inflammatory milieu there is also increased deposition of glycoproteins and proteoglycans, which causes hypertrophy of the vessel wall whilst reducing compliance. Fragmented collagen and elastin molecules are particularly susceptible to glycation and cross-linking, leading to the formation of advanced glycation end-products (AGE),

increasing arterial stiffness both structurally and through further direct stimulation of inflammatory responses, ROS formation and a reduction in NO bioavailability.(57)

As previously described, VSMC play an important role in ECM deposition and maintenance, with interaction coordinated by adhesion proteins and, crucially, integrin receptors. Increased collagen and proteoglycan deposition are facilitated by these interactions, as is the phenotypic switching of VSMC from a contractile to a synthetic phenotype, leading to further proliferation of both the ECM and VSMC layers.(56) There is also evidence that VSMC themselves can stiffen and increase adhesion in response to vessel contraction. VSMC in hypertensive rats have been found to be stiffer than VSMC in normotensive controls, with cytoskeletal adaptations proposed as the underlying mechanism.(58)

1.4.2 Vascular injury

As previously described, endothelial dysfunction results in progressive loss of endothelial integrity, leading to disruption of the endothelial barrier, intimal migration of VSMC and development of chronic vascular pathology such as atherosclerosis and arterial aneurysms. Direct acute mechanical vascular injury usually occurs as the result of trauma, or as the unintentional consequence of intra-coronary stenting or vein bypass grafting.(59)(60) Acute traumatic injury can lead to the loss of endothelial cells, as well as injury to the tunica media and potentially adventitial tissue. When injury does occur, either progressively as with atherosclerosis or directly through iatrogenic or traumatic mechanisms, there are physiological mechanisms which are activated to aid vascular repair.(61) Commonly however, these are overwhelmed by alternate mechanisms, discussed below, which contribute to the development of pathology and limit the effectiveness of medical interventions designed to re-establish vascular patency and tissue perfusion.(62)

1.4.3 Neointima hyperplasia

The predominant response to progressive or direct vascular injury is neointimal hyperplasia: the migration and proliferation of VSMC, and ECM protein deposition within the tunica intima, and if this is excessive can ultimately lead to a reduction in vessel lumen diameter or stent restenosis.(63) The main driver for this has classically been believed to be the VSMC switch from a contractile to synthetic phenotype in response to multiple potential mechanisms,(64) but the exact process is still incompletely understood.

1.4.3.1 Local growth factor release

A key feature of endothelial activation, either through direct insult or ROS production, when considering neointimal hyperplasia is the increased release of growth factors and cytokine production from the activated endothelium. One of the earliest recognised of these was the platelet derived growth factor (PDGF) family. PDGFs are release by endothelial cells, platelets and synthetic VSMC in response to injury. They are a strong chemoattractant for VSMC, and act directly through PDGF receptors, of which there are two types PDGF receptor-alpha (PDGFRα) and PDGF receptor-beta (PDGFRβ).

Activation of either receptor induces phosphorylation of the mitogen activated protein kinase (MAPK) signalling family, including extracellular signal-regulated kinases (ERK) 1/2 and p38 MAPK, as well as upregulating phosphorylation of the PI3K/Akt signalling pathway,(65) and stimulating ROS generation through activation of NADPH oxidase (NOX).(66) Activation of these pathways increases cellular proliferation, migration and has been linked to dedifferentiation of VSMC into the synthetic phenotype. Interestingly a differential response to PDGF stimulation in arterial smooth muscle cells and venous

smooth muscle cells has been evidenced, with greater proliferation seen in venous VSMC in response to PDGF stimulation.(67)

This differential response corresponds clearly in clinical practice, with venous bypass grafts used for coronary artery revascularisation more predisposed to neointimal hyperplasia and subsequent graft failure than arterial grafts in the same patient.(67) The reason for this is yet to be fully elucidated, but it has been shown that there is a differential response to PDGF isoforms between arterial and venous VSMCs, that activation of MAPK and PI3K/Akt pathways may have differing influences on proliferation between the cell types, and that in venous VSMC there appears to be cross activation of epidermal growth factor receptors (EGFR) by the PDGF-BB isoform *in vitro*, which does not appear to occur in arterial VSMC.(65)

The insulin-like growth factors (IGF) and their binding proteins have also been implicated in VSMC proliferation and neointima formation. This will be discussed in detail in section 1.9, and the role played by insulin-like growth factor binding protein-2 (IGFBP-2) will be expanded further in section 1.10.5.

1.4.3.2 Integrins

Integrins are transmembrane receptors, bound to the intracellular cytoskeleton and extracellular matrix protein ligands. They predominantly consist of different alpha and beta subunits, with the composition of these subunits dictating ligand interaction and integrin signalling properties.(47) Integrins play a key role in mediating VSMC adhesion through binding to different ECM components, and as previously described ECM ligand binding to integrins in normal settings contributes to maintenance of the quiescent contractile phenotype.(45)(47) However, in the setting of vascular injury or atherogenesis, alterations in integrin receptor expression and ECM ligand binding can

promote outside-in signalling, contributing to cellular proliferation, migration and de-differentiation to a synthetic phenotype.(47)

Proteins that are not part of the extracellular matrix can also act through integrin binding to influence VSMC structure and function. PDGF synergistically interacts with β1 and αvβ3 integrins, promoting cellular proliferation, and α5β1 and α1β1 integrins play an important role in mediating angiotensin-II induced VSMC proliferation through ERK activation.(68)(69) Another integrin ligand, microfibrillar-associated protein 4 (MFAP4) is secreted by VSMC *in vitro* and binds to αvβ3 integrin, promoting cellular migration through focal adhesion kinase (FAK) activation and cellular proliferation through ERK and PI3K/Akt pathways. It is also associated with VSMC dedifferentiation and loss of contractile markers, although the mechanism for this has not been fully elucidated.(70) Fibronectin–splice variant containing extra domain A (Fn-EDA) is another protein, secreted by synthetic VSMC, which interacts with multiple integrin receptors as well as Toll like receptor 4 (TLR4) to activate multiple signalling pathways including ERK, PI3K/Akt and the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway to induce further phenotypic switching, cytokine expression and cellular proliferation and migration.(63) Additionally, integrin interaction is a primary mechanism through which IGFBP-2 exerts its actions, and will be discussed in greater detail in section 1.10.

1.4.3.3 Progenitor cell contribution

The classical picture of neointimal hyperplasia was one of neointima derived purely from contractile VSMC dedifferentiation and subsequent local proliferation. Two decades ago there were reports to suggest that this simplistic picture may not be the whole story, and that bone marrow derived progenitor cells express smooth muscle markers such as α-SMA and can give rise to synthetic VSMC-like cells in atherosclerotic lesions.(71)

Subsequent studies refuted this,(72) and lineage tracing studies have shown definitively that the differentiation of bone marrow derived progenitor cells into VSMC-like cells during neointima formation is a rare event and plays no meaningful role in neointima development.(73)

Neointima formation is a complex process, meditated through multiple mechanisms by factors expressed by VSMC, activated endothelial cells and platelet aggregation, leading to a cycle of increasing lesion development. Moreover, it is clear that some effects and pathways seen *in vitro* or in animal models of vascular injury may not be relevant to human pathophysiology.(72) However, given these complex and self-perpetuating mechanisms, once the process has begun it becomes increasingly difficult for vascular repair mechanisms to have any effect.

1.4.4 Atherosclerosis formation

Atherosclerosis, arterial aneurysm formation and the other vascular pathophysiological sequelae described below are not the focus of this thesis but all, especially atherosclerosis, are crucial consequences of both endothelial and VSMC dysfunction, and so will be described briefly. Atherosclerosis usually occurs as a result of vascular insult, endothelial instability and progressive endothelial dysfunction. Sites of shear stress due to non-laminar blood flow are prone to migration and proliferation of VSMC, leading to arterial subendothelial intimal thickening, which in turn acts as a soil-bed for progressive recruitment of lipid laden macrophages, inflammatory leukocytes and further migration of proliferative smooth muscle cells, with deposition of extracellular matrix proteins and accumulation of calcium contributing to the development of fibrous atherosclerotic ‘plaques’. Animal models of endothelial dysfunction differ in that the primary result is recruitment and accumulation of inflammatory cells, with VSMC proliferation and migration being a secondary response.(74)

Plaques develop and progress through a complex process of inflammation; shear stress; oxidative damage; endothelial dysfunction and VSMC migration and proliferation.(75) Depending on composition and position, atherosclerotic plaque may be considered stable, leading to chronic tissue ischaemia and cardiovascular conditions such as stable angina or peripheral arterial disease (PAD); or may be unstable and prone to rupture with subsequent tissue infarction.(76)

1.4.5 Arterial aneurysm formation

Arterial aneurysms are characterised by a focal widening of the arterial lumen diameter with an associated reduction in integrity of vascular wall, leading to an increased risk of vascular rupture of the aneurysmal section. Aneurysms can occur throughout the large and medium arterial vasculature and may be a consequence of inherited connective tissue disease affecting the integrity of the extracellular matrix, or as a consequence of chronic endothelial and VSMC damage and apoptosis. Abdominal aortic aneurysms (AAA) are the most prevalent, and rupture of abdominal or thoracic aortic aneurysm (TAA) continues to carry an extremely significant morbidity and mortality risk.(77) Interestingly, although thoracic and abdominal aortic aneurysms have similar morphological and histological appearance, the pathophysiological mechanisms for each appear to be distinct.(78) Activation of matrix metalloproteases represents a fundamental pathway in the development of AAA, with subsequent loss of tunica media integrity secondary to VSMC apoptosis and ECM degradation. This increased MMP activation and synthesis occurs due to increased inflammatory activity, ROS formation and macrophage infiltration of the tunica media, all of which contribute to both MMP production and VSMC phenotypic switching. As with neointima formation and atherosclerosis, this leads to a self-sustaining cycle of inflammation, increased cytokine and growth factor production, and ROS formation.(78)

Thoracic aortic aneurysms have been less well studied than AAA and so less is known about their pathophysiology. TAAs appear to have a stronger genetic component than AAAs, with inherited connective tissue diseases such as Marfans and Ehlers-Danlos Syndrome and the associated abnormal ECM deposition conveying significantly increased risk of TAA. Inflammatory mechanisms, ROS synthesis, activation of MMPs and apoptosis of VSMC all have a significant role in TAA as with AAA, although there has been less elucidation of the pathways involved.(78) It is evident that similar mechanisms involved in aneurysm formation are also factors involved in arterial stiffening, and it has been evidenced that segmental arterial stiffness precedes AAA formation.(79)

1.4.6 Cardiac allograft vasculopathy

Cardiac allograft vasculopathy (CAV) is a condition characterised by the accelerated occurrence of coronary artery disease following cardiac transplantation and is a key reason for mortality following cardiac transplantation. It predominantly appears to be mediated by an inflammatory immune response, leading to endothelial and VSMC dysfunction and subsequent VSMC proliferation, with dysfunctional repair mechanisms exacerbating the rapid development of atherosclerosis.(80) Other contributing factors include classical cardiovascular risk factors such as hypertension, smoking and hyperglycaemia present in either the donor or recipient, as well as opportunistic infections such as cytomegalovirus which provoke an inflammatory immune response as well as impairing eNOS synthesis through the production of eNOS inhibitors.(81) Allograft vasculopathy differs from conventional, focal coronary atherosclerosis as it is characterised by diffuse intimal thickening throughout both the epicardial and intramural coronary arteries, with early fibrofatty deposition followed by late necrotic atherosclerosis and calcium deposition.(81) Although CAV occurs uniquely following transplantation, and the pattern and timeframe of atherosclerosis development differs as described, the pathways activated by the milieu of inflammatory cytokines and growth factors, and the

subsequent endothelial dysfunction, smooth muscle proliferation and excessive ECM deposition are similar to that seen in conventional neointima formation and atherosclerosis.(81)

1.5 Vascular repair

Vascular repair describes the process of re-endothelialization following vascular endothelial injury, re-establishing the quiescent endothelial phenotype and preventing or limiting VSMC infiltration, cellular proliferation and atherosclerotic development.(82) Although there are physiological mechanisms to allow for re-endothelialization, these are not yet fully elucidated, and controversy exists as to the extent murine and *in vitro* experimental findings relate to human physiology. These mechanisms of injury and repair ideally exist in equipoise, but with repeated insult the balance can tipped away from physiological repair and towards vascular damage, with the self-perpetuating nature of the activated endothelium and neointimal formation leading to the development of cardiovascular disease.(83) There has therefore been considerable interest and research effort invested into the investigation and development of a variety of potential therapies aimed at enhancing physiological mechanisms and tipping the balance back towards vascular repair and regeneration.(84)

1.5.1 Re-endothelialization

Re-endothelialization refers to the process by which the endothelial lining of a vessel is restored after disruption of the endothelial monolayer. This can occur after spontaneous rupture of atherosclerotic plaque or after placement of intra luminal stents in the coronary or peripheral vasculature. Re-endothelialization after stent placement is an important part of the healing process, as the incorporation of stents into the vasculature and

autologous endothelial coating reduces the risk of spontaneous thrombus formation at the site of intervention.(85)

There are two primary methods by which it is thought vascular repair may occur in normal physiology: resident endothelial cell recruitment and proliferation adjacent to the area of injury, and endothelial progenitor cell (EPC) mobilisation and vascular incorporation, with significant overlap and interaction between each mechanism. The degree to which each of these contributes in normal conditions has been the subject of debate, disagreement and confusion over the last two decades,(86) with confusion compounded by the wide variety of distinct cell types which have been dubbed ‘endothelial progenitor cells’.(61)

Resident endothelial cell recruitment has been demonstrated to be the primary driver in vascular repair in a number of studies. Hagensen *et al* took carotid artery segments from wild type (WT) mice, subjected them to wire injury and then transplanted into transgenic mice in which both mature endothelial cells and circulating EPCs expressed green fluorescent protein (GFP). They demonstrated that neo-endothelium in the injured carotid artery segments did not express GFP, and therefore must have been derived from adjacent WT endothelial cells, with no evidence of bone marrow derived EPC incorporation.(87) Other studies by Itoh *et al* and Tsuzuki also used GFP fluorescent tagging to demonstrate injured murine artery segments were re-endothelialized by adjacent resident endothelial cell proliferation rather than bone marrow derived cells.(88)(89) Further studies looking at lung and liver endothelial regeneration found that resident endothelial cells were the primary driver in re-endothelialization, although a role for bone marrow derived EPC recruitment and incorporation was identified.(90)(91)

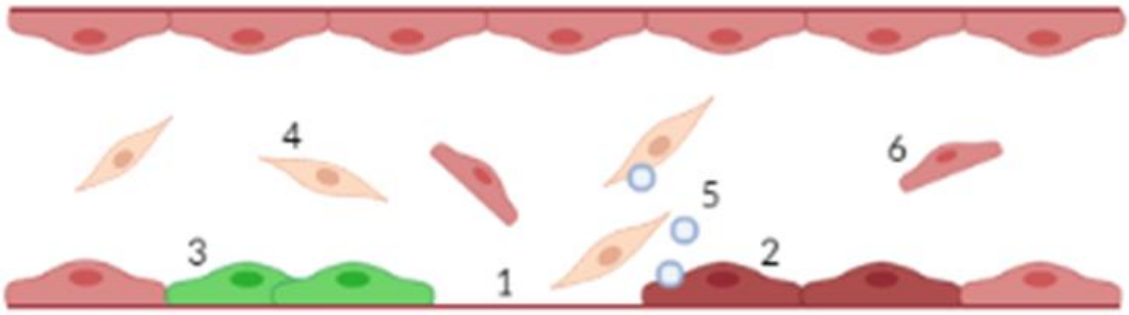


Figure 1-3. Putative re-endothelialization mechanisms. 1. Injured endothelium with removal of endothelial monolayer. **2.** Mature adjacent resident endothelial cell proliferation. **3.** C kit + vascular endothelial stem cells proliferating in response to endothelial activation. **4.** Circulating myeloid angiogenic cells (MACs), which may incorporate into injured endothelium. **5.** Paracrine release of growth factors by MACs, augmenting resident endothelial cell proliferation. 6. Circulating endothelial colony forming cells, which may incorporate into denuded endothelium. Created using Biorender.com

1.5.1.1 Resident endothelial cells

Whilst there is some evidence that mature resident endothelial cells have the capacity to proliferate in response to vascular injury,(90) there are indications that a variety of different endothelial cell subtypes exist within the spectrum of the mature endothelial phenotype which may contribute to vascular repair in differing degrees. In particular it has been hypothesised that there are resident vascular endothelial stem cells (VESC) which are the primary contributors in the adult vasculature to vascular repair and angiogenesis. VESCs have been identified as cells resident within quiescent murine vasculature which, as well as expressing typical endothelial cell surface markers, also express CD117, or c-kit.(92) These c-kit+ cells demonstrate significant ability to expand and proliferate, and it has been demonstrated that an entire, functional blood vessel can be generated *in vivo* from a single c-kit+ VESC. They are therefore likely play a significant role in vascular repair, although the degree to which they are present and their activity in human vascular repair is not yet established (Figure 1-3).(92)

1.5.1.2 Endothelial progenitor cells

A role for EPCs in angiogenesis and vascular repair was first described by Asahara *et al* in 1997. They discovered that spindle shaped cells positive for the endothelial cell surface marker CD34 could be derived from peripheral blood derived monocytes (PBMC). These cells also expressed eNOS and Flk-1, a receptor for vascular endothelial growth factor (VEGF), and so were labelled as having an endothelial cell phenotype.(93) This study found that these cells, when injected into a mouse model of hindlimb ischaemia, were incorporated into the neoendothelium, and so were suggested as potential novel therapeutic agents. A further study by Foteinos *et al* examined the role of bone marrow derived EPCs in normal endothelial turnover in apolipoprotein E-deficient (ApoE) mice, a transgenic mouse model prone to atherosclerosis. Bone marrow transplants in these mice demonstrated that labelled bone marrow derived EPCs were found in atherosclerotic lesion prone areas of the aorta, although they only accounted for 3 to 4% of total endothelial cells in the lesion-prone areas.(94)

These findings were not replicated in other studies however,(87)(95) and the current understanding is that these cells are minimally proliferative, and rather than playing a major role as differentiated endothelial cells, may instead exert proangiogenic and reparative actions through paracrine augmentation of resident EC proliferation, predominantly through the expression of growth factors such as VEGF and hepatocyte growth factor (HGF).(96) These putative EPCs have now been termed Myeloid Angiogenic Cells (MAC), to identify their haematopoietic lineage and differentiate them from other EPC subtypes such as endothelial colony forming cells (ECFC), which will be discussed in detail later (Figure 1-3).(97)

1.6 Vascular regeneration

In utero the *de novo* formation of blood vessels from endothelial precursors is termed ‘vasculogenesis’; a role for vasculogenesis in adults has been hypothesised but is less well delineated.(98) The formation of new blood vessel sprouts from pre-existing vasculature is termed ‘angiogenesis’; with recruitment of VSMC and pericytes and maturation of these neovessels into quiescent vasculature termed ‘arteriogenesis’.(82) Arteriogenesis is also used as a term to describe an increase in diameter of existing collateral vessels, often as a pathological response to occlusion of the predominant supply artery.(99)

In adult physiology the vasculature is largely quiescent, with the exception of the female reproductive system. However, the need for physiological tissue repair and normal cell turnover requires the ability for angiogenic processes to be activated when required in a tightly regulated manner.(100) Thus ‘vascular regeneration’ occurs when neovasculature is required in response to ischaemia and the disruption of pre-existing vasculature. Vascular regeneration is a complex, multi-faceted process that has considerable overlap with vascular repair and the mechanisms involved in vascular repair.(82) Vascular regeneration can therefore be viewed as a physiological response to a pathological process, involving angiogenesis, arteriogenesis and vascular repair. However, angiogenesis itself can be pathological, especially in the context of malignancy and tumour growth, and in certain situations pathological angiogenesis can arise from vascular regeneration processes intended as a physiological response to ischaemia or other insult, such as the neovascularisation seen in proliferative retinopathy.

Vascular regeneration primarily occurs in response to occlusion of the existing vasculature causing ischaemia of the perfused tissue. This is evident in the response seen to chronic occlusion of coronary arteries or limb peripheral arteries, with evidence of both remodelling and enlarging of existing collateral blood vessels, and neovascularisation of the ischaemic tissue.(101)(102). This response is frequently

suboptimal however in restoring adequate perfusion, and for many patients conventional treatment or surgical options to restore perfusion may be limited.(103) Vascular regeneration has therefore been the focus of extensive research into cell based therapies, and therapeutic angiogenesis has long been thought to have the potential to both improve symptoms related to ischaemia and mortality outcomes.(104)

1.6.1 Angiogenesis

Angiogenesis occurs via a stepwise process in response to localised proangiogenic signals and growth factor release. These signals initially cause pericytes to detach and increase endothelial permeability, allowing ECM to extravasate and form a scaffold for migratory ECs. The leading ECs are termed ‘tip’ cells and form filopodia during this initial stage of sprouting angiogenesis. These vascular sprouts develop further, as ’stalk’ cells behind the tip cells elongate the sprout and form a lumen, with signalling differences within these cells reducing additional filopodia formation and preventing chaotic vessel formation.(105) VSMC migration and pericyte attachment follows during arteriogenesis, as mature blood vessels develop into a functioning network to allow blood flow to the newly perfused tissue (Figure 1-4).(106)

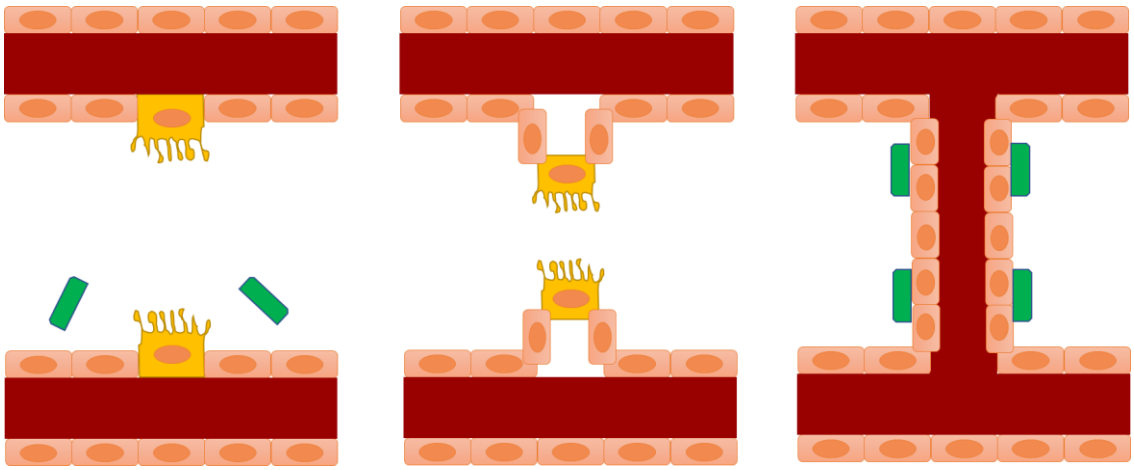


Figure 1-4. Sprouting angiogenesis. Pericytes detach (green), endothelial permeability increases and tip cells with filopodia (yellow) lead the formation of a new blood vessel, with a VEGF gradient allowing the movement of tip cells towards proangiogenic signals. VEGFR-2 downregulation in the following stalk cells prevents unregulated filopodia formation and disorganised vessel formation. Once the new blood vessel forms, pericytes attach and contribute to maturation of the blood vessel, as does shear stress from blood flow within the newly formed vessel.

1.6.1.1 VEGF

The vascular endothelial growth factors are the central mediators of all aspects of angiogenesis. There are five isoforms in the VEGF family, with VEGF-A playing the most prominent and best described role in angiogenesis via interaction with its tyrosine kinase receptor VEGFR-2 (also known as Flk-1).(107) This interaction has been demonstrated to be essential to vascular development, with loss of either VEGF or VEGFR-2 severely retarding vascular development.(108)(109)

VEGF-A and VEGF-C expression is significantly upregulated in hypoxic or inflammatory conditions, the former predominantly mediated by increased Hypoxia Inducible Factor 1 Subunit Alpha (HIF-1α) expression. VEGF-A acts to increase endothelial layer permeability and extravasation of ECM proteins which form the scaffold for vascular sprouts, which migrate via integrin binding as the tip cells lead towards proangiogenic signals, including VEGF-A and VEGF-C. During this process, VEGFR-2 expression is downregulated in stalk cells, preventing them from forming further filopodia. VEGF-A

also plays a role in promoting proliferation of stalk cells, elongating the vascular sprout, as well as contributing to vascular lumen formation.(110) Autocrine expression of VEGF-A by the newly established endothelial monolayer continues to play a role in maintaining normal endothelial cell turnover. Paracrine expression of VEGF-A however, such as that seen with tumour cells, leads to disorganised neovasculature with multiple branches.(106)

1.6.1.2 Additional growth factors; integrins and signalling pathways

Although VEGF is identified as playing a pivotal role in angiogenesis, multiple other growth factors, chemokines and peptides also contribute. Fibroblast growth factors (FGFs), HGF, insulin-like growth factors (IGFs) and angiopoietin-2 (ANGPT2) all play a proangiogenic role, stimulating angiogenesis and increasing vessel sprouting.(100) Insulin-like growth factor binding proteins (IGFBPs) have also been investigated and will be discussed in detail later. The PDGF family and angiopoietin-1 (ANGPT1) play roles in promoting vascular quiescence, through pericyte attachment, and basement membrane deposition respectively. As previously described, integrin binding helps attach proliferating ECs to their ECM scaffold, but additionally will bind to growth factors to further stimulate angiogenesis.(106)

There are multiple signalling pathways other than HIF-1α which can also contribute to angiogenesis. The PI3K/Akt and MAPK tyrosine kinase signalling pathways are both induced by growth factors binding to their upstream receptors as well as integrin binding, and increased phosphorylation of these signalling pathways both cause downstream upregulation of VEGF production.(111)(112) There is therefore considerable cross talk between these pathways and overlap with the mechanisms controlling both vascular repair and neointimal formation.

1.7 Therapeutic strategies for repair and regeneration

1.7.1 Angiogenesis inhibitors

As described, physiological processes for vascular repair and regeneration are complex, and often overwhelmed by the self-perpetuating nature of pathological mechanisms such as neointima formation. Additionally it has been recognised that pathological angiogenesis is a key driver for neoplastic progression and disorders such as neovascular macular degeneration.(113)(114) Significant efforts therefore have been directed towards the discovery of individualised and context-specific therapeutic strategies to modulate vascular repair and angiogenesis. These include attempts to both promote revascularization of ischaemic tissues and inhibit angiogenesis in cancer, ocular, joint or skin disorders. Inhibitors of pathological angiogenesis have been successfully developed which are now used clinically in the treatment of certain cancers and forms of macular degeneration.(115) The initial breakthrough antiangiogenic drugs primarily acted to inhibit VEGF signalling, with more recent medication aiming to inhibit multiple proangiogenic signalling pathways rather than just VEGF.(116)

1.7.2 Therapeutic angiogenesis

Therapeutic angiogenesis describes the upregulation of angiogenesis as a mechanism to repair organ damage and improve tissue perfusion in ischaemic disorders, but harnessing angiogenesis as an effective therapy has proven to be more challenging than inhibiting the angiogenesis process. Cell-based strategies using autologous or modified progenitor cells, as well as systemic and local delivery of proangiogenic factors have all been investigated as potential strategies.(117)

Initial studies used administration of recombinant VEGF and FGF to induce angiogenesis and subsequent collateral artery formation in animal and human models of myocardial

ischaemia, successfully demonstrating improved organ perfusion.(118)(119)(120) Other early studies used cell based therapy, with local delivery of autologous bone marrow derived stem cells in both myocardial infarction and PAD also appearing to improve tissue perfusion and induce local angiogenesis.(121)(122)

However, subsequent larger trials of therapeutic angiogenesis were less successful. The Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis (VIVA) trial was a randomised control trial (RCT) examining intra-coronary recombinant human (rh) VEGF administration in patients with stable but refractory angina, and no significant improvement in myocardial perfusion or exercise tolerance was seen between the treatment and control groups.(123) Subsequent meta-analyses of autologous cell therapy in PAD have also shown that in placebo controlled RCTs there is no benefit to cell therapy.(124)

To overcome the shortcomings of direct protein administration, gene therapy has also been studied, with areas of ischaemia targeted directly by vectors containing VEGF and FGF, allowing for more consistent and longer lasting proangiogenic signalling. Initial trial results in both PAD and myocardial ischaemia were promising,(125)(126)(127) but unfortunately once again larger RCTs with a lower risk of bias demonstrated a lack of significant improvement in perfusion or symptom improvement between control and treatment arms.(128)(129)

The lack of consistent benefit when examined in larger human subject trials means that there has not yet been a proangiogenic therapy licensed for clinical use. However, there remains a need for medical therapy to improve tissue perfusion for patients with ischaemia who are not suitable for mechanical revascularisation. Most studies of therapeutic angiogenesis have only used VEGF or FGF as putative proangiogenic

factors, but there are several other cytokines, growth factors and potential cell-based vectors with significant therapeutic potential.

Two such potential cell-based therapies include endothelial cells differentiated from human pluripotent stem cells (hPSC), and ECFCs derived from peripheral blood mononuclear cell culture. HPSCs can be differentiated into endothelial cells by VEGF-A treatment, and once differentiated continue to demonstrate significantly higher angiogenic and proliferative capability than mature endothelial cells.(130) Administration of these cells into mouse models of ischaemia has been shown to improve perfusion and capillary density of the affected limb.(131) A major limitation of this potential therapy has been low levels of retention and recruitment of these cells into ischaemic tissue.(132) Recently, enhanced delivery of hPSC derived endothelial cells using ECM-mimicking gels has been shown to significantly improve cellular recruitment and long term survival, as these cells were shown to be incorporated in the vasculature and sustaining neovascular formation several months after initial administration.(133) It is important to note however that other cell based and gene therapy treatments have appeared very promising in animal models, but not delivered to their perceived potential when tested in rigorous RCTs.

1.7.3 Endothelial colony forming cells

ECFCs are a form of endothelial progenitor cell, derived from prolonged culture of PBMCs.(97) They have a mature endothelial cell phenotype, exhibit endothelial cell marker expression, lack haematopoietic cell markers such as CD14 and CD45 but have significant proliferative capacity suggestive of progenitor hierarchy. They have been known in the past as Outgrowth Endothelial Cells; Late Endothelial Progenitor Cells and Blood Outgrowth Endothelial Cells, with consensus on nomenclature only reached recently.(97)

There has been significant confusion between these cells and MACs, formally known as early endothelial progenitor cells. Both of these cell types are derived from PBMCs, and may play significant roles in vascular repair and regeneration, but MACs have hematopoietic lineage compared to the endothelial lineage of ECFCs, and likely promote angiogenesis through paracrine factors,(134) whereas ECFCs are recruited directly to areas of vascular injury to assist in re-endothelialization,(135) and additionally have the capacity for tubule formation *in vitro* and new blood vessel formation *in vivo*.(136)(137)

Our group has previously compared ECFC function in South Asian and Caucasian healthy subjects, given the acknowledged increased risk of premature coronary artery disease (CAD) in the South Asian population.(138) It was found that ECFCs from South Asian subjects had reduced capacity for vascular repair and regeneration in murine models of femoral artery wire injury and hindlimb ischaemia.(139) This was found to be due to a down-regulation of the PI3K/Akt pathway, and restoration of this pathway improved their function to that of those ECFCs from healthy Caucasian subjects.(139) This dysfunction was hypothesised to relate to early insulin resistance, as although the South Asian subjects had no history of overt diabetes, they were found to have higher fasting insulin levels than their Caucasian counterparts,(139) and it has been established that ECFCs derived from subjects with diabetes are dysfunctional compared to their healthy counterparts.(140)

It is not yet clear if circulating ECFCs have an intrinsic role in physiological vascular repair and regeneration, as it has only been demonstrated that injection of extrinsically derived ECFCs will hone in on areas of vascular injury or ischaemia and facilitate recovery. By definition it takes weeks to demonstrate significant *in vitro* colony formation, and they only represent a tiny fraction of PBMCs.(86) Their origin is also still debated; it

had originally been thought ECFCs were bone marrow derived,(141) but it is now hypothesised they may result from endothelial monolayer shedding, and so may be the result of *in vitro* proliferation of circulating endothelial cells.(142) It has also been recognised that ECFCs can themselves be separated into distinct groups: high proliferative potential and low proliferative potential ECFCs, which may relate to the type of resident EC they are derived from.(142)

Nevertheless, our group's work and other studies (143) have shown that ECFCs can be studied *in vitro* as a surrogate for human endothelial cell function *in vivo*. Furthermore, ECFCs derived from subjects with insulin resistance demonstrate impaired function, but this function can be rescued through restoration of Akt signalling, improving their capacity to influence vascular repair and regeneration.(139) This ability of ECFCs to incorporate into areas of vascular injury and augment angiogenesis in ischaemic conditions, coupled with the evidence that these capabilities can be restored in individuals with impaired function, has led ECFCs to be touted as having significant potential to be candidates for autologous cell-based therapies for vascular repair and regeneration.(144) While it has been demonstrated that ECFC function can be restored to a normal level by restoration of physiological Akt signalling, it may be that the angiogenic capacity of ECFCs can be augmented further through supraphysiological expression of other relevant growth factors, and this possibility will be explored in later sections of this thesis.

1.8 Cardiometabolic disease

Thus far the discussion has focussed upon vascular structure, repair processes and pathophysiology at the cellular level, as well as introducing the concept of cell-based therapies to enhance vascular recovery. The contribution of traditional cardiovascular risk factors to the development of atherosclerosis and other pathophysiological processes has been described, and the following paragraphs will expand upon the role

played by obesity, insulin resistance and diabetes mellitus, collectively described as cardiometabolic disease, in the development of vascular pathophysiology. Despite vast improvements in improving the global disease burden in modern history, and a recent global reduction in tobacco use, obesity and diabetes continue to increase year on year, creating a cardiometabolic scourge which contributes to ischaemic heart disease as the leading cause of adult mortality.(145)

1.8.1 Obesity

Obesity is a major global epidemic, correlating with a marked rise in cardiovascular disease over the last several decades. The impact of obesity on vascular pathophysiology is complex and multi-faceted. Obesity itself directly impacts on vascular function, and is also associated with other cardiovascular risk factors such as hypercholesterolaemia, hypertension and insulin resistance, manifesting as prediabetes or overt type 2 diabetes mellitus.(146)

White adipose tissue has previously been considered as merely an energy repository, but it is now established that it is in fact an endocrine organ, with the capacity to synthesise and excrete numerous cytokines and growth factors, including inflammatory and growth factors such as TNF-α, interleukin-6 (IL-6), and insulin-like growth factor-I (IGF-I), as well as factors which can be protective of vascular function such as adiponectin and oestrogens.(147) White adipose tissue is therefore an essential organ in normal physiological homeostasis, however an abnormal increase in the amount of white adipose tissue, such as that seen in obesity, leads to adipocyte dysfunction and an imbalance in favour of increased ROS and pro-inflammatory cytokine production.(148) This contributes to the decreased NO bioavailability seen in obese individuals, causing endothelial dysfunction and the sequence of events leading to vascular pathology that have been previously described.(149)

Carotid intimal medial thickness and arterial stiffness has also been demonstrated to be increased in obese humans, including children,(150) suggesting the possibility of alteration in VSMC function and proliferation, and *in vitro* studies have suggested growth factors released by adipocytes stimulate VSMC proliferation.(151) Other causative factors of vascular pathophysiology observed with obesity likely include increased sympathetic nervous system activity, as well as key roles played by the associated factors of insulin resistance, hypertension and hypercholesterolaemia.(147)

1.8.2 Insulin resistance and insulin signalling pathways

'Insulin resistance' describes the impaired response to the actions of insulin in different tissues, and is hypothesised to be a central feature to the development of vascular pathophysiology in obesity, as well as being responsible for the development of prediabetes and subsequent overt type 2 diabetes mellitus, manifesting as persistent hyperglycaemia.(149) As with obesity, insulin resistance is strongly associated with endothelial dysfunction secondary to reduced NO bioavailability, and insulin signalling is not only one of the factors directly responsible for eNOS phosphorylation at physiological levels, but NO itself plays a role in promoting endothelial insulin uptake.(152)

Insulin signalling occurs via the insulin receptor, a transmembrane tyrosine kinase receptor that autophosphorylates on binding of insulin, its ligand. Phosphorylation of the insulin receptor triggers a cascade of further tyrosine phosphorylation via the recruitment of insulin receptor substrates, and subsequent activation of multiple signalling pathways.(153) Cellular glucose uptake, the classical primary function of insulin signalling, occurs through translocation of GLUT4 to the cell surface following activation of the PI3K/Akt pathway, which is also implicated in multiple cellular actions within the vasculature, as previously described.(153) Other consequences of insulin signalling and

Akt activation include protein synthesis in skeletal muscle via mTOR activation, and eNOS activation in the vascular endothelium, enhancing endothelial cell insulin uptake as well as contributing to vascular homeostasis.(152)(153) Another crucial signalling pathway activated by insulin signalling is the MAPK pathway, which plays a primary role in cellular proliferation and differentiation.(154)

Insulin signalling and the effect of insulin resistance and hyperglycaemia in vascular pathophysiology are well described and beyond the scope of this thesis. The related insulin-like growth factor family and its group of binding proteins have also been found to have important contributions to vascular homeostasis and pathophysiology. These have been less extensively studied however, especially the insulin-like growth factor binding proteins and particularly in relation to their effects on vascular biology beyond angiogenesis, which we will attempt to establish.

1.9 The insulin-like growth factor system

The IGF system is a complex hierarchy of growth factors, receptors and binding proteins, and exhibits significant overlap with traditional insulin signalling pathways. Insulin-like growth factors I and II share common ancestry with insulin and have a nearly homologous structure.(155) The IGFs exert their positive actions through binding to the receptor IGF-1R, with a second receptor, IGF-2R playing an inhibitory role by sequestering and degrading IGF-II.(156) Additionally the IGFs will also bind insulin receptors, and insulin can bind to IGF-1R, albeit the affinity for both to the alternate receptor is significantly weaker than to their own receptors, and it is unlikely this occurs at physiological levels in humans.(157)

IGF-I has been repeatedly demonstrated to play an essential role in growth hormone (GH) mediated postnatal development.(158) GH predominantly mediates IGF-I synthesis in the liver, and classically the role of IGF-I in postnatal growth and development was thought to relate entirely to endocrine effects from this mechanism, as liver-derived IGF-I is the principal source of circulating IGF-I. IGF-I is also synthesised in multiple other tissues however, and the importance of hepatic derived systemic IGF-I has been challenged, after separate groups demonstrated normal postnatal growth in a murine model with deletion of the IGF-I gene in the liver alone,(159)(160) positing instead that the effects of IGF-I on growth were entirely related to autocrine and paracrine effects of IGF-I synthesised in target tissues. Other studies have however demonstrated an important endocrine role for systemic IGF-I in postnatal growth, complementing the local autocrine and paracrine actions of IGF-I derived from extrahepatic tissues.(161)

In comparison to the role for endocrine IGF-I seen in postnatal growth and development, in mature adults the effects of IGF-I on cellular growth, proliferation and repair in tissues such as skeletal muscle, bone and VSMC appear to be predominantly related to the autocrine and paracrine effects of locally synthesised IGF-I.(162) Tissue specific IGF-I synthesis may be induced by growth hormone, but also by multiple other factors including cytokines, growth factors and environmental conditions such as hypoxia and mechanical stretch.(162)(163)

Although IGF-I does appear to play a role in blood glucose homeostasis, IGF-I also has distinct metabolic actions which differ from insulin despite its structural homology and analogous signalling pathway activation. This is due in part to differential expression of insulin and IGF receptors in different tissues, affecting their site of action.(157)

Nutritional intake plays a significant role in modulating GH mediated hepatic IGF-I synthesis, with reduced synthesis seen with nutritional restriction.(157) Serum IGF-I levels will negatively feedback on GH production in the pituitary gland to maintain homeostasis.(155) In addition to its stimulating effects on protein synthesis, and inhibition of proteolysis, IGF-I promotes the uptake of free fatty acids into skeletal muscle, preventing excess hepatic free fatty acid flux, a metabolic consequence of GH induced lipolysis, and therefore reducing the associated effects of insulin antagonism and eventual insulin resistance. This has previously been demonstrated in an IGF-I skeletal muscle knockout mouse model which eventually developed type II diabetes mellitus, a consequence rescued by expression of a skeletal muscle fatty acid transporter.(164)

IGF-II expression is independent of GH and, due to negligible expression of the protein in adult rodents, has classically only been considered to have a role in prenatal growth and development (165). However, in humans expression of IGF-II persists postnatally and it continues to play a complimentary role with IGF-I.(166)

Less than 1% of IGF-I is unbound in plasma, with activity tightly modulated by the insulin-like growth factor binding proteins. These are a family of seven structurally similar proteins which bind the IGFs with a higher affinity than the IGF receptors, and therefore act primarily to regulate the activity of IGFs through their binding and transport within the vasculature and into peripheral tissues.(167) This binding can, in the case of IGFBP-3 and IGFBP-5, be as part of a ternary complex with an acid-labile subunit (ALS), therefore having a large molecular mass and an inability to pass into tissue. Other IGFBPs bind to the IGFs as binary complexes which can pass into tissues and act as carrier proteins, stimulating IGF activity by direct transport to the IGF-I receptor,(168) or increasing IGF bioavailability in the pericellular environment.(169)

Evidence is also emerging that the IGFBPs may both potentiate the actions of the IGFs and act independently of IGF activity through a variety of mechanisms.(170)(171) Although each binding protein has complementary structure and binding proteins, these described actions are not seen with all the IGFBPs and both the IGF dependent and independent activity of IGFBPs may be inhibitory or stimulatory depending on the individual IGFBP and cellular environment.(172)

In summary, the IGF family promote cell growth and survival and are indispensable in normal development and whole-body metabolism.(173) However, dysregulation of both IGF activity and IGF-I receptor expression has also been implicated in tumorigenesis, cardiovascular disease and diabetes mellitus.(174)(175)

1.9.1 IGF signalling

Under normal physiological conditions IGF-I and IGF-II predominantly exert their influence through interaction with IGF-1R, a disulphide receptor tyrosine kinase. IGF ligand binding to IGF-1R promotes a phosphorylation cascade and the activation of multiple signalling pathways.(176) The predominantly activated pathways are the PI3K/Akt and Ras/Raf/MEK pathways, which in turn activates the MAPK/ERK signalling pathways. Activation of the PI3K/Akt pathway occurs through phosphorylation of the insulin receptor substrates (IRS), the same intracellular signalling proteins involved downstream of insulin receptor activation.(176) Signalling is also enhanced by IGF-I mediated suppression of phosphatase and tensin homolog (PTEN), a phosphatase which negatively regulates PI3K/Akt signalling.(177)

Activation of these signalling pathways promotes cellular proliferation and survival through multiple mechanisms, as well as protein synthesis through mTOR activation downstream of PI3K/Akt, particularly in skeletal muscle where IGF-I is synthesised

locally and exerts autocrine and paracrine effects. Additionally, glucose homeostasis is affected via Akt/PKB pathway activation and subsequent glycogen synthesis and intracellular glucose uptake.(176) IGF-I stimulation has also been shown to increase eNOS expression through binding to the IGF-I receptor and upregulation of the PI3K/Akt pathway in the same manner as insulin, although this occurs to a lesser degree than insulin and may not occur at physiological concentrations.(178)(179)

IGF-I has also been demonstrated to be proangiogenic through upregulation of VEGF and other angiogenic factors through multiple signalling pathways.(180)(181) Given this array of findings in different tissues, it has been established that the actions of the IGFs and the signalling pathways activated are dependent upon cell type and the cellular environment.

IGF signalling has also been demonstrated to play a major role in both VSMC proliferation as well as atherosclerotic plaque development. IGF-I has been established as a mitogen for vascular smooth muscle cells, promoting cell proliferation and survival *in vitro* through both the PI3K/Akt and MAPK signalling pathways,(182) and increased local expression of both IGF-I and IGF1R have been demonstrated in models of balloon injury induced neointima formation.(183) Interestingly, VSMC within atherosclerotic plaque exhibit reduced IGF1R expression and are more prone to apoptosis. Subsequently, IGF-I has been demonstrated to play a key role in stabilising atherosclerotic plaque, through promotion of VSMC survival via Akt signalling, and potentially preventing pro-atherosclerotic VSMC phenotypic switching.(184) Although exact mechanisms for this have not been fully elucidated, a key role for the integrin receptor α5β1 integrin has been identified.(185)

1.9.2 The insulin-like growth factor binding proteins

A family of seven insulin-like growth factor binding proteins confer spatial and temporal regulation to IGF activity. All the IGFBPs have affinity to both IGF-I and IGF-II, and are expressed by multiple tissues, in response to multiple factors.(186) Gene location and molecular weight for the IGFBPs are listed below in Table 1.

IGFBP	Gene location	Molecular weight (KDa)	Post-translational modifications	Effects of post-translational modification	Functional domains present
IGFBP-1	IGFBP1 Ch7p	27.9	Phosphorylation	Increases IGF-I binding affinity	(RGD)
IGFBP-2	IGFBP2 Ch2q	34.8	Proteolysis	Unknown	RGD HBD1 HBD2 NLS
IGFBP-3	IGFBP3 Ch7p	31.7	Proteolysis Phosphorylation Glycosylation	Proteolysis: increases IGF bioavailability Phosphorylation: releases bound IGF-I Glycosylation: unknown	HBD NLS
IGFBP-4	IGFBP4 Ch17q	27.9	Proteolysis Glycosylation	Proteolysis: reduced affinity for IGF-I Glycosylation: unknown	None known
IGFBP-5	IGFBP5 Ch2q	30.6	Proteolysis Phosphorylation Glycosylation	Proteolysis: increases IGF bioavailability Phosphorylation: alters receptor binding Glycosylation: unknown	HBD NLS
IGFBP-6	IGFBP6 Ch12q	25.3	Proteolysis Glycosylation	Proteolysis: unknown Glycosylation: unknown	NLS
IGFBP-7	IGFBP7 Ch4q	29.1	Proteolysis	Reduces IGF-IR binding	HBD

Table 1. IGFBP gene location, molecular weight and characteristics. KDa: kilodaltons; Ch: chromosome; RGD: arginine-glycine-aspartic acid (integrin binding domain); HBD: heparin binding domain; NLS: nuclear localisation sequence

Whilst the intact structure of the IGFBPs and the IGFBP:IGF complex have not yet been fully elucidated by X-ray crystallography or nuclear magnetic resonance spectroscopy, a number of regional structures have been identified.(187) Three distinct structural regions have been identified which are shared by all IGFBPs: an N-terminal cysteine rich region; a C-terminal cysteine rich region; and a linker region. The N-terminal and C-terminal regions contribute to IGF binding and are highly conserved across the IGFBPs, although the affinity of each to the IGFs varies between the IGFBPs.(188) The linker region is variable, and can contain a variety of functional motifs and binding sites.(189) These functional motifs can be found on several of the IGFBPs and are listed in Table 1. The linker region is also susceptible to post-translational modification. This region contains sites for proteolysis by a range of proteases and proteolytic cleavage occurs in IGFBP -2, -3, -4, -5, -6 and -7. Proteolysis of IGFBPs can have several actions, including the clearance of IGFBPs from the cellular environment, the releasing of intact IGF at the cell surface, and the creation of active fragments which may have distinct, IGF-independent effects.(190) Phosphorylation has been demonstrated in IGFBP-1, -3, -5, -7 and glycosylation in IGFBP-3, -4, -5, -6 and -7. Phosphorylation has been demonstrated to alter the affinity of IGFBP-1 for the IGFs, but no specific role for these post-translational modifications has otherwise been demonstrated.(186) The N-terminal, C-terminal and linker regions will be discussed in more detail in relation to IGFBP-2 specifically in a later passage.

In the circulation, the majority of IGFs are bound to IGFBP-3 in association with an acid-labile subunit (ALS) in a ternary complex, with a molecular mass too large to cross the endothelial barrier. IGFBP-5 also forms a ternary complex with an ALS, with the other IGFBPs forming binary complexes with the IGFs only. These binary complexes allow IGFBPs to primarily inhibit IGF actions, although IGFBPs may also potentiate IGF actions in certain situations.(100) They may additionally stimulate IGF activity by transporting directly to the IGF-I receptor,(168) and certain IGFBPs have reduced affinity for IGF

when bound to the extra cellular matrix, increasing IGF bioavailability in the pericellular environment.(187)

All of the IGFBP family have been implicated in multiple physiological and pathophysiological processes, with actions demonstrated that can be either IGF dependent or independent. Implicated physiological processes include, but are not limited to, normal reproductive physiology in the case of IGFBP-1, neuronal development with IGFBP-2, and osteoblast suppression by IGFBP-3.(191)(192)(193) Metabolic effects have also been seen for the IGFBPs, including enhancing insulin sensitivity and protection against obesity.(194) There has been extensive research of the IGFBPs in the context of malignancy. The IGFBPs have been implicated in a wide variety of neoplastic processes, including cell survival, migration, invasion and tumorigenic angiogenesis, with both inhibitory and stimulatory actions ascribed to the different binding proteins, and IGF dependent and independent effects seen.(171)

Effects of the IGFBPs on multiple aspects of vascular biology have also been demonstrated. Our group have shown that expression of human IGFBP-1 in a transgenic murine model increases endothelial NO bioavailability, via insulin induced PI3K/Akt signalling and subsequently enhanced eNOS phosphorylation, leading to reduced blood pressure and protection from the development of atherosclerosis.(195) The same mouse model has also been demonstrated to have enhanced endothelial regeneration following femoral artery wire injury,(196) and a further study by our group added to these findings by looking at the effects of IGFBP-1 deletion in a transgenic mouse model, finding that IGFBP-1 deletion caused inhibition of endothelial regeneration following vascular wire injury.(197) Interestingly, other studies have demonstrated a possible role for IGFBP-1 in stimulating atherosclerosis development, as gene expression was significantly increased in VSMC within atherosclerotic plaque, positively correlated with inflammatory cytokine production and stimulated VSMC proliferation through ERK1/2 activation.(198)

IGFBP-4 has also been seen to inhibit atherosclerosis via inhibitory binding of IGF-1, whilst IGFBP-5 has been implicated in atherosclerotic VSMC proliferation through the enhancement of locally expressed IGF activity.(189) Multiple studies have also indicated the IGFBPs have effects on angiogenesis in response to an array of factors, with inhibitory and stimulatory actions ascribed to the different binding proteins. The majority of research has been focussed on angiogenesis in the context of malignancy, and the predominant effects of IGFBPs on angiogenesis appear to be mediated through modulation of IGF bioactivity, although important IGF-independent actions are emerging.(100)

A summary of the established actions of the IGFBPs in vascular biology is provided in Table 2. One of the most researched and well described of the IGFBP family is IGFBP-2, with multiple IGF-dependent and independent actions identified, which will be expanded upon further.

Action	**IGFBP-1**	**IGFBP-2**	**IGFBP-3**	**IGFBP-4**	**IGFBP-5**	**IGFBP-6**	**IGFBP-7**
Pro-angiogenic	+	++	+	-	-	-	+
Anti-angiogenic	+	-	+	+	+	+	+
Pro-atherosclerotic	+	?	?	+	+	?	?
Anti-atherosclerotic	+	?	+	-	-	?	?
Pro-endothelial proliferation	+	+	-	-	-	?	?
Anti-endothelial proliferation	-	-	+	+	+	+	+

Table 2. Summary of IGFBP actions on angiogenesis, atherosclerosis and endothelial proliferation. (+ indicates known effect, - indicates no known effect, ? indicates absence of data). As demonstrated, many of the actions of the IGFBPs can be stimulatory or inhibitory depending on the cell type, environment and experimental conditions. Furthermore, there have been several studies examining the association between circulating IGFBP levels and presence of atherosclerosis, which have not been included as a causative mechanism was not established. There remain many areas where the exact role of IGFBPs remain unknown, most notably in the development, and stability, of atherosclerotic plaque.

1.10 IGFBP-2

IGFBP-2 is the second most abundant of the IGFBPs, after IGFBP-3, and expression has been evidenced in a wide range of tissues, including endothelial cells and VSMC. IGFBP-2 exhibits significant effects in a wide range of cell types, although the majority of studies thus far have assessed its role in the context of tumorigenesis. IGFBP-2 appears to have both IGF-dependent and –independent effects, with function and activity affected by cell type as well as receptor activation and nuclear localisation.(100)

1.10.1 IGFBP-2 Structure

IGFBP-2 is a 36kDa protein with three distinct structural regions in common with the other IGFBPs: a N-terminal cysteine rich region, C-terminal cysteine rich region and linker region.(188) The N-terminal and C-terminal both contain an IGF binding domain, and both are required for high affinity biding to the IGFs, with markedly lower binding affinity demonstrated for isolated fragments containing either the N or C-terminals. This has been demonstrated by crosslinking and nuclear magnetic resonance to be due to cooperative IGF binding by the two terminals, with the possibility raised of configurational change of both the IGFs and IGFBP-2 upon binding of the complex.(199)

In addition, IGFBP-2 possesses a number of functional binding motifs which mediate IGF-dependent and IGF-independent interactions. Two heparin binding domains (HBD) have been identified, one in the linker region which is unique to IGFBP-2, and one in the C-terminal region with a similar configuration to heparin binding domains found on

IGFBP-3 and IGFBP-5.(200) Heparin binding domains were first identified on the protease inhibitor antithrombin, as the mechanism through which heparin exerts its anticoagulant effects. HBDs have subsequently been discovered in a variety of growth factors and chemokines, and have been demonstrated to also bind to heparan sulphates, ubiquitous complex polysaccharides, with structural similarity to heparin, that are found on cell membranes and within the pericellular and extracellular matrix.(201) Binding of the HBD in the linker region of IGFBP-2 to the ECM and cell membrane has been demonstrated to exert a variety of effects, predominantly related to cell proliferation and migration.(202) The HBD site in the C-terminal has been less extensively researched but the equivalent region in IGFBP-3 and IGFBP-5 have been determined to bind to the ECM, and it has been confirmed to play a key role in the actions of IGFBP-2 to inhibit adipogenesis.(203)

An integrin binding domain, the highly conserved tripeptide Arginylglycylaspartic acid (Arg-Gly-Asp/RGD), has also been identified in the C-terminal region of IGFBP-2, similar to the RGD domain seen in IGFBP-1.(172) This binding domain predominantly mediates cell membrane integrin receptor interactions, and can subsequently upregulate cell signalling pathways such as the ERK1/2 pathway, with consequential cell invasion, migration and proliferation.(204)

The linker region also contains a nuclear localisation sequence (NLS), which overlaps with the HBD domain.(205) This sequence interacts with carrier proteins called importins, which facilitate nuclear localisation of IGFBP-2 within the cell, following a classical nuclear import pathway, although it has not been determined whether this occurs via extracellular IGFBP-2 through autocrine activity or intracellular synthesised IGFBP-2.(205) Following intranuclear translocation, IGFBP-2 has been shown to directly upregulate *VEGF* mRNA transcription, and subsequently enhance angiogenesis.(206)

Specific actions of these functional motifs of IGFBP-2 in the context of vascular biology will be expanded upon in later sections.

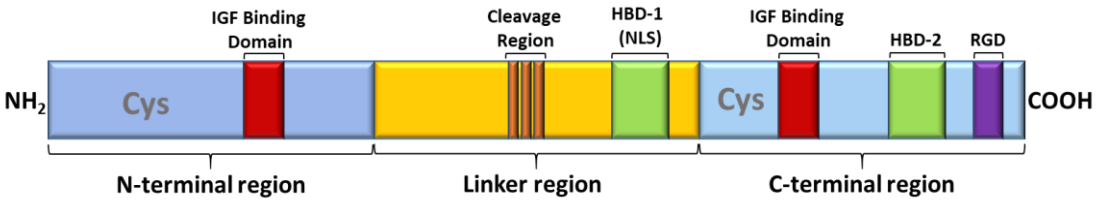


Figure 1-5. IGFBP-2 structure. An IGF binding domain is present in both the N-terminal and C-terminal regions, and both are required for effective IGF binding. A heparin binding domain and integrin binding domain are contained within the C-terminal, with a further heparin binding domain and overlapping nuclear localisation sequence found within the linker region.

1.10.2 Post translational modification

Although post translational modification is a significant factor for some IGFBPs, it appears to have little relevance to IGFBP-2 activity, other than proteolysis. Proteolysis has been noted to occur at a number of cleavage sites within the linker region with a number of implicated proteases including calpain, MMP-7 and Pregnancy Associated Plasma Protein-A (PAPP-A). Proteolysis reduces the binding affinity of IGFBP-2 for the IGFs, and therefore can increase the concentration of free IGFs at their binding sites and increase activation of the IGF-1R.(187) Additionally, a number of studies have used peptide fragments of IGFBP-2 containing individual functional binding sites to demonstrate IGF independent actions of these binding sites, demonstrating that intact IGFBP-2 is not required for all cellular actions.(202)(207)

IGFBP-2 has not been found to be glycosylated. It does have a phosphorylation site, but does not have a recognised phosphorylated isoform and so the significance of this site is unknown.(202) It has been suggested that IGFBP-2 can be altered epigenetically, and

increased DNA methylation of IGFBP-2 in adipose tissue has been linked to the reduced mRNA expression of IGFBP-2 seen in obese subjects.(208)

1.10.3 The role of IGFBP-2 in metabolism

Hepatic expression of IGFBP-2 has been demonstrated to be negatively regulated by insulin,(209) albeit in a slower fashion than IGFBP-1, and levels do not change significantly in response to glucose or post-prandially.(210) Despite this lack of dynamic short term alteration, circulating IGFBP-2 levels have been shown in several studies to inversely correlate with both obesity and insulin resistance.(194)(211)(212) Furthermore, overexpression of IGFBP-2 has been linked to improved insulin sensitivity and reversal of diabetes in mouse models of insulin resistance and deficiency, and IGFBP-2 has also been shown to have protective effects against diet-induced obesity through direct inhibition of adipogenesis.(213) Circulating IGFBP-2 levels have also been inversely associated with plasma triglyceride and very low-density lipoprotein levels, adding to the evidence of a strong association between low IGFBP-2 levels and the metabolic syndrome.(212)

Leptin, a hormone produced by white adipose tissue, has been identified as a powerful regulator of IGFBP-2 expression in both the liver and skeletal muscle, and it has been demonstrated that IGFBP-2 overexpression correlates with improved insulin sensitivity and reduced hyperglycaemia in both leptin-sensitive and leptin-resistant mice, suggesting IGFBP-2 acts downstream of leptin signalling.(214) In skeletal muscle, leptin has been demonstrated to increase intracellular IGFBP-2 and subsequent PI3K/Akt signalling, and silencing of IGFBP-2 reduces leptin and insulin-stimulated glucose uptake, suggesting IGFBP-2 plays a key role in the actions of leptin in peripheral skeletal muscle.(215)

1.10.4 The role of IGFBP-2 in vascular biology

1.10.4.1 Endothelial cells

Endothelial cells were first demonstrated to express IGFBP-2 nearly thirty years ago.(216) Despite this, and despite the established pleiotropic actions, including cell proliferation, migration and angiogenesis, attributed to IGFBP-2 involving PI3K/AKT and ERK signalling in other cell types, there has been little research directly into the effects of IGFBP-2 on vascular endothelial function, with the majority of research in IGFBP-2 involving endothelial cells undertaken in the context of malignancy related angiogenesis.

In this context, IGFBP-2 has been shown as a regulator of angiogenesis in malignant melanomas, and *in vitro* studies of endothelial cells were used to prove this. Das *et al* demonstrated that the addition of human IGFBP-2 (hIGFBP-2) to HUVECs augmented cell proliferation and tube formation *in vitro*. This augmentation was negated by an IGFBP-2 neutralising antibody, confirming IGFBP-2 as the causative factor. IGFBP-2 induction was found to be consequential to HIF-1α induction and PI3K/Akt pathway activation, with the proangiogenic effects of IGFBP-2 occurring through interaction with the integrin receptor aVβ3, and subsequent PI3K/Akt pathway activation and upregulation of VEGF expression.(217) The same group found that Mda-9/syntenin, a protein associated with melanoma progression and metastasis,(218) acted upstream of HIF-1α, and induced its production through ECM interaction and FAK activation. Although the effects of IGFBP-2 were postulated to occur through direct integrin receptor activation, IGF-I receptor knockdown significantly attenuated the effects of IGFBP-2, suggesting an inter-dependent relationship between IGF-I and the IGF-I receptor.(217)

Emerging data from our group demonstrates that external stimulation of HUVECs with IGFBP-2 rapidly upregulates ERK1/2 signalling through integrin β1 interaction with the IGFBP-2 RGD domain,(219) and HUVECs treated in the same manner subsequently demonstrated increased sprouting angiogenesis *in vitro.* Interestingly these effects

appeared to occur independently of VEGF expression, differing from other studies which have demonstrated VEGF upregulation in neuroblastoma cells induced to overexpress IGFBP-2, and a dose-dependent upregulation of VEGF in HUVECs after stimulation with hIGFBP-2.(205)(217) Further effects of IGFBP-2 on angiogenesis in endothelial cells will be discussed in a later section.

1.10.4.2 Vascular smooth muscle cells

Similar to endothelial cells, despite nearly thirty years passing since the discovery that vascular smooth muscle cells express IGFBP-2, there has been very little further research examining the role IGFBP-2 plays in vascular smooth muscle cell function. There has been some debate whether human vascular smooth muscle cells even express IGFBP-2 under normal conditions, as it was shown to be highly expressed in porcine and rat aortic VSMCs, but not initially identified at all in human VSMCs.(220) IGFBP-2 expression has however subsequently been identified in cultured human renal artery vascular smooth muscle cells, although at low levels compared to IGFBPs-3,-4,-5 and -6, and IGFBP-2 did not have an inhibitory effect on IGF-I induced DNA synthesis in human VSMC.(221) IGFBP-2 was seen to inhibit IGF-I induced DNA and protein synthesis in rat derived aortic VSMCs, suggesting differential effects and expression of IGFBP-2 depending on cell origin,(222) an important factor when considering translational impact. The expression of IGFBP-2 in human VSMC may play a larger role in pathophysiology, which will be discussed in more detail later.

It has been demonstrated that IGFBP-2 exhibits an IGF dependent mechanism in porcine vascular smooth muscle cells, with IGFBP-2 found to bind to receptor protein tyrosine phosphatase β (RPTPβ) via its HBD domain.(223) This caused inactivation of RPTPβ and inhibited transcription of the tumour suppressor gene PTEN. Inhibition of PTEN enabled downstream activation of the PI3K/Akt pathway and promoted VSMC proliferation. However, inhibition of IGF-I receptor expression prevented RPTPβ

inactivation, suggesting that effects of IGFBP-2 on PTEN phosphorylation require coordination of IGFBP-2, IGF-I and its receptor.(223)

A key role for IGFBP-2 in the development and progression of lymphangioleiomyomatosis (LAM) has also been demonstrated.(224) This is an unusual disease affecting mainly young women, in which LAM cells – a histologically benign appearing smooth muscle-like cell – proliferate and metastasize to the lungs, causing progressive remodelling and emphysematous-like lung disease.(225) IGFBP-2 was found to accumulate in the nucleus of these cells independently of IGF-1. Accumulation of IGFBP-2 within the nucleus was found to be mediated by oestrogen and oestrogen receptor signalling, although the exact mechanism for nuclear translocation is yet to be elucidated. Knockdown of IGFBP-2 by siRNA reduced LAM cell proliferation, migration and invasiveness, indicating the importance of IGFBP-2 in these tumorigenic actions. Additionally, IGFBP-2 knockdown abrogated MAPK phosphorylation, highlighting a potential mechanism for its tumorigenic effects.(224)

1.10.4.3 The extracellular matrix

The interaction of IGFBP-2 with integrins and ECM components is well established,(210) and many of the actions of IGFBP-2 described in other sections have been shown to be mediated through ECM binding. Binding of IGFBP-2 to the ECM has been demonstrated to be significantly enhanced when IGFBP-2 is in complex with IGF-I or IGF-II,(226) however once bound to the ECM, the binding affinity of IGFBP-2 to the IGFs is markedly reduced.(227) IGFBP-2 can therefore act as a local reservoir for IGF activity and augment local IGF activity when proteolyzed.(202)

A study by Russo *et al* examined the effect of IGFBP-2 on neuroblastoma cells. They determined that IGFBP-2 bound to components of the extracellular matrix *in vitro* and,

through mutagenesis of either the HBD or RGD domains of IGFBP-2, were able to establish that these interactions were mostly mediated through the HBD domain. They went on to describe that both exogenous addition of IGFBP-2 or transduced overexpression of IGFBP-2 within neuroblastoma cells enhanced proliferation, migration and invasion *in vitro.* They again used mutagenesis to demonstrate that these actions were mediated by the HBD domain and proposed that the observed HBD mediated effects were due to interactions with the pericellular and extracellular matrix, as well as local IGF targeting and activation of invasive processes.(228) Within the same study IGFBP-2 inhibited exogenous IGF-I mediated proliferation, likely through IGF-I binding as the effect was similar despite mutagenesis of the RGD or HBD domains, suggesting a dual role through differing, competing pathways.(228)

Although the study by Russo *et al* did not highlight a significant role for integrin binding at the RGD domain of IGFBP-2 in neuroblastoma cells, other studies have demonstrated integrin binding by IGFBP-2 to play a key role in tumour invasion. Holmes *et al* examined glioma progression and linked IGFBP-2 to upregulation of the NF-kB pathway through binding of integrin β1 by its RGD domain, and subsequent activation of integrin-linked kinase (ILK) pathways. This caused upregulation of cell migration, invasion and overall glioma progression.(229) The same group has also previously demonstrated that IGFBP-2 interacts with integrin α5 in glioma cells to promote cell migration, and that this interaction is enhanced by the presence of the ECM glycoprotein fibronectin, despite fibronectin also binding to this integrin receptor, suggesting a co-stimulatory pathway.(230)

Another study by Frommer *et al* identified that exogenous stimulation of breast cancer cells with IGFBP-2 caused upregulation of multiple genes involved in cell proliferation, adhesion and migration. This included genes encoding for integrins, laminin components and ILK. This upregulation was described as IGF-independent, as the cell line used does

not possess IGF-1 receptors, although the pathways leading to the upregulation of these genes was not elucidated.(231)

1.10.5 IGFBP-2 in vascular pathophysiology, repair and angiogenesis

As described previously, despite a multitude of research into cellular effects of IGFBP-2 in a variety of cell types, there has been little research into the actions of IGFBP-2 in vascular physiology, and this is also true for vascular pathophysiology. Despite this, identified actions in other cell types, such as integrin binding and ECM interactions, would also be expected to be play a significant role in vascular pathophysiology, although as IGFBP-2 has been shown to exhibit differing actions depending upon cell type and cellular environment, this cannot be assumed. A small amount of research has identified a possible role for IGFBP-2 in neointima formation, as well as actions of IGFBP-2 in vascular repair processes. A much larger body of work has been undertaken examining the role of IGFBP-2 in angiogenesis, predominantly in the context of malignancy, and the following discussion will expand further on potentially relevant angiogenic actions of IGFBP-2 that have been discovered.

1.10.5.1 IGFBP-2 in atherosclerosis and neointimal development

As previously described, expression of IGFBP-2 has been identified in normal human VSMC but at minimal levels, and no significant actions of IGFBP-2 have been identified, differing from research examining porcine and rat aortic VSMC. However, gene expression of IGFBP-2 has been demonstrated to be upregulated in VSMC derived from coronary artery atherosclerotic plaque and stent restenosis, when compared to normal human VSMC.(232) No specific role for IGFBP-2 was described in this study, but it expanded upon previous work by the same group which had established plaque derived VSMC expressed and secreted increased levels of IGFBP-2, alongside other IGFBPs, and that this increased IGFBP secretion contributed to increased VSMC apoptosis, possibly related to inhibition of IGF-I mediated cell survival.(233) Furthermore, low

density lipoproteins, key components and mediators of atherosclerotic plaque development, increased IGFBP-2 expression in rat derived VSMCs, further suggesting a possible role for IGFBP-2 in atherosclerosis and neointima development,(234) adding to the findings of Shen *et al* that IGFBP-2 increased porcine VSMC proliferation *in vitro*.(223) However, another study looking at carotid atherosclerotic plaque did not find an increase in IGFBP-2 gene expression,(198) suggesting possible differential expression depending on environment.

Marfan syndrome is an inherited connective tissue disease characterised by mutations in the FBN1 gene, which encodes fibrillin, an extracellular matrix protein. It is characterised by ECM remodelling, increased aortic stiffness and subsequent aortic wall weakness, preceding the major complication of aortic dissection. In a murine model of Marfan Syndrome, marked upregulation of IGFBP2 mRNA transcription was identified, with a proposed key role in ERK1/2 signalling, a known factor in Marfan syndrome disease progression.(235)

1.10.5.2 IGFBP-2 in vascular repair

There has been very little research examining the potential for IGFBP-2 to enhance vascular repair, despite its established positive actions on cell proliferation and migration. In addition to the effects of increased cell proliferation previously described in HUVECs, Feng *et al* demonstrated that overexpression of IGFBP-2 in HUVECs promotes adhesion of EPCs to a HUVEC monolayer *in vitro*. The same group also found that overexpression of IGFBP-2 by HUVECs promotes incorporation of EPCs into HUVEC tubules *in vitro*. This was demonstrated to occur via integrin binding at the RGD domain of IGFBP-2, and although these were *in vitro* findings they do raise the possibility that IGFBP-2 could play a role in vascular repair and neovascularisation through enhanced action of EPCs.(236)

IGFBP-2 has also been seen to play an important role in the support of haematopoietic stem cell (HSC) activity, supporting the survival and cell cycling of HSCs in the bone marrow,(237) and therefore potentially supporting EPC signalling in vascular repair.

In contrast to these findings, as yet unpublished work by our group has demonstrated reduced re-endothelialization after femoral artery wire injury in a murine model of global IGFBP-2 overexpression, suggesting that overexpression of IGFBP-2 may also inhibit vascular repair, although a mechanism for this possible inhibitory function has not yet been identified.

1.10.5.3 IGFBP-2 in angiogenesis

IGFBP-2 has established proangiogenic effects across a range of cell types, and some of these actions have been described in previous sections of this thesis. A proangiogenic role for IGFBP-2 has been seen in breast cancers, with IGFBP-2 shown to positively modulate IGF-I activation of IGF-1R on endothelial cells, promoting endothelial cell recruitment in metastatic angiogenesis. These effects were negated following suppression of IGFBP-2 via the micro-RNA miR-126, a known suppressor of breast cancer metastasis.(238) Interestingly, IGFBP-2 also downregulates IGF-mediated proliferation of cells in the context of breast carcinoma through integrin receptor interactions, suggesting that observed effects may be determined by environmental milieu.(239)

The importance of IGFBP-2 in vascular development has also been mooted, as IGFBP-2 knockdown led to impaired sprouting angiogenesis in zebrafish embryos,(240) an effect proposed to be through paracrine actions, although a mechanism was not defined. Unpublished work from our group has also found that increased neonatal retinal angiogenesis and enhanced vascular perfusion after hind limb ischaemia is evident in a

murine model of IGFBP-2 overexpression specific to the vascular endothelium. Increased ERK1/2 signalling via integrin interaction with the IGFBP-2 RGD domain has also been demonstrated. This has been shown to be independent of VEGF expression, indicating an ERK1/2 mediated, VEGF independent mechanism for the increased angiogenesis seen.

Our group did not find that IGFBP-2 localised to the nucleus of endothelial cells. However, others have shown IGFBP-2 will localise to the nuclei of neuroblastoma cells, subsequently promoting angiogenesis through upregulation of VEGF mRNA transcription as well as upregulating activation of other protumorigenic genes.(241) This upregulation was only seen in the presence of intracellular IGFBP-2, with no role observed for IGF-I or its receptor. The same group later showed that nuclear translocation was mediated through a nuclear localisation signal sequence within the IGFBP-2 link domain, and that an IGFBP-2 mutant that could not enter the nucleus was unable to upregulate VEGF expression in the same fashion.(205) Nuclear translocation of IGFBP-2 was also seen in breast and prostate cancer cells in addition to neuroblastoma, indicating the potential for a similar role across several cancer types.(205)

IGFBP-2 expression is positively regulated in some cell types by hypoxic conditions and HIF-1α,(242)(243) a growth factor strongly associated with a metastatic phenotype.(244) This association would be consistent with the role of IGFBP-2 in tumour angiogenesis and Das *et al* described how HIF-1α induction in response to Mda-9/syntenin signalling upregulated IGFBP-2 transcription, subsequently augmenting angiogenesis in chorioallantoic membrane neovascularisation assays and increased angiogenic tube formation in co-cultured HUVECs.(217) IGFBP-2 expression is significantly upregulated in neurons following hypoxic brain injury, and appears to play a role in facilitating IGF-I transport to the areas of ischaemia.(245) However, other studies have demonstrated no

link between IGFBP-2 and HIF-1α, suggesting this interaction may be cell type specific.(246)

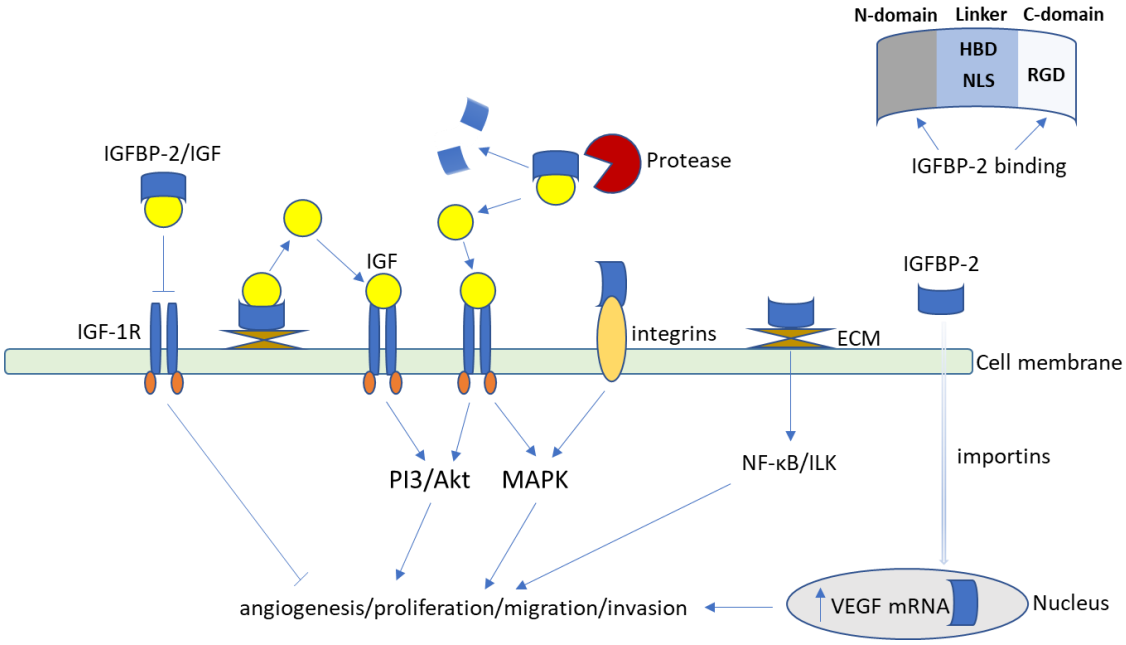


Figure 1-6. Proposed IGFBP-2 signalling mechanisms. IGFBP-2 can act through numerous mechanisms, depending on cell type and environmental conditions. These signalling pathways can be dependent upon IGF-I interaction with the IGF-1R; or IGFBP-2 can act independently of IGF-I, through binding of its functional motifs to cell membrane receptors, and potentially through nuclear translocation and direct action of IGFBP-2 on VEGF mRNA transcription.

1.11 Summary and Objectives

Blood vessels are predominantly comprised of endothelial cells, vascular smooth muscle cells and the extracellular matrix. A number of vascular insults – including ischaemia, physical damage and repeated damage due to cardiovascular risk factors – lead to vascular remodelling via functional and phenotypic changes in endothelium and vascular smooth muscle cells, and increased ECM deposition.

IGFBP-2 has been demonstrated to have a wide variety of actions in a range of cell types, including changes in cellular proliferation, adhesion, and migration. Through modulation of the activity of IGFs and direct IGF-independent actions, IGFBP-2 is implicated in a range of physiological and pathological processes including reproduction,

bone metabolism and cancer. Work by our group and others has shown that IGFBP-2 has important effects in whole body metabolism, with a murine model of global hIGFBP-2 overexpression demonstrating improved insulin sensitivity and protection against obesity. More recently, circulating IGFBP-2 has been identified using proteomic analysis and polygenic risk scoring as a protective mediator of cardiometabolic disease, as well as being identified as a potential druggable target.(247) In contrast to these well described roles, potential effects of IGFBP-2 in the vasculature have received little attention.

Evidence is emerging that IGFBP-2 is a driver of angiogenesis, and expression of IGFBP-2 as well as other IGF binding proteins has been described in constituent cells of the vascular wall. Increased expression of IGFBP-2 has also been demonstrated in tissue after ischaemic injury and in atherosclerotic plaque, suggesting a possible contributory role both in response to hypoxic injury and in the progression of atherosclerosis.(232)(245)

It is recognised that IGFBP-2 contains structural moieties which can interact with cell surface integrins and extracellular matrix constituents: for example an RGD-containing domain, the counterpart of which in another member of the binding protein family – IGFBP-1 – has been demonstrated to influence vascular function and blood pressure regulation.(195) Interaction of IGFBP-2 with cell-surface receptors on vascular smooth muscle cells is known to modulate cellular signalling pathways, yet effects of IGFBP-2 on smooth muscle cell function are poorly understood.

Collectively, these observations lead to our hypothesis to be addressed in this thesis, that IGFBP-2 is an important player in vascular biology as well as in metabolism – providing a potential link between cardiovascular and metabolic disease. Additionally,

the stimulatory effects of IGFBP-2 on angiogenesis, ischaemia recovery and endothelial cell adhesion also suggest a possible role for IGFBP-2 in the context of augmented cell-based therapies targeting endothelial repair and vascular regeneration, although molecular mechanisms for this remain unclear. This thesis describes a series of complimentary experiments in constituent cells of the vessel wall and in gene modified mice to help define the role of IGFBP-2 in vascular biology.

Fundamental hypothesis:

The effects of IGFBP-2 on cells of the vessel wall play an important role in vascular pathophysiology by modulating fundamental aspects of vascular biology, including vasomotion, angiogenesis and neointimal response to injury.

Objectives:

1. Determine the influence of IGFBP-2 on vasomotor function and blood pressure regulation.

This will be ascertained with experiments that examine vasomotor function *ex vivo*, using aorta harvested from mice with overexpression of human IGFBP-2 in either all tissues or in the vascular endothelium. Organ chamber apparatus will be used to examine the vasodilatory and vasoconstrictive responses of these aortas to stimuli. Further *in vivo* experiments will be performed to examine blood pressure and aortic distensibility in the same mice.

2. Investigate the effect of IGFBP-2 on vascular smooth muscle cell phenotype and the neointima response to injury

In vivo models of vascular injury will be used in mice with conditional overexpression of hIGFBP-2 to examine the effect of IGFBP-2 overexpression on neointimal formation. These will complement *in vitro* experiments examining the effect of external stimulation with IGFBP-2, as well as transduced overexpression of IGFBP-2, on the proliferative actions of VSMC. Western blotting and real time polymerase chain reaction will be used to determine if IGFBP-2 can alter common proliferative pathway signalling, and if there is increased expression of IGFBP-2 following vascular injury.

3. Examine the influence of IGFBP-2 overexpression on angiogenesis in endothelial cells and endothelial colony forming cells.

*S*prouting angiogenesis will be studied *in vitro* in HUVECs and ECFCs. These will be transduced to constitutively overexpress hIGFBP-2 and the effects on proangiogenic signalling pathways will be elucidated by Western blotting. Sprouting angiogenesis experiments will be replicated in pulmonary endothelial cells derived from mice with conditional overexpression of hIGFBP-2 in the vascular endothelium alone.

Chapter 2 General Methods

2.1 Animal husbandry

All murine work was carried out following favourable opinion of the University of Leeds Animal Welfare and Ethical Review Committee, and in accordance with The Animals (Scientific Procedures) Act 1986 (amended in 2013 to incorporate EU Directive 2010/63)**,** under the project licence P144DD0D6, and personal licence I3662974D. Mice were housed in The University of Leeds animal facility and were kept in standard conditions, including a 12 hour sleep/wake cycle, and free access to standard chow diet and water. Ear-notching was performed on all mice at weaning and the notched tissue was used for genotyping by a commercial vendor (TransnetYX®) using real-time polymerase chain reaction. Mice were weighed each week using a standard laboratory balance. Mice were culled by a suitable method as described in Schedule 1 of the Animals (Scientific Procedures) Act 1986 (Amended 2013) at differing ages depending on experimental requirements, as detailed in individual chapters. Only male mice were used for experiments; female mice were not used due to the smaller diameter of the female murine vasculature limiting experimental reliability, and the potential varying effects of oestrogen on endothelial cell function throughout the oestrous cycle (248)(249), and so were culled at weaning by a suitable method as described in Schedule 1 of the Animals (Scientific Procedures) Act 1986 (Amended 2013) unless required for breeding.

2.2 Histological methods

2.2.1 Carotid cuff placement

All murine surgery was kindly performed by Dr Nadira Yuldasheva. Mice were induced with tamoxifen at ten weeks in the same manner as described for the aortic relaxation

assays. At 14 to 16 weeks, they were shaved and hair removal cream was used to clear fur from the femoral and carotid areas, the day before operating.

All procedures were performed under isoflurane anaesthetic using a Vet-Tech delivery system, with the mouse placed in a supine position. A Zeiss microscope with 7.2% magnification was used throughout the procedure.

Carotid cuff placement was performed first.(250) A 1cm midline incision was performed over the suprasternal cricoid process. Blunt dissection of soft tissue was performed with tweezers and the left sternocleidomastoid muscle was isolated to identify the carotid triangle. The left common carotid (LCC) was then separated from the vagus nerve and the bifurcation of the internal and external carotid was identified. A 2mm silicone cuff (Dow Corning) was placed around the LCC inferior to the bifurcation, and the cuff was secured proximally and distally using non-dissolvable 7.0 silk sutures. Cuff placement restricted arterial blood flow, before complete thrombus occlusion after approximately 48 hours.

A sham procedure was then performed with exposure of the right common carotid but no intervention. The midline incision was then closed with a 6.0 coated Vicryl (Ethicon) continuous mattress suture.

2.2.2 Femoral wire injury

Femoral injury was performed immediately following carotid cuff placement, under the same anaesthesia.(251)

A 2-3 centimetre incision was made over the left inguinal region, followed by blunt dissection of the surrounding tissue to isolate the common femoral artery (CFA). The CFA was then separated from the femoral nerve and vein, and an 8.0 Vicryl suture was placed loosely under the CFA to temporarily aid haemostasis during wire introduction. The superficial femoral artery (SFA) was identified and two further 8.0 Vicryl sutures were placed proximally and distally to the site of arteriotomy to prevent bleeding post wire insertion, with the distal suture tightened prior to wire insertion, and the proximal suture tightened after wire removal to prevent blood loss.

The SFA bifurcation was identified and tension applied to the CFA haemostatic suture. An arteriotomy was performed between the proximal and distal SFA sutures using iris scissors, and an angioplasty guide wire (0.25mm HI-Torque Cross-IT guide wire, Abbott) was inserted into the SFA and moved proximally towards the CFA, up to the iliac bifurcation. The wire was moved back and forth several times to ensure de-endothelialization was achieved. The wire was then removed, CFA haemostatic suture was released, the proximal suture tightened, and sterile saline was used to clean the procedure site, ensuring haemostasis had been achieved. A 6.0 coated Ethicon Vicryl continuous mattress suture was again used to close the skin incision.

A sham procedure was then performed on the right with exposure of the right CFA only, and the incision was closed in the same manner. Buprenorphine 0.1mg/kg and 45µl saline was administered to the mouse to aid recovery.

2.3 *In vitro* cell culture

2.3.1 ECFC culture

Healthy human subjects were identified and recruited locally. Subjects were male, aged between 18-40 years, free of chronic illness, non-smokers and not taking any regular medication. All participants provided written informed consent, according to the declaration of Helsinki and ethical approval was provided by the Harrogate and Leeds (Central) NHS research ethics committees (06/Q1107/32 and 11/YH/0030).

Parameter	**Median**	**Interquartile Range**
Age (years)	31	27.75 – 34
Body Mass Index	22.75	20.98 – 25.15
Blood Pressure (mmHg)	120/75	116/71 – 127/82
Fasting Glucose (mmol/L)	5	3.4 – 5.2
Fasting Insulin (mmol/L)	4.03	2.58 – 4.38
HbA1c (mmol/mol)	34	31 – 35
Total Cholesterol:HDL ratio	3.05	1.9 – 3.7
Creatinine (µmol/L)	79	62 – 92.5

Table 3. Key characteristics of ECFC donors.

Approximately 35 millilitres (mL) of human peripheral blood was collected in Ethylenediaminetetraacetic acid (EDTA) tubes (Greiner Bio-One 455036). Density gradient separation was performed with Ficoll-Paque PLUS (GE Healthcare 17144003). This separated red blood cells from plasma, and a 'buffy' layer of PBMCs formed on top of the Ficoll layer. This buffy layer and remaining plasma was aspirated into clean 50 mL centrifuge tubes, mixed with Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich D8537) and centrifuged to create a cell pellet. The supernatant was removed, cells were resuspended in PBS and centrifuged again to clean all Ficoll from the cells. After

centrifugation cells were resuspended in Endothelial Cell Growth Medium 2 (EGM-2) basal media supplemented with EGM-2 SingleQuots endothelial cell growth medium (Lonza CC-3162) and 20% HyClone foetal bovine serum (FBS) (GE Healthcare 10117272). Cells were separated into 3 wells of a 0.1% gelatin coated 6 well plate. After 48 hours, wells were washed with PBS and new media added for 5 consecutive days. After this, half media changes were performed on alternate days.

After around 14 days of culture, colonies of cells with a mature endothelial cell phenotype began to become evident. Once these colonies reached a total of three 4x microscope fields in size, cells were detached with 0.05% trypsin/EDTA solution (Gibco 25300062) and moved to a single 0.1% gelatin coated well. Once the cells were confluent within the well the process was repeated and cells were transferred first into a T25 flask, and then a T75 flask, both of which were also coated with 0.1% gelatin. Once cells were confluent within a T75 flask they were detached with 0.05% trypsin/EDTA, centrifuged, resuspended in cryomedium (Cryo-SFM) (Promocell C-29910) and placed in a Nalgene freezing container in -80 degrees Celsius for 24 hours, before transfer to liquid nitrogen storage.

Cells were characterised as endothelial colony forming cells by the appearance of a typical phenotype after established ECFC culture methodology. Our group has previously published findings using ECFCs cultured in the same manner which were shown to express the cell surface markers CD31, CD144, CD146, and CD309 consistent with mature endothelial cells, and lacked leukocyte and monocyte surface markers CD45 and CD14 respectively (139).

2.3.2 PEC culture

Pulmonary endothelial cells were cultured from hIGFBP2$^{iEC\text{-}tg}$ mice using MACS magnetic cell sorting (Miltenyi Biotech) (252). Mice were injected with tamoxifen, as described in Section 3.2.1.2, at 3-4 weeks of age. At 5-6 weeks of age, mice were culled and lungs harvested. The lungs were then minced in 300 units/mL Type II collagenase-Hanks' Balanced Salt Solution (HBSS) (Gibco 24020-091) using scalpel blades. The lung tissue pulp was then transferred into a 15 mL centrifuge tube in 10 mL of collagenase-HBSS solution, and incubated at 37° C on MACSMix rotator (Miltenyi Biotech 130-090-753) for 45 minutes.

After incubation the mixture was transferred to a 50 mL centrifuge tube and filtered through a 70 micrometre (µm) cell sieve (Greiner Bio-one 542070) . The sieve was then rinsed with 10 mL of a 0.5% PBS/Bovine Serum Albumin (BSA) solution (Sigma A8412), and any tissue remnants was discarded. This mixture was then centrifuged at 400 times gravity (xg) for five minutes to pellet the cells. The supernatant was discarded and the pellet washed with 0.5% PBS/BSA, before centrifuging again and discarding the supernatant again. The cell pellet was resuspended in 300 microlitres (µL) of cold 0.5% PBS/BSA and transferred into a 1.5 mL Eppendorf, on ice. Twenty µL of LSEC CD146 microbeads (Miltenyi Biotech 130-092-007) were added and the sample was incubated using the MACSMix rotator at 4° C for 20 minutes. After this incubation, the sample was washed by adding 500 µL of 0.5% PBS/BSA, spun at 500xg for 5 minutes, and the supernatant was discarded. This step was then repeated before the pellet was resuspended in 500 µL 0.5% PBS/BSA.

Magnetic separator columns (Miltenyi Biotech 130-042-201) were attached to a MACS multistand (Miltenyi Biotech 130-042-303). A 30 µM pre-separation filter (Miltenyi Biotech 130-041-407) was placed on top of the column, and a 15 mL centrifuge tube underneath it. The column was prepared by adding 500 µL 0.5% PBS/BSA to the top and allowing

buffer to run through, before the cell suspension was added to the column and allowed to run through.

The endothelial cells were then collected by placing the magnet in a 15 mL centrifuge tube, adding 1 mL of 0.5% PBS/BSA to the column and immediately flushing out the fraction into the tube by firmly applying the column plunger. The sample was centrifuged and the supernatant discarded. The purity of the cells was improved by resuspending the pellet thoroughly in 500 µL 0.5% PBS/BSA and running them through the MiniMACS magnets again. After this they were resuspended in Endothelial Cell Growth Medium MV 2 (ECGM-2) (Promocell C-22022) and plated into a 0.2% gelatin coated T75 flask. Once the cells reached confluency they were trypsinised and used in the Cytodex bead sprouting angiogenesis assays described in Section 6.2.2.

2.3.3 HUVEC culture

Pooled donor human umbilical vein endothelial cells (HUVEC) were purchased from Promocell (C-12203). They were brought to confluency in ECGM-2 in T75 flasks and passaged twice before being aliquoted into Cryo-SFM and stored in liquid nitrogen. When required they were brought up in Medium 199 (M199) (Sigma M4530), supplemented with 20% FBS, 2% 1M HEPES (Sigma H0887), 1% 100x Antibiotic-Antimycotic solution (Sigma A5955), 2% sodium pyruvate (Sigma S8636), 0.5% 1000 units/mL heparin sodium (Wockhardt) and 0.5% endothelial cell growth supplement (Sigma E2759), passaged once and then maintained in M199 with 10% FBS until experimental use.

2.3.4 VSMC culture

Human primary VSMC samples were kindly donated by Dr Karen Porter. The samples were obtained from human saphenous vein and human internal mammary artery culture

(253). Initial harvest was performed by Mr David O'Regan at Leeds General Infirmary, from patients undergoing coronary artery bypass grafting, with appropriate informed patient consent and local ethical committee approval (LREC CA01/040) in accordance with the Declaration of Helsinki. VSMC isolation and culture was performed by Dr Karen Hemmings and Dr Emily Clark, and VSMC were identified by typical phenotype. VSMC were maintained in culture with Dulbecco Modified Eagle Medium (DMEM) (Gibco 21969035), 10% FBS (Biosera), 1% 100x Antibiotic-Antimycotic solution (Sigma A5955), and 1% glutamine (Gibco A2916801).

2.4 Western immunoblot assays

Western blotting was performed on both cultured cells and harvested tissue. After harvesting, tissue was immediately placed in dry ice before transferring to storage at -80 °Celsius (C). To lyse tissue, 500 µL of cell extraction lysis buffer (CEB) (Invitrogen FNN0011), supplemented with 1 µL protease inhibitor cocktail (Sigma P8340) was added to samples in U-bottomed Eppendorf tubes. Two stainless steel beads (Qiagen 69990) were added to the tubes, and samples were lysed at 25Hz for two minutes (TissueLyser II Qiagen). Tubes were then centrifuged and the lysate aspirated and stored at -80 °C.

For samples derived from cell culture, cells were washed with PBS and the same lysis buffer was added. A cell scraper was used to remove cells from the well, transferred into an Eppendorf, and left on ice for 30 minutes, before centrifuging at 17G and 4°C for 10 minutes. The supernatant was then removed and frozen at -20°C.

Bicinchoninic acid (BCA) standards (Pierce BCA Protein Assay Kit) (Thermo Scientific 23227) were used to create a curve of known protein concentrations. A BCA assay was then performed to establish accurate protein concentrations of samples.

NuPage™ loading buffer and reducing agent (Invitrogen NP0007 & NP0009) were added to 10-20 µg of sample, at 1:4 dilution and 1:10 dilution respectively. Samples were heated at 95°C for five minutes to fully denature the protein. Samples were then loaded on 4-12% Bis-Tris Gels (Invitrogen) and protein electrophoresis was then performed in 2-(N-morpholino)ethanesulfonic acid buffer (Invitrogen NP0002) at 180 volts for 60 minutes to separate the proteins by molecular weight, with a Precision Plus Protein Western C Standards ladder (Bio-Rad 161-0376) added to identify known molecular weight levels.

Membrane transfer was then performed using a Trans-Blot® Turbo™ Transfer system BioRad). Nitrocellulose membranes (BioRad 1704270) were soaked in Trans-Blot® transfer buffer (BioRad 1704270).

The membrane was then cut to separate target proteins by molecular weight. The membrane was then blocked with either a 2% milk solution in 0.1% tris-buffered saline-tween20 (TBS-T) (10mM Tris.HCl, 15mM NaCl, and 0.1% Tween® 20, adjusted to pH 7.5), or if phospho-antibodies were used then a blocking buffer of 3% BSA in 0.1% TBS-T was used.

The membrane was soaked in blocking buffer for an hour, then washed with TBS-T, before adding primary antibodies for each protein of interest (see specific methods within relevant chapters), and incubated overnight at 4°C.

Membranes were washed the next day in TBS-T, before secondary antibodies were added for an hour (see specific methods within relevant chapters), and then again washed in TBS-T.

Finally, Immobilon Western Chemiluminescent HRP Substrate (Millipore WBKLS0500) was added to the membranes, and imaged in a Syngene GBOX using Genesys software. Images were saved as both Tiff and Syngene files, with analysis performed using ImageJ.

2.5 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was used in murine arterial samples to quantify IGFBP-2 expression and markers of contractile smooth muscle.

2.5.1 RNA isolation

RNA isolation from tissue samples was performed using the Monarch Total RNA Miniprep Kit (New England BioLabs T2010S), which contained all described reagents, buffers and columns. DNA/RNA protection reagent, Protein K reaction buffer and Protein K were added in quantities advised in the kit protocol. Samples were then mechanically lysed at 25Hz for two minutes (TissueLyser II Qiagen), and incubated at room temperature for thirty minutes. Samples were then transferred to gDNA removal columns, spun for thirty seconds and the flow-through saved. An equal quantity of ethanol was added to the flow-through samples and mixed thoroughly. The samples were then added to RNA purification columns and spun for thirty seconds again. The flow through was this time discarded, as the RNA is contained within the purification column. RNA priming buffer was added to the columns before spinning for thirty seconds and discarding the flow through. RNA wash buffer was then added twice and spun through. Finally 50 µl of nuclease free water was added to elute the RNA. A NanoDrop Spectrophotometer (Thermo Scientific) was used to quantify the RNA concentration, and samples were stored at -80°C.

2.5.2 Reverse transcription

Reverse transcription of RNA to cDNA was performed using the LunaScript® RT SuperMix Kit (E3010). LunaScript RT SuperMix (5x) was added to RNA sample and nuclease-free water in MicroAmp tubes (Applied Biosystems N8011535). Quantities varied depending on the experiment performed due to varying quantities of RNA extracted, and will be detailed in specific chapters. These reactions were then incubated in a 96 well thermocycler (Veriti Applied Biosystems) in the following stages: primer annealing (25°C for 2 minutes); cDNA synthesis (55°C for 10 minutes); heat inactivation (95°C for 1 minute).

2.5.3 Quantitative polymerase chain reaction

The following primers (BioRad) were used in qPCR experiments: IGFBP2 Hsa (qHsaCID0012428); IGFBP2 Mmu (qMmuCID0006519); MYH11 Mmu (qMmuCID0019272); ACTA2 Mmu (qMmuCID0006375); ACTB Mmu (qMmuCED0027505) and GAPDH Mmu (qMmuCED0027497). GAPDH was used as the house-keeper gene, ACTB was also trialled but found to not be reliable. 1 µL of each primer was added to 9 µL of iTAQ Universal SYBR® green Supermix (BioRad 1725120), nuclease free water and cDNA, to give a final cDNA concentration of 0.5 ng/µL or 1 ng/µL depending on quantity of RNA initially obtained prior to reverse transcription.

This mastermix was then added to 96 well plates, in duplicate for each primer, for a final concentration of 10 ng/well. Real-time qPCR was then performed in a Lightcycler 480 II (Roche) with the following cycle conditions: pre incubation (95°C for thirty seconds); amplification (45 cycles of 95°C for 15 seconds and 60°C for one minute); melting curve (95°C for five seconds, 65°C for one minute, 65-97°C for 10 minutes at a 0.11 ramp rate).

A cycle threshold of below 30 cycles was used to identify true results, and linear amplification was used to accurately assess for false readings.

2.6 Experimental design

All animal experiments were designed using the 3Rs principles of replacement, refinement and reduction,(254) and the design of individual experiments was based on previous lab experience. Later experiments were limited by constraints created by the Covid-19 pandemic, and sample sizes were not always optimal. This is discussed in more detail within each individual chapter and in my conclusions.

Formal randomisation was not required, as comparisons were being made between genotypes. Therefore, wherever possible, harvesting, experiments and analysis were performed blinded to genotype. However, when specific mouse genotypes were required for even sample sizes, blinding was not always possible, as all analysis had to be performed by myself. This represents a limitation of my findings and is discussed in further detail later.

All murine experiments were performed using different biological replicates for each N number. The PEC cytodex bead sprouting assay, VSMC cell counting assays and ECFC wound closure assays also used different biological replicates. Due to significant time constraints imposed by the COVID-19 pandemic, it was required to use technical replicates for the transduced ECFC and HUVEC experiments. Each sample was separately cultured, transduced and individual assays were performed, and so these were treated as individual technical replicates.

2.7 Data analysis and statistics

Imaging was performed using ImageJ software unless otherwise stated. Data were collated using Microsoft Excel. Data were inputted into GraphPad Prism 9 software to generate graphics and for all statistical analysis. Statistical tests used were dependent upon the most appropriate test for each condition observed, and will be outlined in each methods section for individual experimental chapters.

P<0.05 was considered statistically significant, and denoted by * on graphs. P<0.01 is denoted as **, p<0.001 as *** and p<0.0001 as ****. Data are presented as mean +/- standard error of the mean (SEM).

Chapter 3 The effects of IGFBP-2 overexpression on general phenotype of transgenic murine models

3.1 Background

Genetically engineered mouse models have been used for decades to gain further understanding of disease pathophysiology, and the physiological actions of individual proteins, through genetic manipulation to either overexpress or knockout a gene of interest.(255) Mice have traditionally been used as they have a high degree of genetic conservation with humans, and breed reliably with high fecundity and a short gestational period.(255) Our group has previously used a variety of genetically manipulated mouse models to investigate multiple aspects of the insulin and IGF axes, and the actions of the IGFBPs.(195)(213)(256)

Three separate transgenic mouse models of hIGFBP-2 overexpression were employed in experiments in this thesis, and generation of these models will be described in Section 3.2.1.

The hIGFBP2^{global} transgenic mouse overexpresses hIGFBP-2 globally under its native promoter, and has been described previously in publications by our group.(213) It was first established several years ago however, and so it was felt prudent to re-establish the degree of hIGFBP-2 expression observed, given the established risk of genetic drift within long established inbred transgenic lines.(257)

The hIGFBP2$^{iEC\text{-}tg}$ transgenic mouse model exhibits conditional hIGFBP-2 overexpression driven by the VE-cadherin promoter, which drives expression

predominantly in vascular endothelium.(258) This mouse model has been examined by our group in work that to date has only been published in abstract form.(259) A previous thesis from our group using this mouse model confirmed increased hIGFBP-2 expression in vascular endothelium compared to controls, with no significant difference in bodyweight between hIGFBP-2 overexpressing mice and the control group.(260)

The hIGFBP2$^{iGLOBAL\text{-}tg}$ line is novel, exhibiting conditional global overexpression of hIGFBP-2 in all tissues and characterised for the first time in this thesis. We therefore set out to confirm secretion of hIGFBP-2 in plasma and hIGFBP-2 expression in selected tissues. Murine bodyweight was also assessed, given the established effects IGFBP-2 has in lowering bodyweight.

3.1.1 Experimental overview

The secretion of hIGFBP-2 by all three transgenic mouse models was assessed by serum analysis using an enzyme-linked immunosorbent assay (ELISA). Human IGFBP-2 expression as assessed by immunoblotting was established in both highly vascularised and less vascularised tissue from the hIGFBP2$^{iGLOBAL\text{-}tg}$ line. Mouse bodyweights from the hIGFBP2$^{iGLOBAL\text{-}tg}$ line were taken at weekly intervals using standard laboratory scales before and after injection with tamoxifen.

3.1.2 Aims

To provide characterisation of the hIGFBP2$^{iGLOBAL\text{-}tg}$ line and establish that human IGFBP-2 is secreted and present in the circulation of all transgenic murine models utilised in this thesis.

3.1.3 Objectives

1. Establish hIGFBP-2 secretion in all transgenic murine models
2. Establish hIGFBP-2 expression in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line
3. Establish the effects of global hIGFBP-2 expression on murine growth

3.2 Methods

3.2.1 Generation of transgenic murine models

3.2.1.1 Global hIGFBP-2 overexpressing mice

Global human IGFBP-2 overexpressing mice were originally generated through microinjection of a human IGFBP2 cosmid clone into the pronucleus of single cell FVB/N mouse embryos, which were in turn implanted into oviducts of CD-1 females. Transgenic offspring were bred with wild type FVB/N mice to establish transgenic lines, and colonies were subsequently established through the crossing of male mice heterozygous for hIGFBP2 with wild type female FVB/N mice.(213) Subsequent generations were back-crossed to C57BL/6 mice, and donated to The Jackson Laboratory (JAX strain #008222). We established new breeders to use in this thesis from frozen embryos held by The Jackson Laboratory. Wild type litter mates were used as controls. For the purposes of this thesis these mice will be labelled as $hIGFBP2^{global}$.

3.2.1.2 Tamoxifen inducible vascular endothelial hIGFBP-2 overexpressing mice

Mice with conditional expression of human IGFBP-2 in the vascular endothelium alone, utilising cre-lox recombination, were commissioned from GenoWay, Lyon, France. This involved the creation of transgenic mice with a C57BL/6 background, by knock-in of cDNA encoding hIGFBP2 into the ROSA26 locus. The hIGFBP2 coding sequence was flanked by a floxed STOP codon preventing transcription (Figure 3-1). These mice were termed $ihIGFBP_{tg}$.

These mice were then bred with Cdh5-CreERT2 mice (MGI:3848982),(261) which were a kind gift from Professor Helen Arthur at Newcastle University. These mice are intended to express Cre recombinase selectively in the endothelium following administration of tamoxifen and were created by generation of an artificial chromosome carrying the gene for VE-Cadherin (Cdh5), with insertion of Cre/ERT2 cDNA at the start codon for Cdh5.

This artificial chromosome is then integrated into the genome of mice from a C57BL/6 background.(262) Breeding with the Cdh5-CreERT mouse created double transgenic Cdh5-CreERT.ihIGFBP2$_{tg}$ mice. Litter mates that did not express the Cdh5-CreERT gene were used as control subjects (Cre -ve).

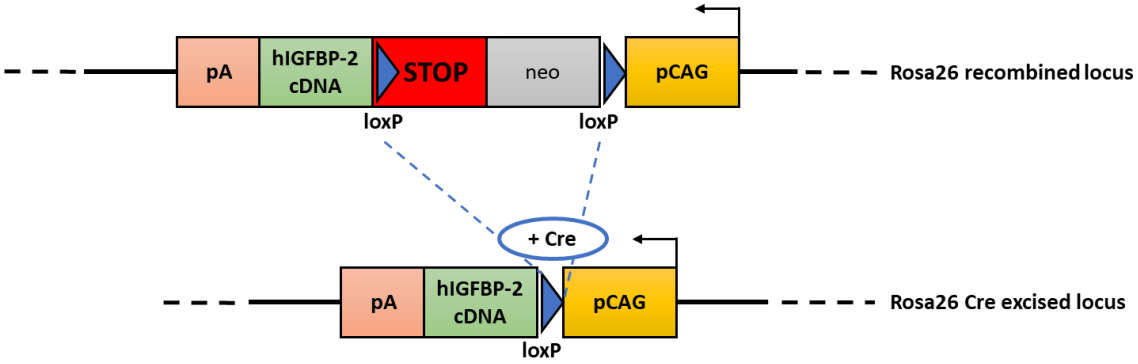


Figure 3-1. Cre/loxP system for hIGFBP2 gene expression. The hIGFBP-2 coding sequence was inserted at the Rosa26 locus, followed by flanking loxP sites around a STOP codon. This prevented transcription of hIGFBP2 until the injection of tamoxifen led to activation of Cre recombinase, causing cleavage of the STOP codon and subsequent upregulation of hIGFBP2 transcription.

All mice were then injected with tamoxifen at variable ages depending on the experiments planned. Tamoxifen injection activated Cre recombinase expression, cleaving the stop codon and allowing the transcription of hIGFBP-2 in the vascular endothelium alone. Injections were via an intraperitoneal route with 1mg/day of tamoxifen (Sigma T5648 dissolved in Corn Oil Sigma C8267) for five consecutive days. For the purposes of this thesis, these mice will be labelled as hIGFBP2$^{iEC\text{-}tg}$. Control mice were also injected with tamoxifen, but as they did not contain Cre recombinase this did not lead to IGFBP-2 overexpression.

3.2.1.3 Tamoxifen inducible global hIGFBP-2 overexpressing mice

The same ihIGFBP$_{tg}$ mice were also bred with CAGGCre-ER mice, obtained from The Jackson Laboratory (JAX strain #004682). These mice, with a C57BL/6 background, also

have a tamoxifen inducible cre-recombinase system, in this case driven by a chicken beta actin promoter/enhancer coupled with a cytomegalovirus (CMV) immediate-early enhancer.(263) This cross breeding created a CAGG-CreER.ihIGFBP2$_{tg}$ line which, following tamoxifen injection, allowed for upregulation of hIGFBP-2 in all tissues. Litter mates that did not express the CMV-CreERT2 gene were used as control subjects, and were also injected with tamoxifen. For the purposes of this thesis, these mice will be labelled as hIGFBP2$^{iGLOBAL\text{-}tg}$.

3.2.2 Enzyme-linked immunosorbent assay (ELISA)

Mice were culled by exsanguination using ventricular puncture under terminal anaesthesia. Blood samples were centrifuged at 13G for 5 minutes, separating plasma and red blood cells. Plasma was removed and stored at -80°C.

An IGFBP-2 human ELISA kit (Abcam ab100540) was used. All diluents and solutions were provided with the kit. 50 ng/mL IGFBP-2 stock standard was prepared by adding 400 µL Diluent A to the IGFBP-2 standard provided in the kit. Serial dilutions of the stock standard were prepared at the following concentrations: 6000 pg/mL, 2000 pg/mL, 666.7 pg/mL, 222.2 pg/mL, 74.07 pg/mL, 24.69 pg/mL, 8.23 pg/mL and 0 pg/mL.

100 µL of each standard and sample (diluted between 1:200 and 1:1000 depending on sample genotype) were added in duplicate to the appropriate wells of a 96 well plate supplied with the kit. The wells were covered and the plate was gently shaken overnight at 4°C.

The next day the solution was discarded and the plate was washed 4 times with wash solution. 100 µL of 1X biotinylated IGFBP-2 detection antibody was added to each well,

and the plate was incubated for one hour at room temperature with gentle shaking, before the wash steps were repeated.

100 µL of 1X HRP-Streptavidin solution was added to each well. The plate was incubated for 45 minutes at room temperature, before the wash steps were again repeated. Finally 100 µL of TMB One-Step substrate reagent was added to each well, the plate was incubated for 30 minutes in the dark at room temperature, before 50 µL of Stop Solution was added, and the plate was read at 450 nm as per kit instructions.

Data were collated in Microsoft Excel, analysed using Graphpad Prism and statistical analysis was performed using unpaired Student's t-test.

3.2.3 Immunoblotting

Immunoblotting was performed as described in section 2.3. A goat primary antibody for hIGFBP-2 (Santa Cruz sc-6001) was used, with an anti-goat secondary antibody (Dako P0160). Beta-actin (Cell Signalling Technologies #4967) was used as a house keeping standard when assessing aortas and organs, which differed to immunoblotting of *in vitro* cultured cells for which heat shock protein 90 (HSP90 Santa Cruz SC-13119) was used, as our group has previously found HSP90 to be inconsistent when assessing lysed aortic tissue.

Data were collated in Microsoft Excel, analysed using Graphpad Prism and statistical analysis was performed using unpaired Student's t-test.

3.3 Results

3.3.1 General characteristics

Transgenic mice bred from all three models were morphologically normal, bred normally and did not exhibit unusual behaviours. No mice required culling for morphological abnormalities.

Breed	**Total litters**	**Pups/litter**	**Gender** (Male/Female)	**Genotyping** (hIGFBP2 negative/hIGFBP2 positive)
hIGFBP2^{global}	152	4.2	328/306 52%/48%	169/172 (50%/50%)
hIGFBP2$^{iEC\text{-}tg}$	109	4.3	236/234 50%/50%	140/130 (52%/48%)
hIGFBP2$^{iGLOBAL\text{-}tg}$	92	4.9	216/233 48%/52%	164/172 (49%/51%)

Table 4. Breakdown of br**e**eding characteristics for each mouse model used.

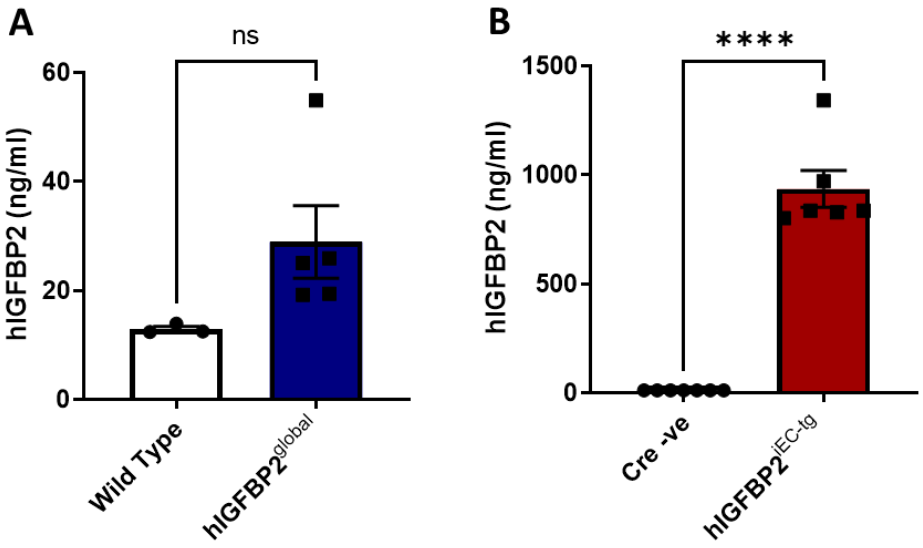
3.3.2 hIGFBP-2 expression and secretion

3.3.2.1 hIGFBP-2 plasma concentration

Murine plasma samples were obtained and ELISAs performed as described in section 3.2.2. A trend to increased plasma hIGFBP-2 was seen in the hIGFBP2^{global} mice that was not significant, although sample size was small (Figure 3-2 A). There was a highly significant difference in plasma concentrations between hIGFBP2$^{iEC\text{-}tg}$ mice that were positive and negative for Cre recombinase (Figure 3-2 B). Similarly, hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice showed markedly elevated plasma hIGFBP-2 levels compared to Cre negative hIGFBP2$^{iGLOBAL\text{-}tg}$ mice (Figure 3-2 C). Of note, low levels of hIGFBP-2 were

detected in both wild type and Cre negative mice, suggesting a degree of cross-reactivity with murine IGFBP-2.

Additionally, it was noted during the course of experiments that Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice had reduced bodyweight compared to their Cre negative counterparts, prior to tamoxifen administration, with divergence seen soon after weaning (Figure 3-5). Plasma hIGFBP-2 was therefore also checked in these mice pre tamoxifen injection and was found to be markedly higher than in Cre negative mice, with levels roughly half that of levels seen post injection (Figure 3-2 C). This suggests that there is a degree of Cre activation before injection, with further activation after tamoxifen injection.



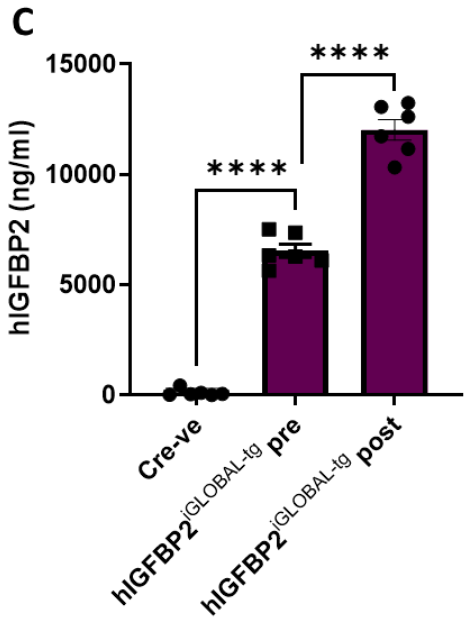


Figure 3-2. Secretion of hIGFBP-2 assessed by ELISA of the plasma derived from hIGFBP2^{global}, hIGFBP2^{iEC-tg} and hIGFBP2$^{iGLOBAL-tg}$. A: the hIGFBP2^{global} strain did not show a significant difference in IGFBP-2 plasma secretion between transgenic and control mice (p=0.12 n=3,5). **B**: hIGFBP2^{iEC-tg} mice showed a marked increase in hIGFBP-2 secretion in Cre positive mice compared to Cre negative mice (p=<0.0001 n=6,6). **C**: hIGFBP2$^{iGLOBAL-tg}$ mice showed a marked increase in hIGFBP-2 secretion in Cre positive mice compared to Cre negative mice, both before and after injection with tamoxifen (p=<0.0001, n=6,6). Note different Y axis scales are used in each panel.

3.3.2.2 hIGFBP-2 expression in hIGFBP2$^{iGLOBAL-tg}$ organs

Tissue was harvested from hIGFBP2$^{iGLOBAL-tg}$ mice at 2 weeks post tamoxifen injection. Heart, kidney, bladder and skeletal muscle were used as representative tissue. Expression of hIGFBP-2 was significantly upregulated in all organs of Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice, with no expression seen in Cre negative mice (Figure 3-3 A-D).

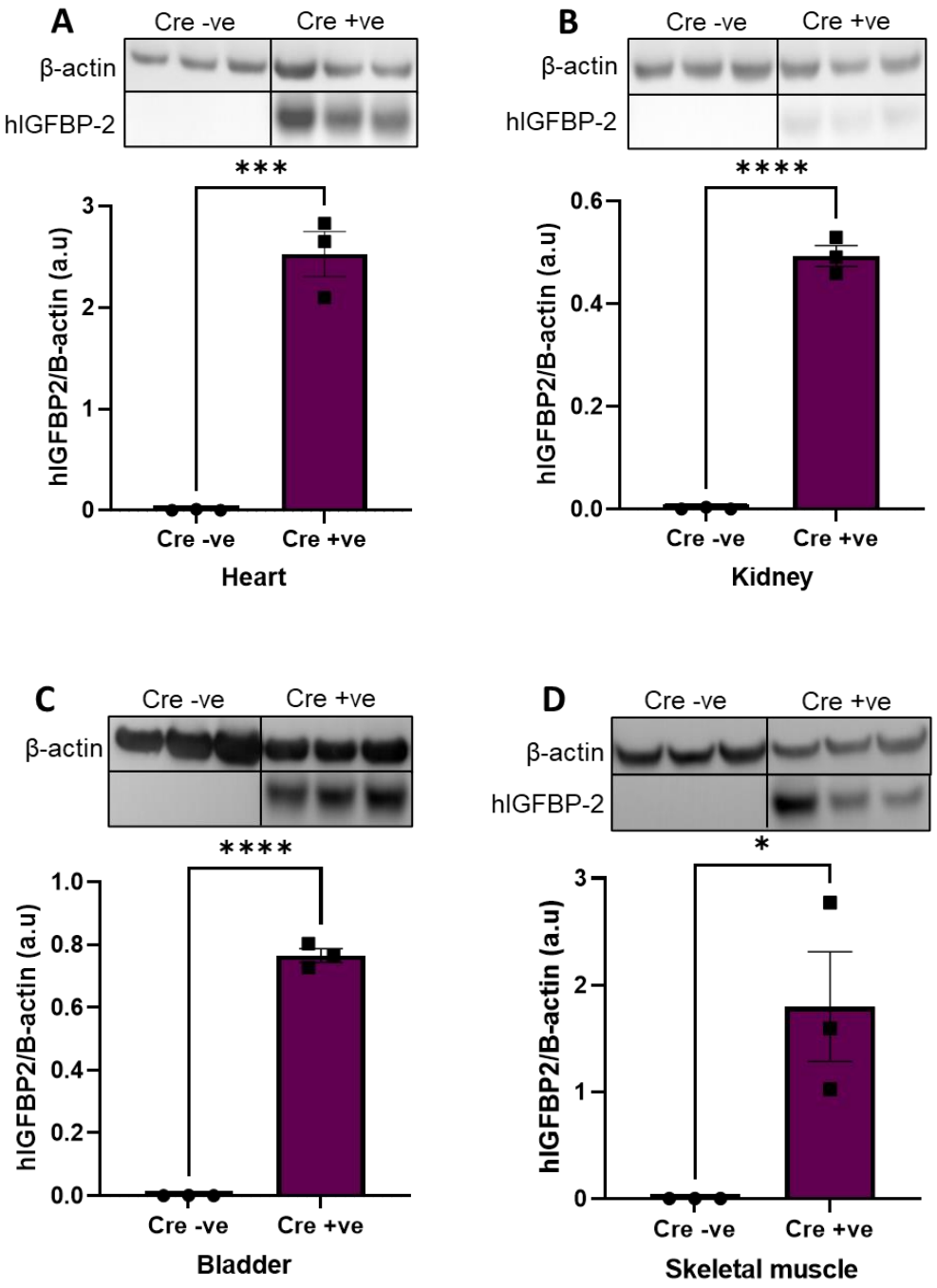


Figure 3-3. Expression of hIGFBP-2 in different organs of the hIGFBP2$^{iGLOBAL-tg}$ mouse as assessed by immunoblotting (Western Blotting). Expression of hIGFBP-2 was significantly upregulated in all organs of Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice, with no expression seen in Cre -ve mice (n=3,3). Beta-actin was used as a loading control.

3.3.3 hIGFBP2$^{iGLOBAL-tg}$ bodyweight change

It was noted during the course of experiments that Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice appeared to have a lower body weight than their Cre negative counterparts. This was

not a feature seen with either the hIGFBP2^{global} mice or the hIGFBP2$^{iEC\text{-}tg}$ strain. We therefore tracked mouse bodyweight at weekly time-points, and saw an early divergence in bodyweight after weaning, with a significant difference in weight at all time points including pre tamoxifen injection. The weight difference became more marked after tamoxifen injection at seven to eight weeks, suggesting the increased hIGFBP-2 exposure may have an additive effect (Figure 3-4).

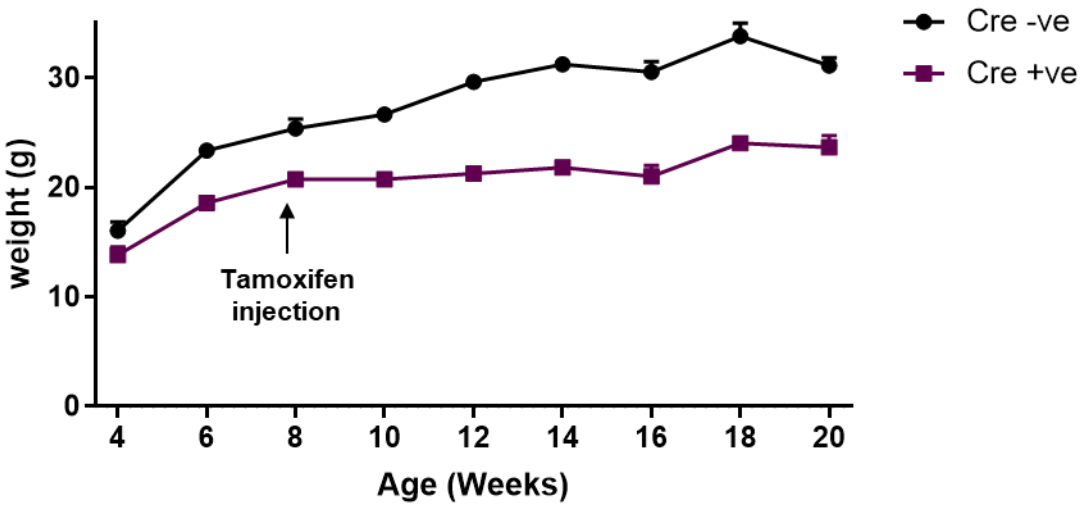


Figure 3-3. hIGFBP2$^{iGLOBAL\text{-}tg}$ mouse bodyweight from weaning up to 20 weeks. A significant difference in weight was seen at all time points including pre tamoxifen injection (p=<0.0001, assessed by mixed model 2-way ANOVA), with a more pronounced divergence after tamoxifen injection at 7-8 weeks.

3.3.4 hIGFBP2$^{iGLOBAL\text{-}tg}$ organ weights

Organs were harvested from mice culled at 6 weeks post tamoxifen injection, and weighed using a standard laboratory balance. Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice demonstrated significantly reduced heart, kidney and epididymal adipose tissue weights, with no difference observed for spleen and liver samples (Figure 3-5).

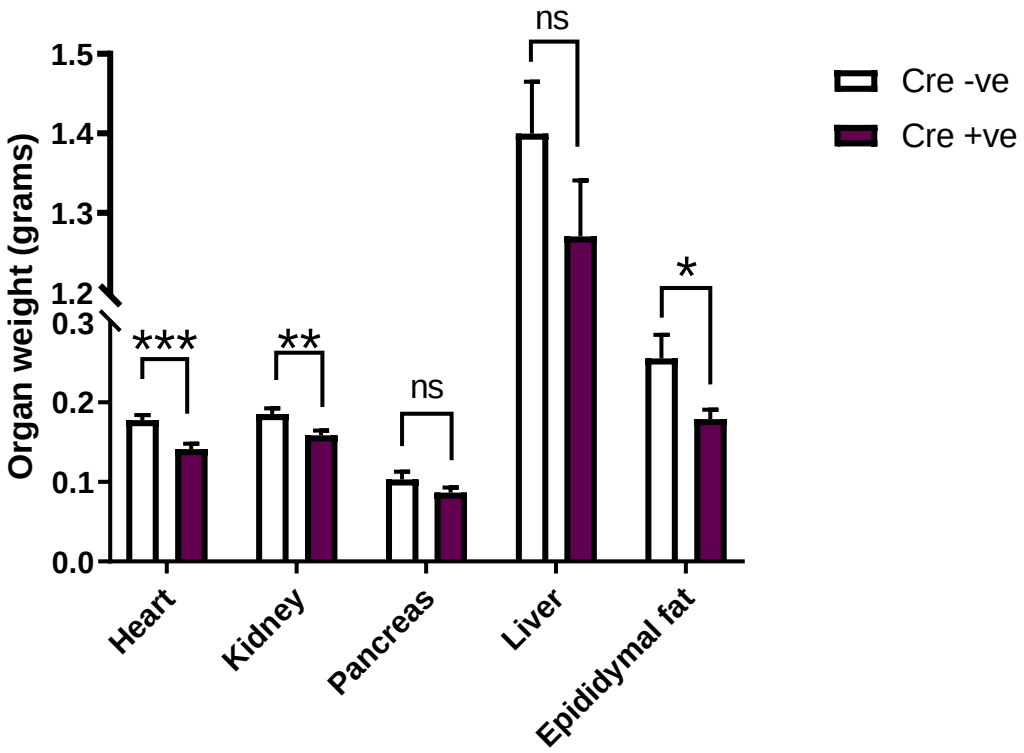


Figure 3-5. Individual organ weights from hIGFBP2$^{iGLOBAL\text{-}tg}$ mice. Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice demonstrated significantly reduced heart (p=0.0007), kidney (p=0.0075) and epididymal adipose tissue (p=0.0141) weights, with no difference observed for spleen and liver samples (n=13,17).

3.4 Discussion

The hIGFBP2^{global} mouse line has been previously demonstrated by our group to exhibit a 2.2 fold increase in total circulating IGFBP-2, which was associated with increased insulin sensitivity and conferred a reduced propensity to obesity when fed a high fat diet, compared to wild type littermates.(213) We had therefore planned *a priori* to use these mice for this thesis and compare findings to the inducible mouse lines. However, when we established a new colony of the hIGFBP2^{global} mouse line, we found that there was no longer a significant upregulation in hIGFBP-2 secretion, although low levels of hIGFBP-2 were detected in the sera of the wild type mice, suggesting a degree of cross-reactivity with murine IGFBP-2 (Figure 3-2 A). These findings are at odds with prior published findings, and may reflect a phenotype change due to transgenic mice being crossed with C57Bl6 mice rather than the FVBN mice used in the original studies.(213) Another potential explanation for the observed loss of IGFBP-2 secretion may be genetic drift, an established sequelae in long established colonies, particularly when retired genetically altered breeders are replaced with offspring serially during long-term colony maintenance.(257)

Conversely, secreted levels of hIGFBP-2 in both the hIGFBP2$^{iEC\text{-}tg}$ and the hIGFBP2$^{iGLOBAL\text{-}tg}$ lines were substantially elevated compared to Cre negative controls, and supra-physiological compared to levels seen in human circulation, which have been reported as approximately 500 ng/mL in normal subjects.(264)(265) Expression of hIGFBP-2 was also markedly upregulated in each organ analysed in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line, confirming global overexpression (Figure 3-2 B-C). Secretion in both conditional models was markedly higher than that seen in the original hIGFBP2^{global} mouse line, potentially related to the enhanced expression expected with VE cadherin and CMV promoters in the hIGFBP2$^{iEC\text{-}tg}$ and the hIGFBP2$^{iGLOBAL\text{-}tg}$ lines respectively, compared to the native promoter in the hIGFBP2^{global} line.

IGFBP-2 is well established to be expressed and secreted by multiple tissues in normal conditions, with liver the primary source of IGFBP-2 expression in adult humans, along with cardiac, pancreatic and reproductive tissue.(266) Acute regulation of IGFBP-2 occurs in response to both insulin and leptin (168) and, as previously described, secreted IGFBP-2 has been demonstrated to exert endocrine, autocrine and paracrine effects both in an IGF-dependent and independent manner, depending upon the conditions examined.(202) Supraphysiological expression and secretion of IGFBP-2, independent of normal physiological regulation, may therefore exert its effects through a multitude of pathways, and these will be explored further in subsequent chapters.

It was noted that Cre positive mice in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line were smaller in bodyweight than their Cre negative counterparts from early after weaning, with a more pronounced change in bodyweight noted after tamoxifen injection (Figure 3-4). We found that there was unintentional hIGFBP-2 expression and secretion in Cre positive mice prior to tamoxifen injection, and this likely contributed to the difference in bodyweight, given the established effects of IGFBP-2 overexpression in protecting against murine obesity and reducing postnatal murine bodyweight.(213)(267) Although the exact cause for these effects are not known, the former has been theorised as a complex process relating to improved insulin sensitivity and reduced adipogenesis,(168) whilst the latter was postulated to relate to a reduction in IGF bioavailability due to increased sequestration by IGFBP-2. Although the reduced organ weight also seen in the Cre positive mice points to a reduction in lean body mass rather than adiposity, this was not ascertained accurately, and could be a focus for future study.

There is an acknowledged risk in transgenetic mice with inducible Cre-recombinase that there may be a degree of Cre recombinase activity prior to tamoxifen injection.(268)(269)

We could have tested for this by crossing the Cre recombinase mouse lines with a floxed mTmG reporter line, in which tissue fluorescence alters following Cre activation.(270) We did consider including this reporter line in our crosses, however both the mTmG and hIGFBP2 lines were knock-ins at the ROSA26 locus, which affected genotyping and so we weren't able to proceed.

Unintentional overexpression of hIGFBP-2 in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line prior to tamoxifen injection may have had effects in the vasculature of these mice from the point of embryogenesis, which was not initially desired. There was however a stepwise change in bodyweight after tamoxifen injection, and a doubling of hIGFBP-2 secretion as assessed by serum ELISA, indicating that there may be an additive effect derived from increasing hIGFBP-2 expression. Furthermore, despite their reduced bodyweight, Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice were healthy and did not exhibit any unusual behaviours. It was therefore felt appropriate to further investigate the effect of hIGFBP-2 overexpression in vascular pathophysiology using this mouse line, when compared to their Cre negative littermates which did not express any hIGFBP-2.

Chapter 4 IGFBP-2 overexpression and its effects on vascular physiology

4.1 Background

As discussed in Chapter 1, there are acknowledged effects of IGFBP-2 on the different components of the vasculature, including promotion of vascular smooth muscle cell proliferation *in vitro*, proangiogenic effects in endothelial cells and links to aberrant extracellular matrix formation in inherited connective tissue diseases such as Marfan Syndrome.(217)(223)(235) There has however been little investigation into the effects of IGFBP-2 on overall vascular function, and it is not yet known if IGFBP-2 overexpression has any effect on vascular function *in vivo*, or in which tissue it primarily exerts its effects.

4.1.1 Vascular physiology

As described, blood vessels, excepting the capillary network, consist of three layers: the tunica adventitia, tunica media, and tunica intima. The vascular smooth muscle cells, adventitial cells and extracellular matrix that compose the outer two layers have a complex interplay between themselves and the endothelial monolayer within the tunica intima. This interplay is designed to provide a quiescent balance to the vasculature, allowing for a functioning vascular network without aberrant vessel formation, and counteracting proinflammatory signals which, if unchecked, contribute to the development of pathophysiology.

IGFBP-2 expression has been demonstrated in each component of the vasculature, including endothelial cells, VSMC and the adventitia.(216)(221) Additionally, multiple interactions of IGFBP-2 with components of the extracellular matrix have been identified, driving cellular proliferation and migration through several signalling pathways.(210) Key

pathways include the PI3K/Akt and ERK signalling pathways, through which IGFBP-2 interaction has been demonstrated to promote both sprouting angiogenesis and cellular proliferation through both IGF-I dependent and independent mechanisms.(218)(219)

4.1.1.1 Vasomotor function

Vasomotor function describes the regulation of vascular tone through arterial vasoconstriction and dilatation, and is an essential component of normal vascular function. Assessment of vasomotor function can therefore serve as a proxy for overall endothelial and VSMC function and dysfunction, as co-ordinated interplay between the two are required for both constriction and dilatation.(271)

The endothelium secretes several factors which act to regulate vascular tone through their actions on underlying VSMC. Endothelin-1 and thromboxane both act to induce vasoconstriction, whilst secreted vasodilators include prostacyclin, endothelium derived hyper-polarising factor, and nitric oxide.(9) Of these, nitric oxide is considered the most crucial, due to its myriad roles in vascular protection, and reduced NO expression is considered a central part of early endothelial dysfunction and the development of vascular pathophysiology.(26) A detailed description of the mechanisms underpinning NO production can be found in Section 1.2.1.

Our group has demonstrated that overexpression of IGFBP-1 in transgenic mice led to aortic hypocontractility and reduced blood pressure, related to a basal increase in NO production.(272) Furthermore, IGFBP-1 overexpression in mice with genetic insulin resistance led to increased endothelial cell nitric oxide production and reductions in blood pressure, atherosclerosis and aortic contractility in the presence of insulin. This increase in NO production was found to be secondary to increased eNOS synthesis via upregulation of PI3K/Akt signalling in an IGF-I independent manner.(195) Our group also

demonstrated that IGFBP-1 enhanced insulin sensitivity and insulin secretion in obese mice, with this effect mediated by the RGD integrin-binding domain contained within IGFBP-1.(273) IGFBP-2 contains an RGD domain homologous to IGFBP-1, therefore raising the possibility overexpression of IGFBP-2 may enhance NO secretion and affect vascular physiological function in a similar manner to IGFBP-1.

As previously described, vasoconstriction and vasodilatation depend upon the action of contractile VSMC within the tunica media. These actions occur under multiple influences: the aforementioned factors secreted by the endothelium; autocrine/paracrine humoral factors such as acetylcholine; circulating factors such as adrenaline and noradrenaline and mechanical influences such as shear forces and mechanical stretch.(9)(274) The intracellular calcium mediated mechanisms for VSMC contraction and relaxation are discussed in detail in Section 1.3.1.

Together, these agents influence the action of contractile VSMC during physiological function, with dysregulation of these factors creating the potential to influence phenotypic switching to a proliferative, non-contractile synthetic VSMC phenotype, with reduced expression of contractile VSMC markers such as alpha smooth muscle actin and smooth muscle myosin heavy chain.(52) The actions of synthetic VSMC and sequelae of phenotypic switching will be discussed in more detail in later chapters.

The influence of IGFBP-2 on the physiological actions of contractile VSMC is unknown, as any prior investigation of IGFBP-2 and VSMC has focussed on synthetic properties such as cellular proliferation, or its presence within pathophysiological environments such as atherosclerotic plaque and neointimal restenosis.(223)(232) IGFBP-2 has however been demonstrated in other cell types to increase intracellular calcium levels,

and stimulate calcium/calmodulin-dependent protein kinase activation, raising the possibility it may also have direct effects on VSMC vasomotor function.(275)(276)

4.1.1.2 Blood pressure

As previously described in Section 1.3.1.4, blood pressure homeostasis is maintained through alterations in cardiac output and in systemic vascular resistance by peripheral vasoconstriction or dilatation of resistance vessels.(48) In this manner vascular smooth muscle cells play a crucial role in blood pressure homeostasis, as does the endothelial monolayer, with control of physiological blood pressure reliant on balanced production of both vasoconstrictive and vasodilatory factors.(277)

Several signalling pathways known to be influenced by IGFBP-2 may play a role in alterations of physiological blood pressure. The mitogen-activated protein kinase signalling family, including ERK1/2 and p38 MAPK, are associated with increased VSMC proliferation, reducing luminal area of resistance arteries, as well as increased contractile response, and therefore increasing blood pressure. However, the increased VSMC proliferation observed is in response to a phenotypic switch to the dedifferentiated synthetic phenotype, which has a lower capacity for contraction, and therefore may eventually lead to reduced vasoconstriction as the proportion of contractile VSMC is reduced.(278) This reduction in vasoconstriction is unlikely to lead to a reduction in systemic blood pressure however, given the concurrent reduction in luminal area and subsequent increase in systemic resistance, as well as increasing arterial stiffness, and the potential role of the MAPK family in the development of vascular pathophysiology will be explored in more detail in later chapters.

IGFBP-2 has been associated with increased MAPK signalling, including endothelial cells in as yet unpublished work by our own group, whilst others have seen increased

ERK activity in glioma cells.(279) It is not known if IGFBP-2 can effect MAPK signalling in VSMC, although a reduction in MAPK phosphorylation with IGFBP-2 inhibition has been seen in lymphangioleiomyomatosis cells, tumour cells with a VSMC phenotypic appearance.(224)

IGFBP-2 has also been demonstrated to activate the PI3K/Akt pathway, through a number of mechanisms and in a number of different cell types, including endothelial cells and VSMCs as previously described in detail in Section 1.10.4. This signalling pathway plays a critical role in eNOS activation, and is activated downstream of multiple mechanisms, including insulin activation, shear stress and VEGF activation. Given its role in NO production, it is considered a vital factor in blood pressure homeostasis and the maintenance of vascular quiescence.(112)(280) Activation of this pathway can lead to enhanced NO production, and subsequently reduced blood pressure as seen with IGFBP-1 overexpression.(195) However, disproportionate expression may potentially upset the balance of homeostasis, with increased concurrent ERK activation leading to increased VSMC proliferation, endothelial dysfunction and increased blood pressure. While the effects of IGFBP-2 have not yet been investigated in systemic blood pressure, markedly raised levels of IGFBP-2 have been discovered in both pulmonary endothelial cells and pulmonary smooth muscle cells in patients with pulmonary arterial hypertension, correlating with disease severity.(281)

4.1.1.3 Aortic distensibility

Aortic distensibility relates to the regulation of pulsatile blood flow during cardiac systole, allowing this to become continuous flow in the peripheral resistance vessels.(50) As previously described, distensibility is achieved through aortic compliance, related to the proportion of components of the extra cellular matrix, primarily elastin.(51) A variety of mechanisms can affect the ratio of elastin to other ECM components as well as fibroblast and VSMC content, and so in turn affect aortic compliance.

Because IGFBP-2 interacts with ECM, as described in section 1.10.4.3, and is upregulated in transgenic murine models of Marfan's Syndrome (235) it is plausible that IGFBP-2 may influence aortic compliance. It has not however been established if IGFBP-2 has any effect on aortic distensibility *in vivo*, either acutely or over a prolonged period of time.

4.1.2 Experimental overview

This chapter describes a series of complementary experiments designed to explore the effects of IGFBP-2 on vascular physiology. Transgenic mice expressing human IGFBP-2, described in section 3.1, were used to ascertain how hIGFBP-2 affects vascular function. Vasomotor function *ex vivo* was assessed using organ chamber apparatus. Subsequent experiments were designed to examine the effect of global hIGFBP-2 overexpression on the vasculature *in vivo*, utilising tail cuff blood pressure monitoring and aortic distensibility experiments. Finally, immunoblotting and real time polymerase chain reaction were used to assess signalling pathway proteins and contractile VSMC gene expression respectively.

4.1.3 Aims

The primary aim of this chapter was to investigate the influence of IGFBP-2 overexpression on vascular physiology.

4.1.4 Objectives

1. Investigate the effects of hIGFBP-2 overexpression on arterial vasomotor function.
2. Investigate the effects of hIGFBP-2 on blood pressure and aortic distensibility.

3. Investigate how hIGFBP-2 overexpression influences VSMC contractile gene expression.
4. Investigate how hIGFBP-2 overexpression influences aortic kinase signalling pathway protein expression and phosphorylation.

4.2 Methods

4.2.1 Aortic vasomotor assay

Aortic vasomotor assays were performed using an eight chamber organ bath apparatus (Panlab). This apparatus allows for exposure of aortic tissue to different compounds *ex vivo*, and allows for multiple segments of aorta to be assessed simultaneously (Figure 4-1). Vasodilatory and vasoconstriction responses can continue to be elicited as the aortic rings are placed in oxygenated chambers containing a physiological buffer, and has been used by our group in several studies.(195)(197)(256)

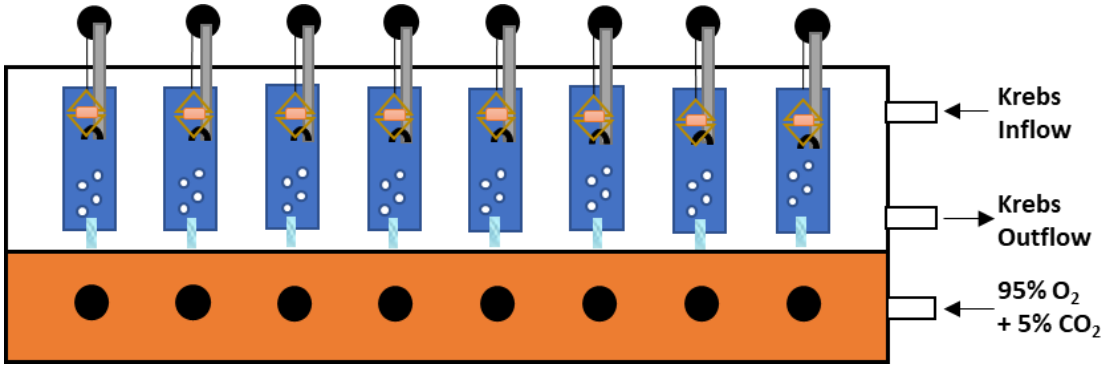


Figure 4-1. Organ bath apparatus. Aortic rings were cleaned, carefully placed between 2 wire triangles and hung on a pressure transducer system. These rings were then placed in chambers filled with Krebs-Henselheit buffer solution, warmed to 37°C and perfused with 95% O_2. All described vasomotor assays took place in these conditions.

Mice were culled at 12-13 weeks of age by exposure to rising concentrations of carbon dioxide, followed by confirmation of permanent cessation of circulation by removal of the heart, as described in Schedule 1 of the Animals (Scientific Procedures) Act 1986 (Amended 2012). For tamoxifen-inducible lines, mice were injected with tamoxifen at 7-8 weeks of age as detailed in Section 3.2.1.2. Continued overexpression of hIGFBP-2 at the time of harvest was confirmed with western blotting of cultured pulmonary endothelial cells. The thoracic aorta was then carefully removed, ensuring it was not over-stretched, which can cause damage to the endothelium and VSMC.

Aortas were transferred in chilled Krebs-Henselheit solution (Table 5), a physiological buffer optimised to ensure prolonged viability of the aorta during the experiment. The aorta was then cleaned under a dissecting microscope as quickly as possible to avoid prolonged time without oxygen perfusion, and then divided into 5mm rings. These aortic rings were then placed between two wire triangles under the microscope; hung on a pressure transducer; and suspended in a chamber with 10 mL of Krebs-Henselheit buffer, with consistent oxygen perfusion and warmed to 37°C.

Compound	g/L
NaCl	6.95
KCl	0.35
KH2PO4	0.16
NaHCO3	2.10
MgSO4.7H2O	0.29
Glucose	1.98
CaCl2.2H2O	0.37

Table 5. Components of Krebs-Henselheit buffer.

Passive tension was increased gradually to three grams and equilibrated to stabilise rings before commencing experiments. The pressure transducers had been calibrated prior to this to ensure consistency between rings.

Aortic ring viability was assessed with the replacement of Krebs buffer with a 0.04M potassium chloride (KCl) solution. A 10% increase in tension from baseline was required to demonstrate viability. This was performed twice with the first exposure to KCl acting

as a primer for the rings. If a 10% increase in tension was not observed then those rings were not included in analysis, with presumed damage to VSMC. Vasodilation was then studied using phenylephrine (PE) pre-constriction and subsequent ascending concentrations of acetylcholine (Ach) (See Table 6 for full concentrations of all agents used). If end-vasodilation was less than 50% of the original tension, or more than 140%, then that ring was also excluded due to likely endothelial damage.

A full constriction curve was then performed with ascending concentrations of phenylephrine. NO bioavailability was assessed by eNOS inhibition with NG-Methyl-L-arginine (L-NMMA) and repeating the phenylephrine constriction curve. Finally, sodium nitroprusside (SNP) was used to assess endothelium independent vasodilation (Table 6). Chambers were washed several times with Krebs buffer and rings equilibrated back to 3g of tension between each curve.

Agent used	**Concentrations applied (µmol/l)**
Potassium Chloride	$4x10^{4}$
Acetylcholine	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10
Phenylephrine	0.3 pre-constriction 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10
NG-Methyl-L-arginine	100 pre constriction curve
Sodium Nitroprusside	$1x10^{-5}$, $3x10^{-5}$, $1x10^{-4}$, $3x10^{-4}$, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10

Table 6. Concentrations of all agents used during aortic vasomotor assays

Labchart Pro Software (ADInstruments) was used to directly gather dose response tension curves from the organ bath apparatus. These data were then manually extracted using Microsoft Excel, and inputted into GraphPad Prism for graph formation and statistical analysis. Difference between substrate response was assessed using area under the curve (AUC). Additionally, the half maximal effective concentration (EC50) of each substrate was assessed, as was the difference in maximal tension (Emax).

Statistical analysis was performed with GraphPad Prism, and unpaired Students t-tests were used to assess for statistical significance between groups.

4.2.2 *In vivo* blood pressure measurement

In vivo blood pressure measurements were taken using the non-invasive tail cuff CODA system (Kent Scientific). Mice were placed in appropriately sized holders for 15 minutes prior to obtaining measurements to allow for acclimatisation, and were kept in a heating chamber at 37°Celsius. Two occlusion tail cuffs were placed on the mouse and taped in place to ensure consistent readings (Figure 4-2). The first cuff automatically inflated to impede tail blood flow, and then deflated slowly whilst the second cuff, incorporating a volume-pressure sensor, measured tail swelling secondary to returning arterial blood flow. Systolic blood pressure was automatically measured when tail swelling was first detected and diastolic blood pressure was measured at the point when tail swelling ceased. Measurements were taken at 0, 2 weeks and 6 weeks after tamoxifen injection.

Statistical analysis was performed with GraphPad Prism, and 2-way ANOVA was used to assess for statistical significance between groups.

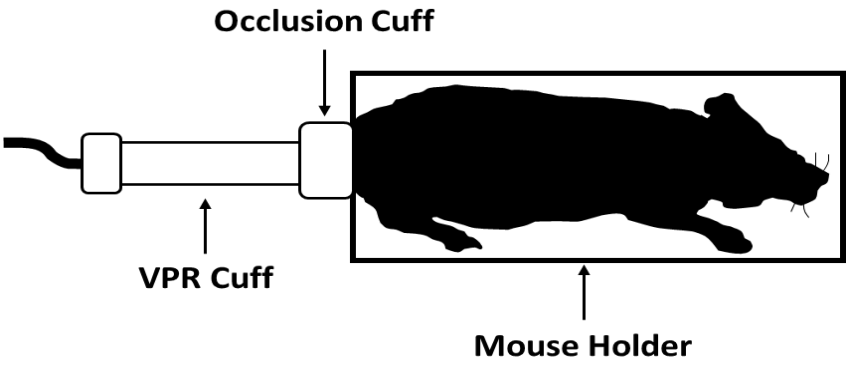


Figure 4-2. Representative image of mouse tail cuff blood pressure apparatus. Mice were kept in holders within a heating chamber at 37°Celsius. Two occlusion tail cuffs were placed on the mouse. The first cuff inflated to impede blood flow whilst the second cuff measured tail swelling secondary to returning arterial blood flow as the first cuff deflated.

4.2.3 *In vivo* aortic distensibility

In vivo ultrasound scanning (USS) of the abdominal aorta was performed on anaesthetised animals at two and six weeks after tamoxifen injection using the Vevo 2100 high resolution, high frequency pre-clinical micro ultrasound system (Fujifilm VisualSonics). Twenty four hours prior to imaging, the mice were shaved over their thorax and abdomen and hair removal cream (Veet) was used to ensure optimal imaging.

Mice were anaesthetized throughout scanning using isoflurane and placed on a heated monitoring pad. Electrodes on the monitoring pad allowed continuous ECG and respiration rate monitoring. Core body temperature was continuously monitored by rectal probe.

An MS-550D transducer (Fujifilm VisualSonics) at 40MHz frequency was used to obtain images. M-mode imaging was used to measure aorta diameter in systole and diastole. B-mode imaging was then used to obtain a cross-sectional view of the abdominal aorta, with acquisition of multiple electrocardiographic gated images over multiple cardiac cycles to create a single representative cine loop, known as ECG gated Kilohertz

visualisation (EKV) imaging. Serial sections of the abdominal aorta were imaged in this manner, with three to four images used per mouse. M-mode images were then analysed using Vevo Lab software to measure aortic diameter, and EKV images were used for an estimation of aortic distensibility, by assessment of lumen area change in systole and diastole, and quantified as 1/Megapascal (1/MPA). Aortic distensibility was an estimation only, as it was not possible to simultaneously measure animal blood pressure, a parameter required for definitive aortic distensibility calculation. Therefore, pressure values pre-determined by the VevoLab software of 120mmHg and 100mmHg were used for systolic and diastolic pressures respectively.

Statistical analysis was performed with GraphPad Prism, and unpaired Students t-test was used to assess for statistical significance between groups.

4.2.4 Immunoblotting

Immunoblotting was performed as described in section 3.3. Aortic tissue was used, and Beta-actin (Cell Signalling Technologies #4967) was used as a house-keeping standard as this is the established standard for aortic tissue in our labs. It was noted however during analysis that beta-actin was consistently down-regulated in hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre +ve aortas (not shown). Our approach was therefore modulated to assess phosphorylation ratios for each signalling protein and the data is presented in this manner. Signalling protein specific antibodies used are presented in Table 7. Statistical analysis was performed with GraphPad Prism, and unpaired Students t-test was used to assess for statistical significance between groups.

Antibody	**Details**
Akt – rabbit	(Cell Signalling Technologies #9272)

Phospho-Akt (Ser473) – rabbit	(Cell Signalling Technologies #4060)
P44/42 MAPK (ERK1/2) – rabbit	(Cell Signalling Technologies #4695)
Phospho-p44/42 MAPK (ERK1/2) – rabbit	(Cell Signalling Technologies #4370)
p38MAPK – rabbit	(Cell Signalling Technologies #8690)
Phospho-p38MAPK – rabbit	(Cell Signalling Technologies #4511)
Beta-actin – rabbit	(Cell Signalling Technologies #4967)

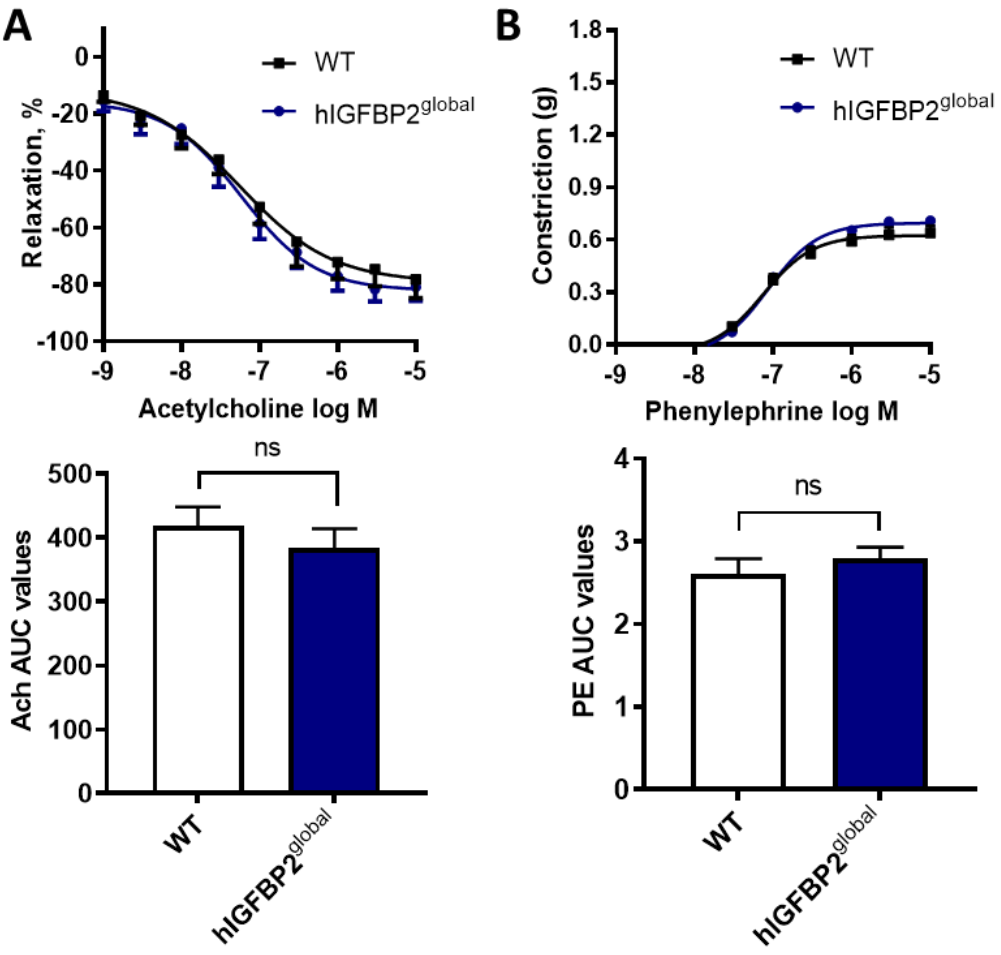
Table 7. List of antibodies used to assess signalling pathways

4.3 Results

4.3.1 Vasomotor function

4.3.1.1 hIGFBP2^{global} vasomotor function

Aorta vasomotor assays were performed using organ bath apparatus as outlined in Section 4.2.1. No significant difference was seen in vasodilation in response to acetylcholine (Figure 4-3 A). There was also no difference in vasoconstriction in response to stimulation with phenylephrine (Figure 4-3 B). NO inhibition with L-NMMA did not demonstrate any difference in NO bioavailability between transgenic mice and their WT littermates (Figure 4-3 C), and there was no difference in vasodilatory response to the NO donor sodium nitroprusside (Figure 4-3 D).



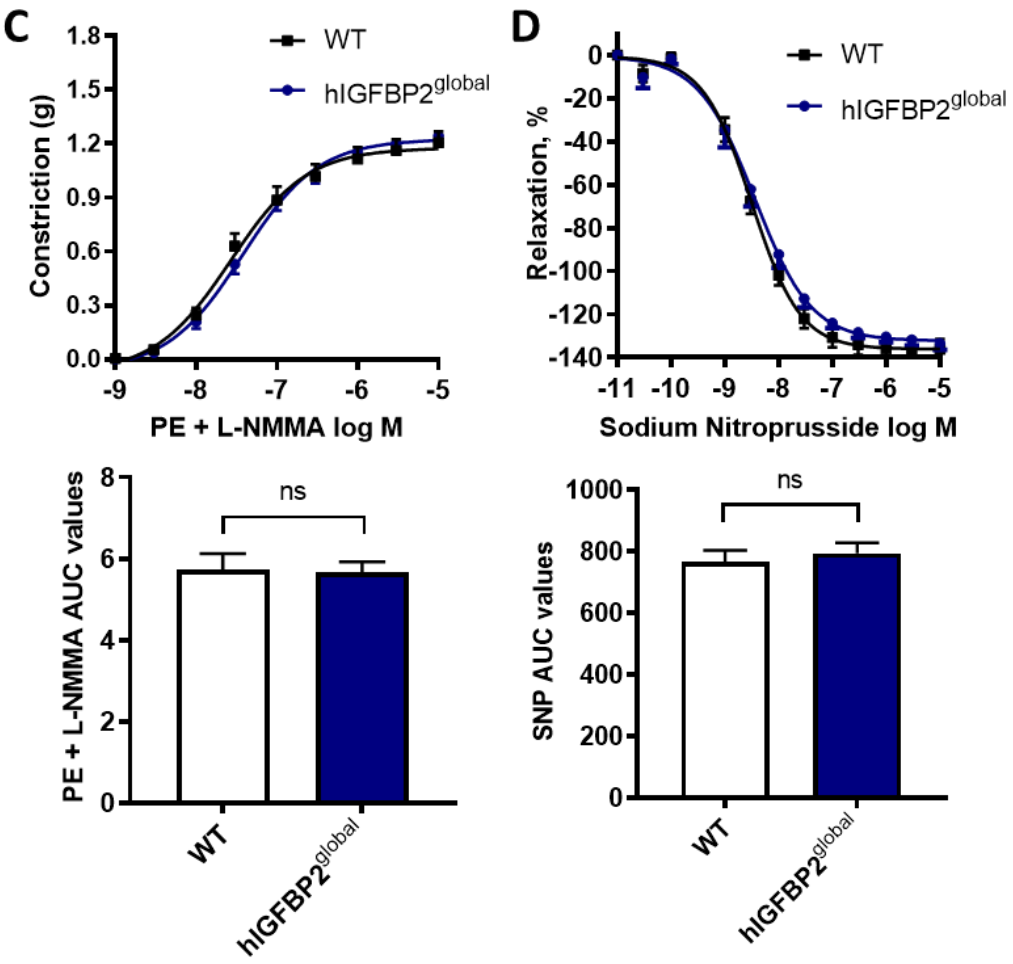


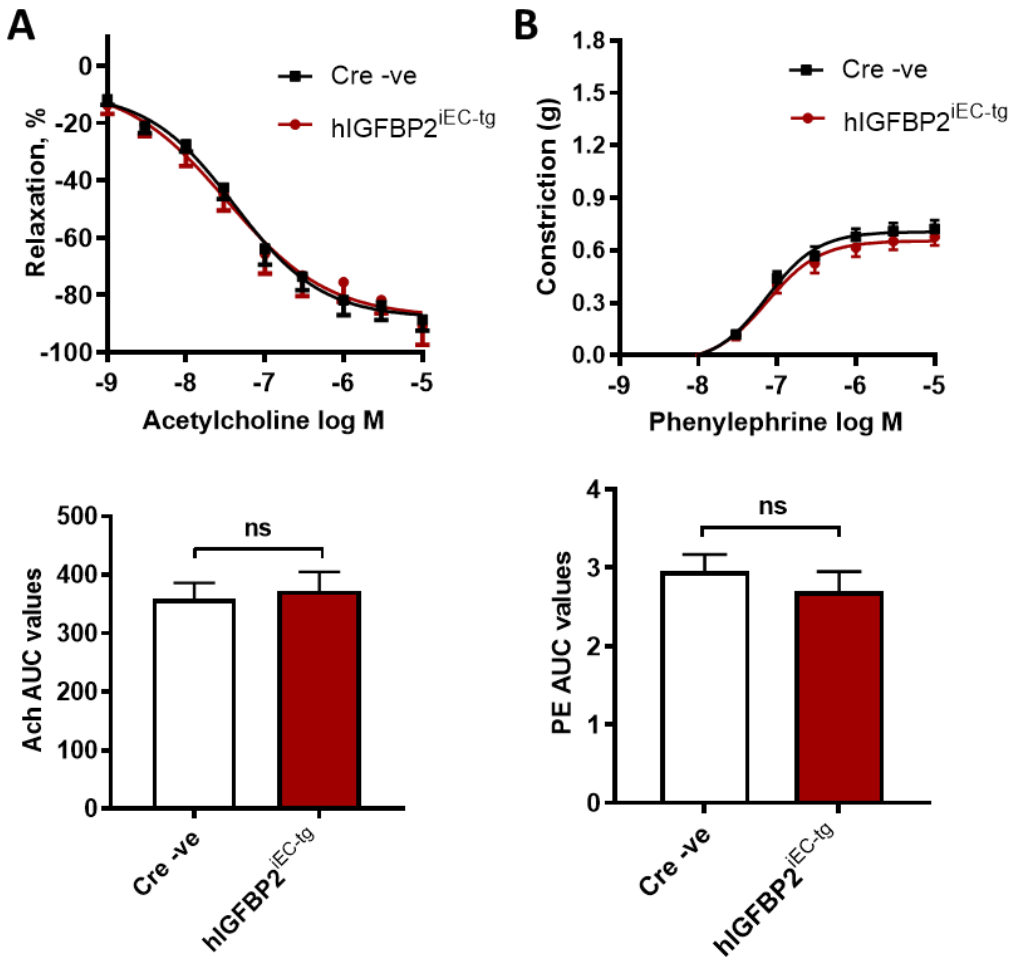
Figure 4-3. Vasomotor function of the hIGFBP2^{global} mouse line assessed using aortic response to physiological stimuli. No significant difference was seen in response to acetylcholine, phenylephrine, L-NMMA incubation or sodium nitroprusside. (n=10,10)

Agonist (unit)	LogEC50/IC50 Wild Type	LogEC50/IC50 hIGFBP2^{global}	Emax Wild Type	Emax hIGFBP2^{global}
Ach (%)	-7.253	-7.236	67.58	66.48
PE (g)	-7.126	-7.066	0.6718	0.7583
LNMMA + PE (%)	-7.571	-7.423	1.252	1.286
SNP (g)	-8.502	-8.443	135.6	131.9

Table 8. LogEC50/IC50 and Emax values for hIGFBP2^{global} dose response curves

4.3.1.2 hIGFBP2$^{iEC\text{-}TG}$ vasomotor function

As with the hIGFBP2^{global} mouse line, no significant differences were seen between Cre positive hIGFBP2$^{iEC\text{-}tg}$ mice and their Cre negative controls under any of the conditions assessed: vasodilation or vasoconstriction in response to acetylcholine and phenylephrine stimulation respectively (Figure 4-4 A-B); and no change in aortic response with NO inhibition or donation by L-NMMA and sodium nitroprusside respectively (Figure 4-4 C-D).



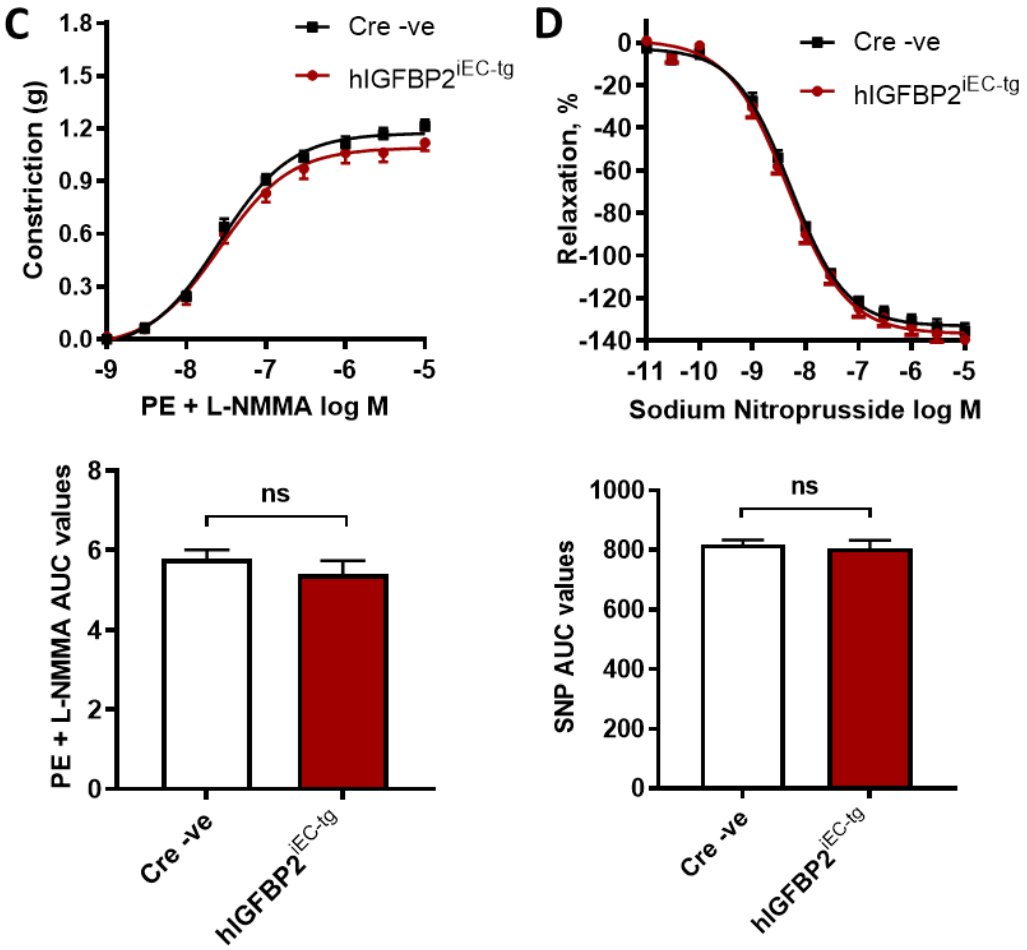


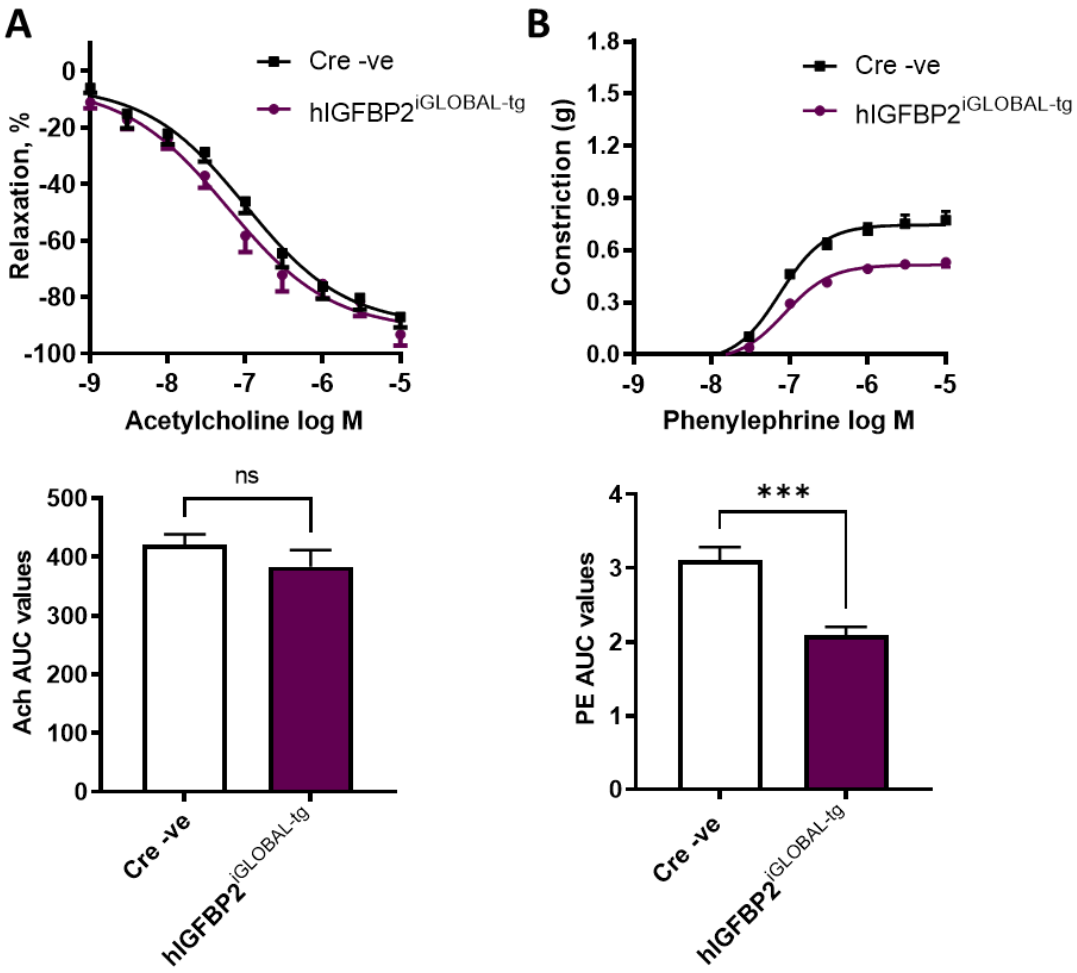
Figure 4-4. Vasomotor function of the hIGFBP2^{iEC-TG} mouse line assessed using aortic response to physiological stimuli. No significant differences were seen in response to acetylcholine and phenylephrine, or with L-NMMA incubation or NO donation by sodium nitroprusside. (n=7,9)

Agonist (unit)	LogEC50/IC50 Cre -ve	LogEC50/IC50 Cre +ve	Emax Cre -ve	Emax Cre +ve
Ach (%)	-7.385	-7.482	78.89	81.6
PE (g)	-7.134	-7.13	0.7536	0.6989
LNMMA + PE (%)	-7.581	-7.576	1.243	1.137
SNP (g)	-8.284	-8.344	130.7	138

Table 9. LogEC50/IC50 and Emax values for hIGFBP2^{iEC-tg} dose response curves

4.3.1.3 hIGFBP2$^{iGLOBAL-tg}$ vasomotor function

No difference between groups was seen in vasodilatory response to acetylcholine (Figure 4-5 A). A significant difference was seen in vasoconstriction in response to phenylephrine, with reduced vasoconstriction seen in Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice (Figure 4-5 B). A significant difference in vasoconstriction was also seen after phenylephrine administration following L-NMMA incubation, and the difference between groups was not altered compared to the difference in vasoconstriction without L-NMMA, suggesting the change was not related to alterations in NO bioavailability between groups (Figure 4-5 C). No difference in vasodilation in response to sodium nitroprusside was observed between groups (Figure 4-5 D).



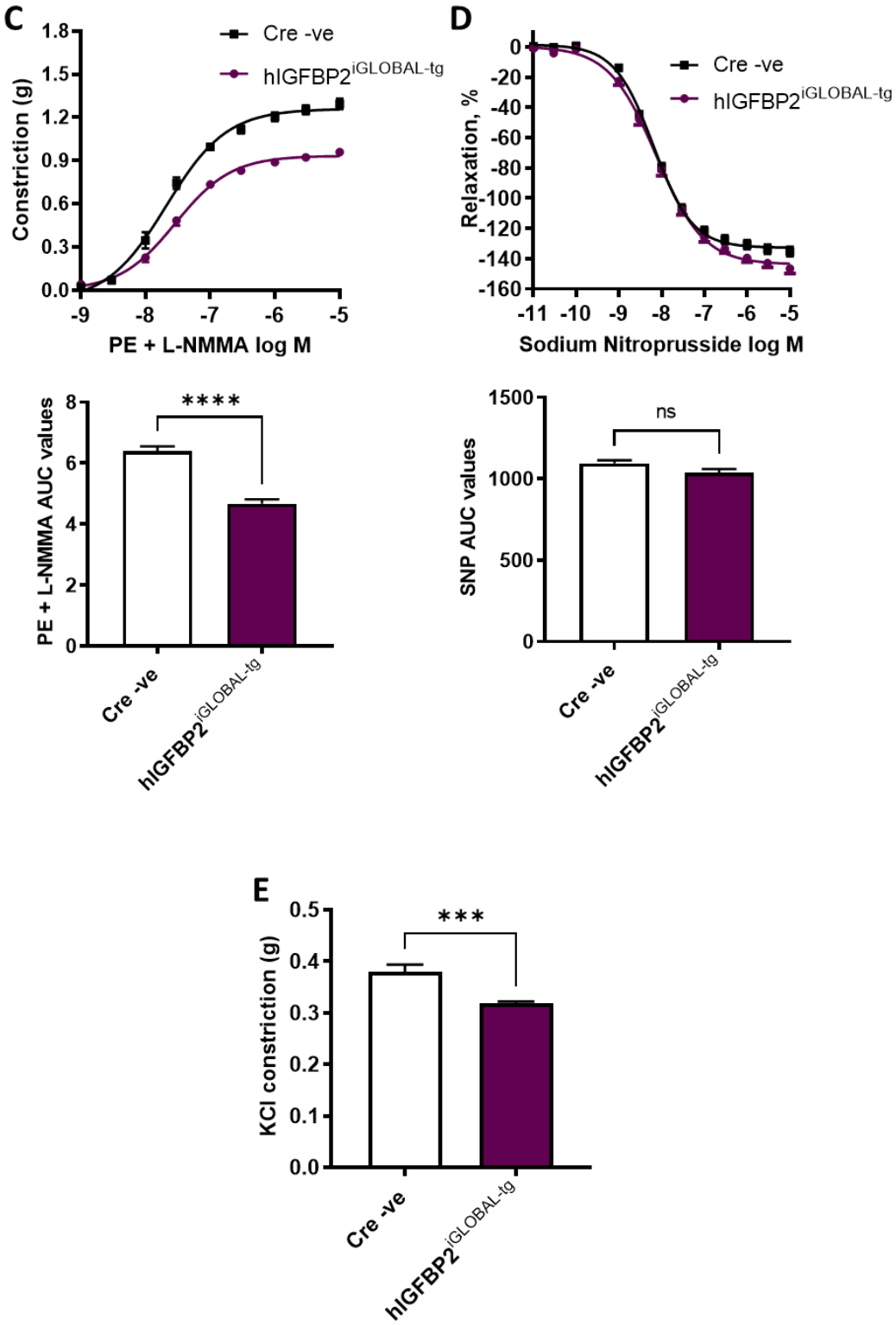


Figure 4-5. Vasomotor function of the hIGFBP2$^{iGLOBAL-tg}$ mouse line assessed using aortic response to physiological stimuli. A: no difference between groups was seen in vasodilatory response to acetylcholine. **B:** a significant difference was seen in vasoconstriction in response to phenylephrine, with reduced vasoconstriction seen in Cre +ve hIGFBP2$^{iGLOBAL-tg}$ mice (p=0.001). **C:** a significant difference in vasoconstriction was also seen after L-NMMA administration (p=<0.0001). **D:** no difference in vasodilation in response to sodium nitroprusside was observed between groups. **E:** a significant difference in vasoconstriction was seen after potassium chloride administration (p=0.0002) (n=8,10).

Agonist (unit)	LogEC50/IC50 Cre -ve	LogEC50/IC50 Cre +ve	Emax Cre -ve	Emax Cre +ve
Ach (%)	-6.996	-7.225	84.95	86.67
PE (g)	-7.127	-7.063	0.7896	**0.5542****
LNMMA + PE (%)	-7.676	-7.536	1.341	**0.9347****
SNP (g)	-8.186	-8.125	134.3	144.1

Table 10. LogEC50/IC50 and Emax values for hIGFBP2$^{iGLOBAL-tg}$ dose response curves. Emax values for PE and LNMMA + PE were significantly less for the Cre +ve mice compared to the Cre -ve mice (p=<0.0001).

4.3.2 *In vivo* tail cuff blood pressures

Tail cuff blood pressures of hIGFBP2$^{iGLOBAL-tg}$ mice were taken pre tamoxifen injection, two weeks post injection and six weeks post injection, using the methods described in section 4.2.2. Systolic, diastolic and mean arterial pressure (MAP) readings were taken. At baseline pre injection, there was no significant difference in blood pressure by any parameter. At two weeks post injection, there was no effect on blood pressure in Cre positive mice or Cre negative mice when assessed by 2-way ANOVA, and at six weeks blood pressures in both groups continued to show no significant difference (Figure 4-6).

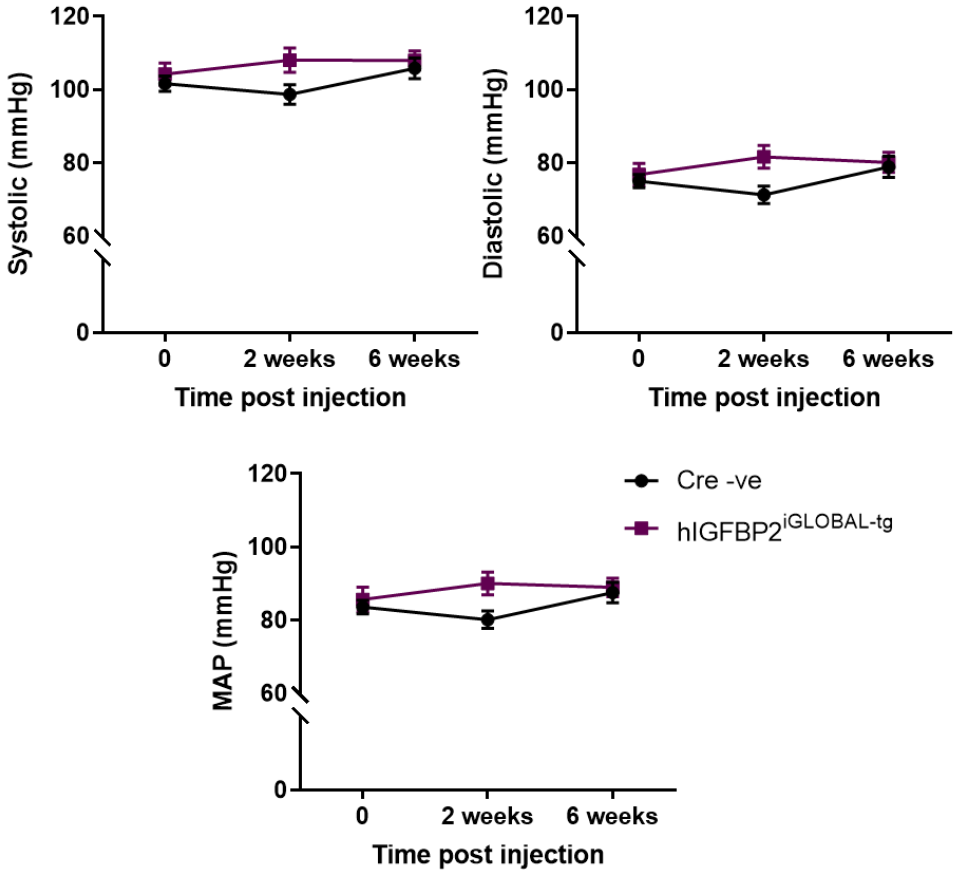


Figure 4-6. Tail blood pressures of hIGFBP2$^{iGLOBAL-tg}$ mice. No significant difference between groups was seen in blood pressure at any time point (n=12,14).

4.3.3 *In vivo* aorta width and aortic distensibility

Abdominal aortas were imaged in M-mode at six weeks post tamoxifen injection as described in Section 4.2.3 (Figure 4-7 A shows a representative image). Serial images of the abdominal aorta were taken, and measurements of internal lumen diameter were taken at the point the aortic diameter was largest during systole, and smallest, at end-diastole. A small but significant reduction in aortic lumen diameter of Cre positive hIGFBP2$^{iGLOBAL-tg}$ mice was seen in both systole and diastole (Figure 4-7 B-C).

Aortic distensibility was measured at two and six weeks using EKV gated images as described in Section 4.2.3 (Figure 4-8 A shows a representative image, with the

abdominal aorta highlighted by the red arrow). At two weeks, distensibility was significantly higher in Cre positive mice compared to Cre negative controls. This difference in distensibility was maintained at six weeks, although there was a significant decrease in distensibility in the Cre positive group at six weeks compared to two weeks, with no significant interval change seen in the Cre negative group (Figure 4-8 B).

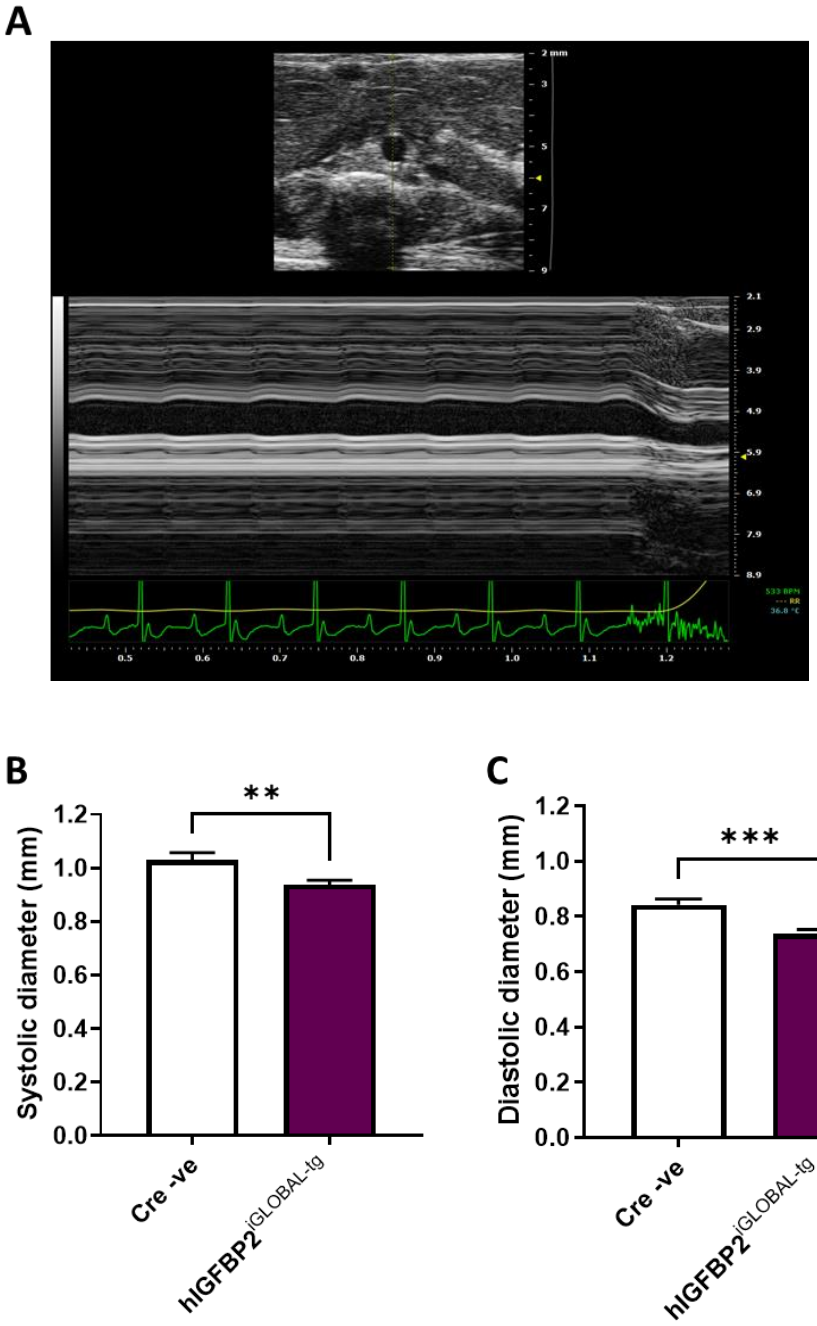


Figure 4-7. Systolic and diastolic diameter of *in vivo* abdominal aortas. A: representative images of 2D view and M mode. **B-C**: a small but significant reduction in aorta diameter of Cre +ve mice was seen in both systole (p=0.006) and diastole (p=0.0006). (n=11,14).

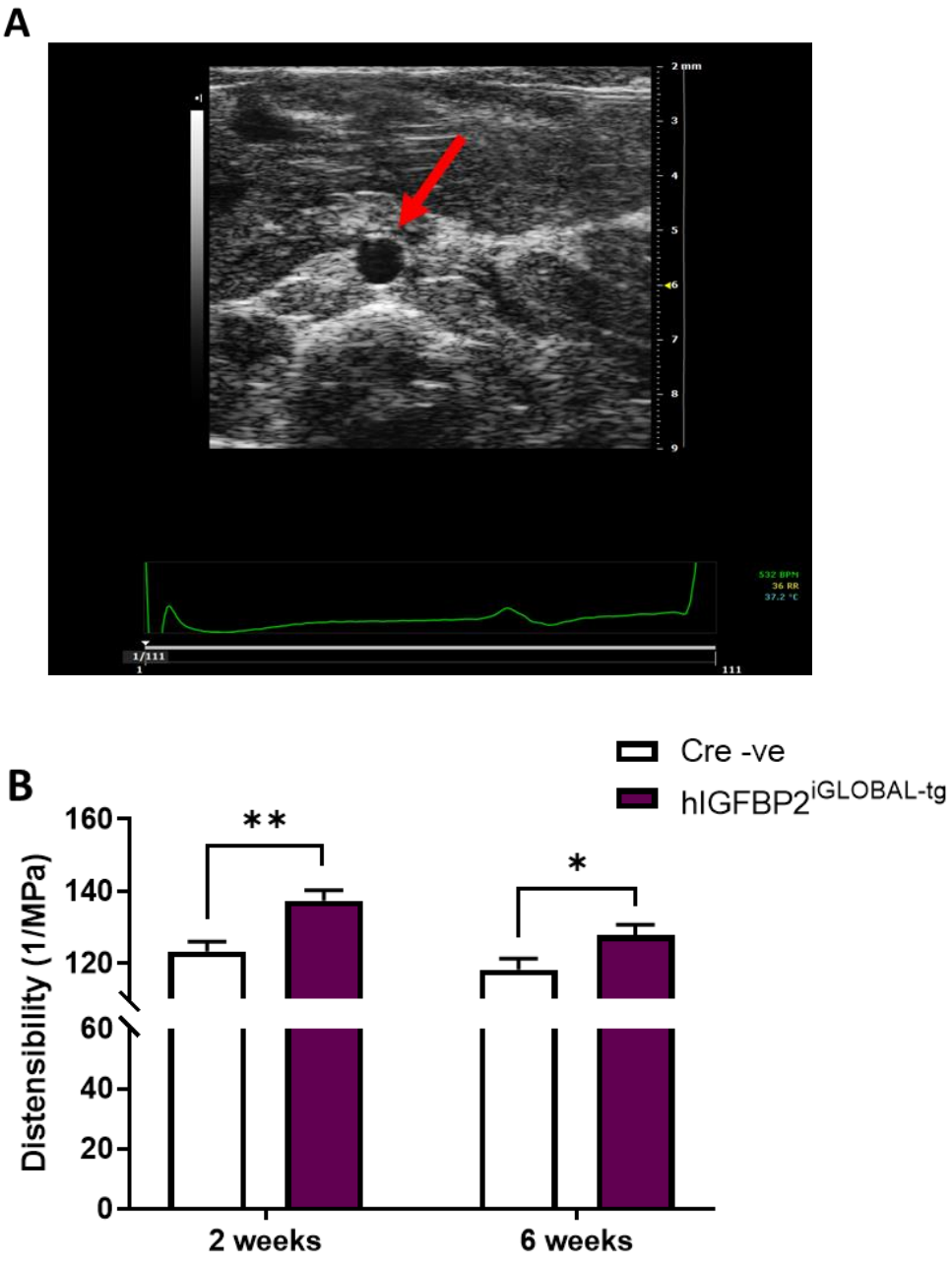


Figure 4-8. Aortic distensibility of the hIGFBP2$^{iGLOBAL-tg}$ mouse line assessed using ultrasound scanning. A: representative still image of EKV gated imaging. **B**: at both 2 weeks (p=0.0019) and 6 weeks (p=0.0379) post tamoxifen injection, distensibility was significantly higher in Cre +ve mice compared to Cre -ve controls. A significant reduction in distensibility was seen between the 2 and 6 week time points in the Cre +ve mice (p=0.0259), with no significant reduction in distensibility in Cre -ve mice (n=11,14).

4.3.4 Contractile VSMC gene expression

Aortas were removed from hIGFBP2$^{iGLOBAL-tg}$ mice at six weeks post tamoxifen injection.

RNA was isolated and real-time quantitative PCR was performed as described in Section

2.4. The contractile smooth muscle related genes ACTA2 and MYH11 as well as human IGFBP2 were assessed, and gene expression was normalised to GAPDH. There was a significant down regulation in both ACTA2 and MYH11 gene expression in the Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice when compared to Cre negative controls, with significant upregulation in hIGFBP2 gene expression, as anticipated (Figure 4-9).

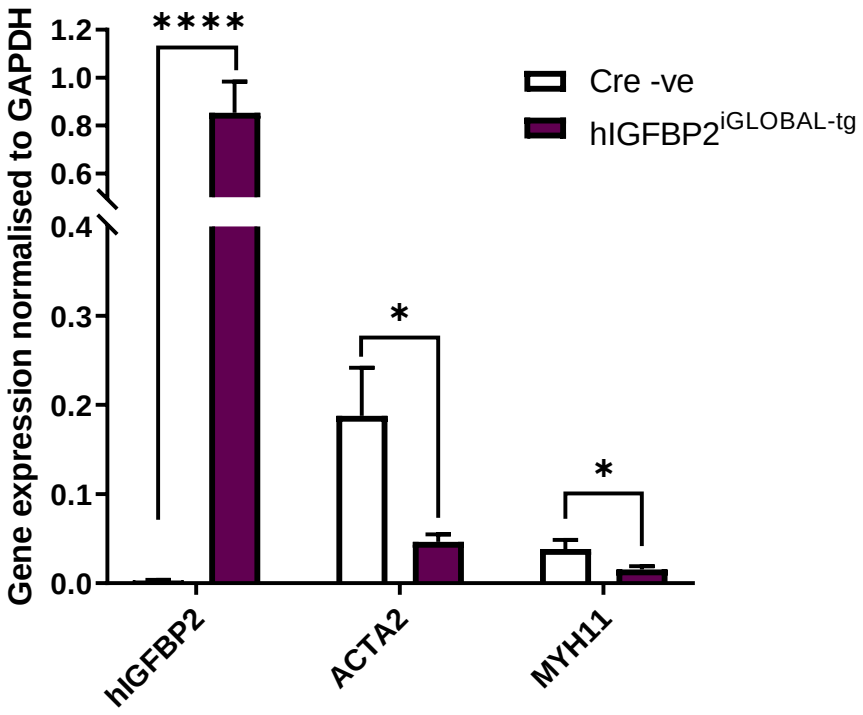
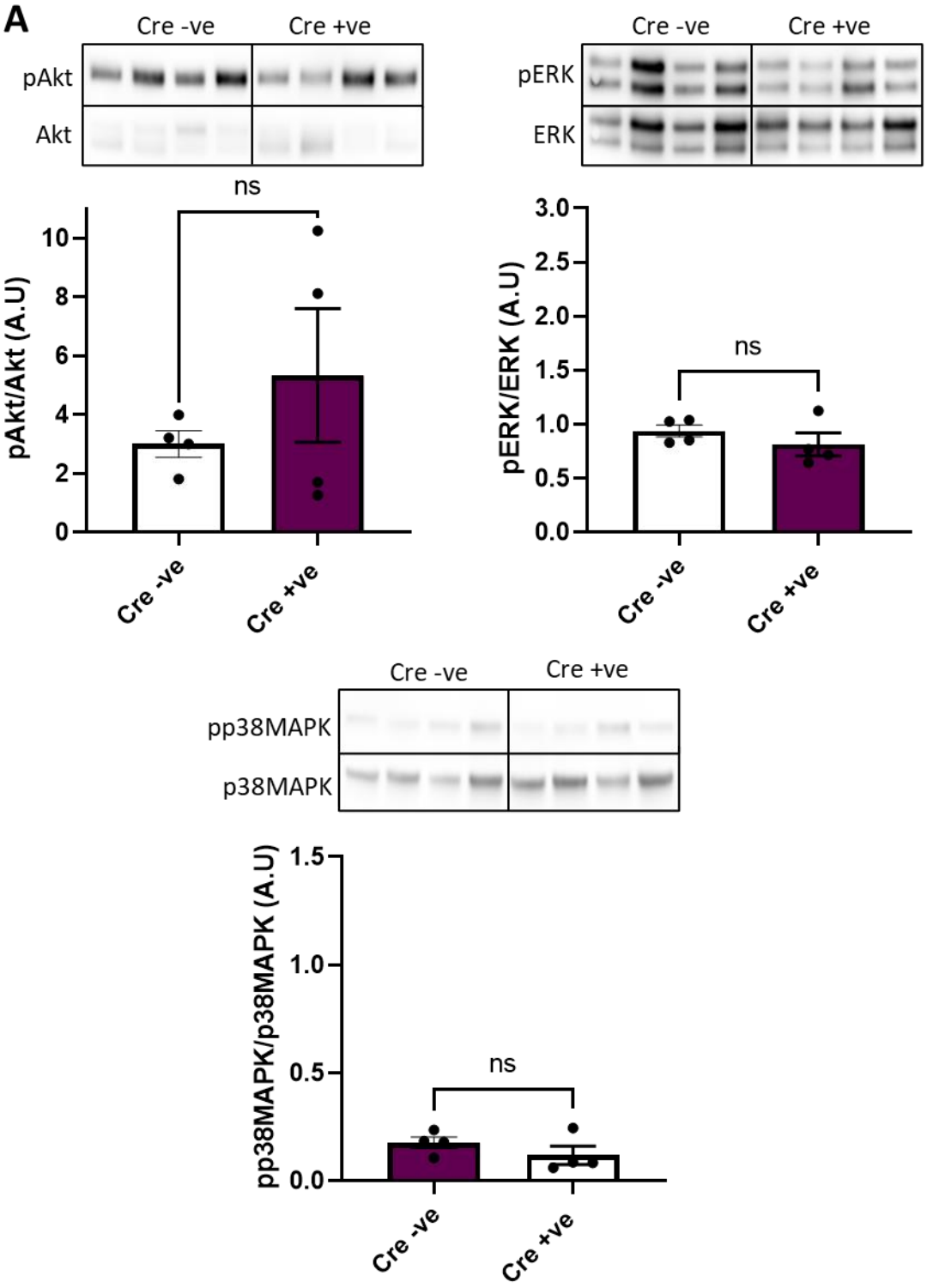


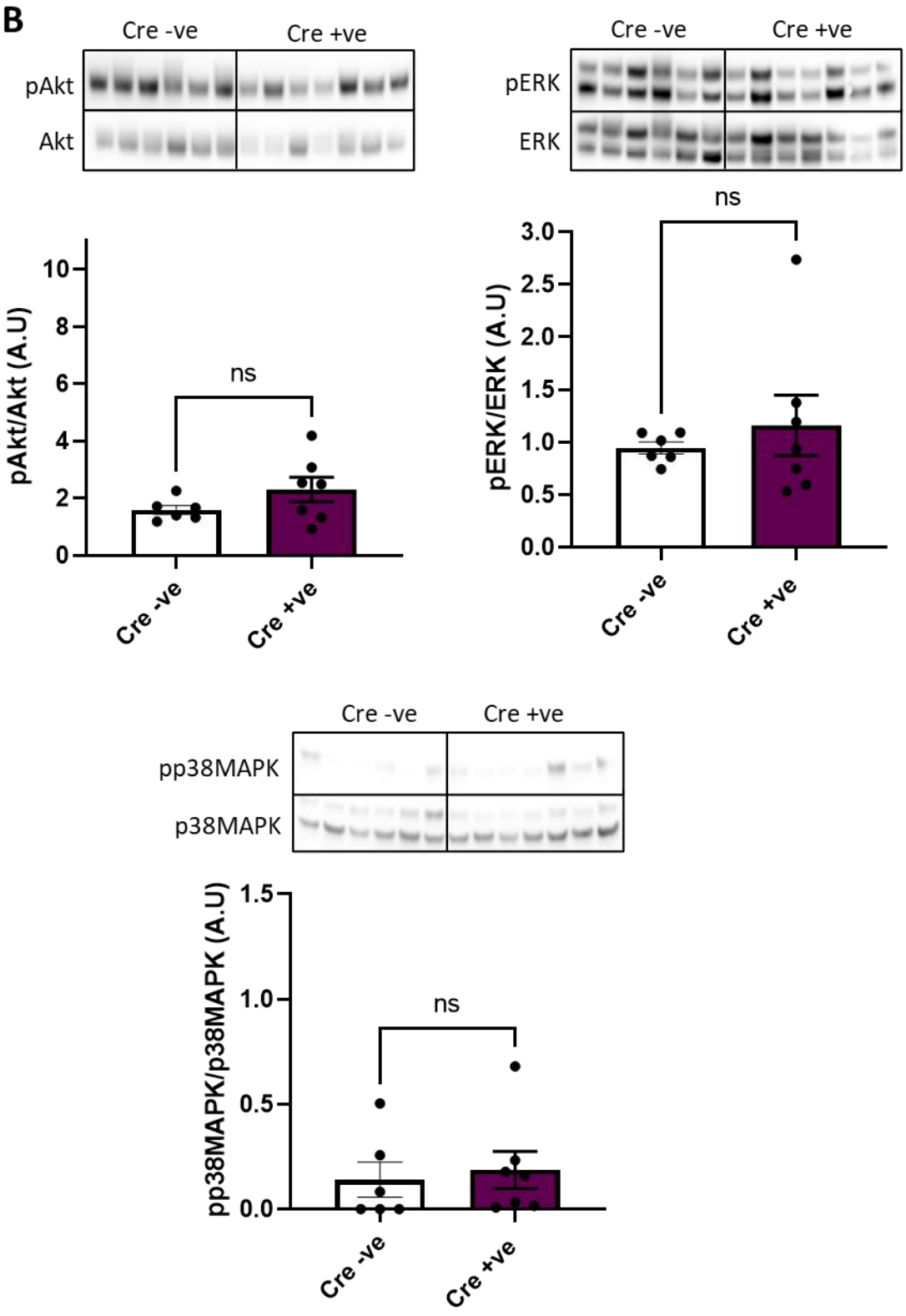
Figure 4-9. Contractile VSMC gene expression in aortic tissue from hIGFBP2$^{iGLOBAL\text{-}tg}$ mice. Gene expression was assessed using RT-qPCR at 6 weeks post tamoxifen injection. At 6 weeks there was a significant down regulation in both ACTA2 (p=0.01) and MYH11 (p=0.04) in the hIGFBP2$^{iGLOBAL\text{-}tg}$ mouse line when compared to Cre -ve controls (n=7,9).

4.3.5 Kinase signalling pathways

Thoracic aortas from hIGFBP2$^{iGLOBAL\text{-}tg}$ mice were harvested, lysed and immunoblotting was performed as described in Section 2.3. Data are presented as the ratio of phosphorylated protein to total protein. Akt, ERK and p38 phosphorylation were assessed at three separate time points: prior to tamoxifen injection, two weeks post tamoxifen injection and six weeks post tamoxifen injection. No significant differences were seen in phosphorylation of Akt, ERK or p38 MAPK pathways in aortas harvested

prior to tamoxifen injection or two weeks post tamoxifen injection (Figure 4-10 A-B). At six weeks post tamoxifen injection there was a significant difference in Akt phosphorylation, with no significant difference in ERK or p38 MAPK phosphorylation seen (Figure 4-10 C).




B
Cre -ve
Cre +ve
pAkt
Akt
pAkt/Akt (A.U)
ns
Cre -ve
Cre +ve
pERK
ERK
pERK/ERK (A.U)
ns
Cre -ve
Cre +ve
pp38MAPK
p38MAPK
pp38MAPK/p38MAPK (A.U)
ns
Cre -ve
Cre +ve

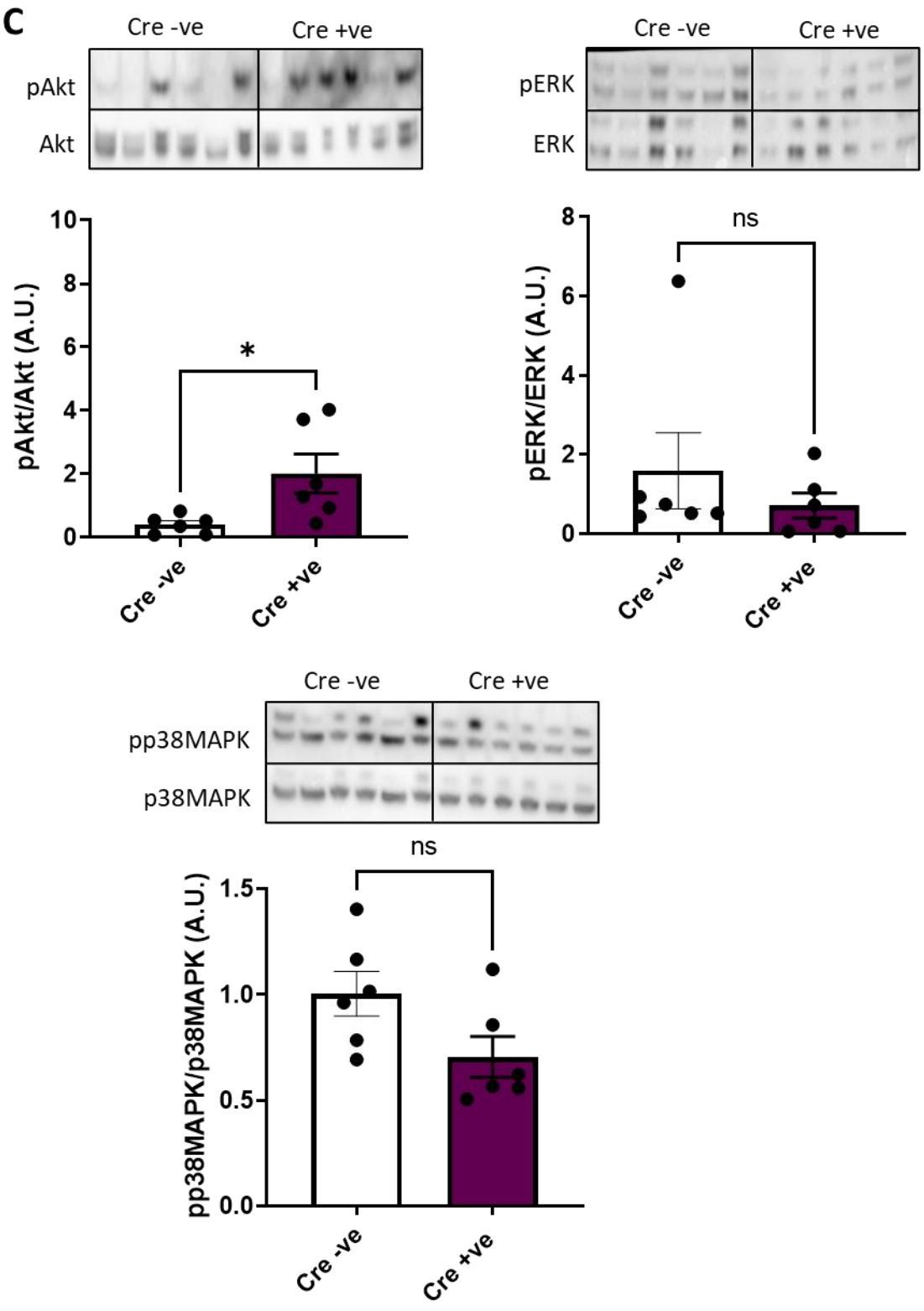


Figure 4-10. Kinase signalling phosphorylation pathways assessed by immunoblotting of aortic tissue derived from hIGFBP2$^{iGLOBAL\text{-}tg}$ mice. No significant differences were seen in phosphorylation of Akt, ERK or p38 MAPK pathways in Cre -ve versus Cre +ve aortas harvested prior to tamoxifen injection (n=4,4) or after two weeks (n=6,7)**.** At six weeks post tamoxifen injection there was significantly greater Akt phosphorylation in aortas derived from Cre +ve mice (p=0.04), with no significant difference in ERK or p38 MAPK phosphorylation seen (n=6,6).

4.4 Discussion

In this chapter, the modulatory effects of IGFBP-2 overexpression on vasomotor function *ex vivo*, blood pressure and aortic distensibility *in vivo*, and potential signalling pathways through which IGFBP-2 can exert its influence, were studied.

4.4.1 Vasomotor function

Vasomotor function was assessed using an established aortic ring vasomotor assay. This assay, using isolated segments of murine aorta and exposure to incremental doses of vasoconstricting and vasodilating agents, has been well established for a number of years and has been published by our group on several occasions.(195)(197)(282) Our work examined the effects of murine overexpression of IGFBP-2, and how this affected vasodilatory response to acetylcholine, vasoconstrictor response to phenylephrine, and what effect exposure to the nitric oxide synthase inhibitor L-NMMA had in enhancing the vasoconstrictor response to PE.

Acetylcholine acts on endothelial cells through muscarinic receptors on the endothelial cell membrane, activating eNOS via a calcium dependent process, and has recently been demonstrated to occur as an autocrine process induced by local shear stress.(274) Vasodilation in response to acetylcholine depends on an intact and functional endothelium, and so has been established as a marker of normal endothelial function, with differential vasodilatory response predominantly related to NO bioavailability.(283) Differential acetylcholine response has been used to demonstrate impaired endothelial function in insulin resistance, and the beneficial endothelial effects of superoxide inhibition.(284)(285) We found that endothelial response to acetylcholine was unaffected by IGFBP-2 overexpression, whether it was non-conditional, overexpressed in the endothelium alone, or conditionally expressed in all organs (Figure 4-3 A, 4-4 A, 4-5 A). We also found that the addition of L-NMMA did not differentially alter contractile response

to phenylephrine in any of the three mouse models assessed, suggesting that IGFBP-2 overexpression has no *ex vivo* effect on NO bioavailability (Figure 4-3 C, 4-4 C, 4-5 C). This contrasts with our previous findings when investigating IGFBP-1, which showed increased NO bioavailability with overexpression or preincubation with IGFBP-1.(195) IGFBP-1 and IGFBP-2 share homologous functional motifs such as the RGD motif, and so may be expected to have similar actions on signalling pathways. As the same increase in NO bioavailability was not observed with IGFBP-2 overexpression, this may suggest that the differing three dimensional structure of IGFBP-2 alters the activity of these functional motifs compared to IGFBP-1, or that these actions are environment dependent. This could be tested in the future using molecular dynamics modelling to investigate predicted interactions, and how they are affected by molecular structure.

IGF-1 is also known to augment vasodilation through increasing NO production,(286) so our findings here may suggest, under *ex vivo* conditions, that the marked increase in IGFBP-2 in our transgenic models does not negatively or positively influence the role of IGF-1 in terms of NO production and eNOS activity.

Vasoconstriction was tested by examining an incremental dose response to phenylephrine, an established sympathomimetic agent which activates alpha adrenergic G protein-coupled receptors on vascular smooth muscle cells, eventually releasing intracellular calcium and inducing cell contraction.(287) Despite no apparent increase in nitric oxide bioavailability, marked hypocontractility in response to PE was observed in the hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive line. This was in contrast to the findings in our other two mouse models, of non-conditional whole body IGFBP-2 overexpression (hIGFBP2^{global}) and conditional endothelial specific IGFBP-2 overexpression (hIGFBP2$^{iEC\text{-}tg}$), in which no change in contractile response was observed (Figure 4-3 B, 4-4 B, 4-5 B).

As described in Chapter 3, it was found that there is no longer a significant increase in IGFBP-2 overexpression in the hIGFBP2^{global} line, so the absence of any difference in contractile response was not unexpected. The unchanged contractile response in the hIGFBP2$^{iEC\text{-}tg}$ line suggests that the hypocontractility seen in the hIGFBP2$^{iGLOBAL\text{-}tg}$ mouse line is endothelium independent, which is supported by the lack of differential contractile response with L-NMMA incubation.

Contractile response is unaffected when IGFBP-2 is overexpressed in endothelial cells alone but expression in all tissues, including VSMC, causes a hypocontractile response. This suggests that the actions of IGFBP-2 in this setting are intracellular or autocrine in nature, and are unlikely to relate to paracrine or endocrine activity. This is supported by the additional finding that in the hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice there was also a hypocontractile response to potassium chloride (Figure 4-5 E), a direct vasoconstrictor of VSMC via voltage gated calcium channel induction, without G protein-coupled receptor activation.(288)

We cannot exclude exogenous influence of IGFBP-2 on aortic function whilst *in vivo* and prior to our experiments. Ideally we would have tested this with the addition of exogenous IGFBP-2 to aortic tissue from wild type mice, however a prohibitive quantity of IGFBP-2 would have been required to perform replicative experiments in the 10 mL volume of individual organ bath chambers, and therefore direct exposure to hIGFBP-2 was not assessed. There was also consideration for further experiments with endothelium denuded aortic segments to assess if the observed contrasting contractile response persisted, however mice were not available as other experiments took priority. Finally, it would have been interesting to assess microvascular function in response to the same agents, either with an *ex vivo* approach using pressure myography, or *in vivo*

assessment with laser doppler imaging, but these were beyond the scope of this project.(199)

There was a difference in both bodyweight and aortic dimensions, as assessed by ultrasound, between the hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre negative control mice and the Cre positive mice overexpressing hIGFBP-2. The difference in aortic diameter was minimal however, at around 0.1mm (Figure 4-7 B,C). Additionally, although we did not have bodyweight data for all mice used in the vasomotor assays, close scrutiny of individual data points (not shown) revealed that the lowest bodyweight Cre positive mice had some of the greatest contractile responses to phenylephrine, and therefore differences in bodyweight and aortic diameter were not felt to be a cause for the hypocontractile response observed.

One potential explanation for the observed hypocontractility in hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice would be an IGFBP-2 induced dedifferentiation of VSMC phenotype, from contractile to synthetic, which is discussed further below. Synthetic VSMC have less capacity for contraction, and a reduction in L-Type voltage gated calcium channel expression, explaining the reduced response to both PE and KCl.(289) We demonstrated an upregulation in Akt phosphorylation at 6 weeks post tamoxifen injection (Figure 4-10 C), and an upregulation in PI3K/Akt signalling has been implicated in VSMC phenotypic switching (290) as well as inducing VSMC proliferation and migration *in vitro*,(223) providing a potential pathway for this to occur. However PI3K/Akt signalling is also associated with protecting contractile VSMC from dedifferentiation,(291) so effects may depend upon environmental conditions. ERK1/2 and NF-κB signalling are more consistently associated with VSMC phenotypic switching,(291)(292) and IGFBP-2 has been shown to upregulate signalling in both of these pathways in different cell types.(279)(293) However we did not see evidence of increased ERK1/2 phosphorylation here in our experiments (Figure 10 A-C), and did not examine NF-κB

signalling. Further investigation into these signalling pathways in VSMC may therefore be warranted.

4.4.2 Blood pressure

Given the positive findings of alterations in vasomotor function with the hIGFBP2$^{iGLOBAL\text{-}tg}$ line, with no difference seen in the other two mouse lines, it was decided to proceed with *in vivo* assessment of blood pressure and aortic distensibility in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line. The same mice were used to assess both blood pressure and aortic distensibility, to assess if there was any correlation between the two, and to minimise the number of mice used in experiments as per the 3R guidelines.

Mammalian blood pressure is influenced by a complex interplay of cardiac output, arterial elasticity and peripheral vascular resistance.(294) There are therefore multiple possible alterations that could affect blood pressure, and a change in one aspect may be compensated for by alterations in another. Elastic resistance of the aorta is a factor in the generation of blood pressure, with a reduction in elasticity leading to an increase in systolic blood pressure, and is a major pathophysiological factor in the development of hypertension.(295) Previous work by our group demonstrated both aortic hypocontractility in response to PE and reduced blood pressure measurements in mice overexpressing IGFBP-1, with increased NO bioavailability identified as the unifying cause for this.(195)(272) It was therefore felt appropriate to investigate if the *ex vivo* aortic hypocontractility seen in the hIGFBP2$^{iGLOBAL\text{-}tg}$ mice correlated with any alterations to *in vivo* blood pressure.

Baseline tail cuff blood pressure measurements were obtained from these mice prior to tamoxifen injection, after a period of acclimatisation. No significant difference in systolic

or diastolic blood pressure was observed at baseline, two weeks, or six weeks (Figure 4-6).

It is important to note that there was a significant difference in bodyweight between the hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive and Cre negative mice, as previously discussed. Differences in tail and body size may well have had an effect on blood pressure measurements obtained, both artefactual related to cuff placement and real due to murine stress related to differences in space for movement within tube holders. These potential limitations need to be taken into consideration, and there are other invasive methods of blood pressure measurement, such as blood pressure telemetry, which may be more accurate to assess small alterations in blood pressure.(293)

4.4.3 Aortic distensibility

Aortic distensibility was assessed using the Vevo 2100 high resolution, high frequency pre-clinical micro ultrasound system. This imaging system uses ECG gated kilohertz visualisation imaging to measure the alterations in abdominal aortic area during systole and diastole, and estimate distensibility.(296) As it was not possible to simultaneously measure blood pressure at the time of ultrasound imaging, only an estimation of aortic distensibility was possible, using a blood pressure standard of 120mmHg systolic and 100mmHg diastolic for each mouse. Ultrasound imaging can additionally be used to measure aortic lumen diameter in cross sectional M-Mode, and we found, corresponding to a reduced bodyweight and in agreement with prior studies examining rodent aorta size and bodyweight, that both systolic and diastolic luminal width were marginally reduced in Cre positive mice (Figure 4-7 B,C).(297)

Aortic distensibility is an important aspect of its function, allowing for the conversion of pulsatile blood flow generated by cardiac systole into continuous flow at the level of the

resistance vessels.(49) As previously described in Section 1.4.1, aortic stiffness and reduced distensibility is an early marker of both endothelial and VSMC dysfunction, and contributes to systemic hypertension and heart failure as well as aortic pathophysiological processes such as aortic aneurysm formation.(55)(79) Increased aortic stiffness is also a hallmark of inherited connective tissue disorders.(298) Vascular smooth muscle cell switching from a contractile to a synthetic phenotype would also be expected to contribute to increased arterial stiffening and reduced distensibility, through increased ECM deposition and intimal hyperplasia.(56)

We found that distensibility was in fact higher in hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice compared to Cre negative controls (Figure 4-8 B). We can only speculate on why this difference was seen, and it may be that changes in aortic composition, such as alterations to ECM deposition, contributed. However, there was a small but significant difference in aortic diameter, and therefore there will be differences in wall tension related to this. Larger aortic diameters are subject to higher wall tension, as per LaPlace's law of wall tension, and increased wall tension will reduce compliance and distensibility.(299) This may well therefore account for the differences seen in these experiments, rather than any intrinsic difference in aortic compliance due to alterations in composition or function. Differing blood pressures between groups at the time of imaging may also contribute to differences in distensibility, however when tail cuff blood pressure was assessed separately no difference was observed between groups, making it less likely to be a contributing factor.

4.4.4 Contractile VSMC gene expression and kinase signalling pathways

Contractile VSMC is characterised by its spindle-like shape and expression of specific contractile markers, notably smooth muscle myosin heavy chain and alpha smooth muscle actin, encoded by the genes MYH11 and ASMA respectively. Dedifferentiated VSMC do not have specific identifying markers, and so have classically been difficult to

define. Loss of contractile VSMC markers is therefore commonly used as an indication of de-differentiation to a synthetic phenotype.(300)

We harvested aortas from hIGFBP2$^{iGLOBAL\text{-}tg}$ mice six weeks after tamoxifen injection, corresponding with timing for vasomotor function studies, and immediately following the *in vivo* blood pressure and aortic distensibility studies. Using qRT-PCR, we found that both MYH11 and ASMA were downregulated in Cre positive mice (Figure 4-9), consistent with our hypothesis that hIGFBP-2 overexpression induces phenotypic switching in VSMC. Another possibility is that overexpression of IGFBP-2 within VSMC increases VSMC apoptosis through inhibition of IGF-I action, as previously described in plaque derived VSMC.(233) Significant aberrant VSMC apoptosis would, however, be likely to induce pro-inflammatory cytokine production, and thus would be expected to induce endothelial dysfunction, or reduce aortic distensibility, neither of which were seen.(301) There are several other specific VSMC contractile markers that could be used to identify contractile VSMC, however ASMA and MYH11 have been used in our lab previously, were identified as reliable and accurate markers, and the downregulation of both is consistent with dedifferentiation of VSMC.(300)

IGFBP-2 is involved in stimulation of multiple phosphorylation signalling pathways, notable the MAPK family and PI3K/Akt, both of which contribute to cell proliferation, migration and angiogenesis. This stimulation in activity has been seen predominantly acutely after IGFBP-2 exposure,(279)(293) and the effects of chronic over-exposure to hIGFBP-2 are not known. Additionally, ERK signalling has been linked to both increased VSMC phenotypic switching and increased VSMC contractility.(278) PI3K/Akt upregulation is linked to increased NO production through an upregulation in eNOS phosphorylation, and therefore both hypocontractility and reduced blood pressure. PI3K/Akt pathway signalling has also been linked to VSMC phenotypic switching, and so may have differential effects depending on the environment.(290)(302) Another part of

the MAPK family are the p38 MAPK proteins. These are predominantly involved in the induction of pro-inflammatory and cytokine cascades, but have also been shown to play a role in VSMC phenotypic switching, as a pathway induced by leptin derived from perivascular adipose tissue.(300)

We examined PI3K/Akt, ERK1/2 and p38 MAPK signalling in aortas harvested pre tamoxifen injection, at two weeks and at six weeks post injection. We did not see a significant difference in ERK or p38 MAPK phosphorylation at any of these time points (Figure 10 A-C). There was a significant difference in Akt phosphorylation at six weeks (Figure 10-C). This may point towards increased PI3K/Akt signalling in VSMC as a pathway to phenotypic switching, and this correlates with findings by Shen et al, who found PI3K/Akt pathway upregulation increased porcine VSMC proliferation.(223) IGFBP-2 overexpression is likely to have different effects in different cell types and environments however, as will be covered in later chapters. We originally used beta-actin as a housekeeper protein in initial experiments (not shown) but found a consistent reduction in beta actin expression in Cre positive mice, and therefore instead examined ratios of phosphorylated to unphosphorylated protein in the presented immunoblotting series. The relevance of this reduction in beta-actin, a cytoskeletal component commonly used as a housekeeper protein, is unclear, but warrants further attention in future experiments.

4.4.5 Summary

Aortas derived from mice with conditional whole body overexpression of hIGFBP-2 demonstrated a hypocontractile response to phenylephrine and potassium chloride *ex vivo*. Endothelial function and NO bioavailability were unchanged in these mice, and hypocontractility was not observed in transgenic mice with hIGFBP-2 expression limited to the vascular endothelium only, suggesting the mechanism for this hypocontractile

response was endothelium independent, and potentially related to changes in VSMC phenotype.

This interpretation was supported by our observation that contractile VSMC gene markers were down-regulated in Cre positive mice six weeks after tamoxifen injection. Furthermore, there was a significant upregulation in Akt phosphorylation at this same time point.

No significant difference in blood pressure between groups was observed, despite significantly greater aortic distensibility in Cre positive mice. Although aortic distensibility affects blood pressure, this may have been compensated for by other factors, for example alterations in cardiac output or systemic vascular resistance. The most likely explanation for the greater distensibility observed in Cre positive mice is reduced wall tension related to a reduced aortic lumen diameter. The effects of IGFBP-2 overexpression on cardiac stroke volume and microvasculature were not assessed, but could be a focus for future investigation.

Chapter 5 IGFBP-2 action in vascular remodelling and repair

5.1 Background

Alterations in endothelial cells, vascular smooth muscle cells and the extracellular matrix all contribute to vascular disease and the ability of blood vessels to remodel and repair. Little is known about how these processes are influenced by IGFBP-2, but extrapolation from research in other settings suggest that IGFBP-2 could potentially have both detrimental and favourable effects. This chapter and the following (Chapter 6) focus on the effects of IGFBP-2 on neointima formation, re-endothelialization and angiogenesis.

5.1.1 Neointima formation

Neointimal hyperplasia relates to the proliferation and migration of VSMC and increased extracellular matrix deposition within the tunica intima. It predominantly occurs as a response to vascular injury, whether that be chronic injury, occurring as part of atherosclerosis development, or acute injury, such as the delivery of a coronary stent.(63) A key driver for this action is VSMC dedifferentiation to a synthetic phenotype which then proliferates, migrates and contributes to excessive ECM deposition.(64) A detailed description of neointima formation can be found in Section 1.4.3.

IGFBP-2 is expressed in healthy human VSMC at very low levels and has not been considered to have clear actions physiologically.(221) However, gene expression of IGFBP2 in VSMC derived from atherosclerotic plaque and in stent restenosis samples has been found to be upregulated compared to healthy samples,(232) suggesting a possible role of IGFBP-2 in propagating VSMC proliferation and migration. Additionally, IGFBP-2 stimulation increases proliferation of porcine VSMCs *in vitro* (223) and IGFBP-

2 expression was increased in VSMC exposed to low density lipoproteins, key mediators of atherosclerosis development.(234) As yet, there has not been any research looking at direct actions of IGFBP-2 on the development of neointima *in vivo*, and so this was one of the aims of this chapter.

Many of the actions of IGFBP-2 on cellular proliferation and migration are thought to be related to binding of IGFBP-2 with components of extracellular matrix via its heparin binding domain.(228) However, effects of IGFBP-2 on ECM deposition and structure are unknown. Marked upregulation of IGFBP-2 gene expression has been described in a murine model of Marfan Syndrome, but a causative role of IGFBP-2, or the pathway through which IGFBP-2 may contribute to abnormal ECM remodelling, has not been elucidated.(235)

5.1.2 Vascular repair

Re-endothelialization is the process of endothelial recovery after vascular injury, which limits neointimal infiltration and re-establishes vascular quiescence.(82) Endothelial recovery is thought to occur through two pathways: resident endothelial cell recruitment and proliferation, as well as endothelial progenitor cell mobilisation and vascular incorporation.(86) The process of re-endothelialization and possible physiological mechanisms are described in-depth in Section 1.5.1.

There is emerging evidence that IGFBP-2 overexpression in endothelial cells may facilitate vascular endothelial repair. Overexpression of IGFBP-2 in HUVECs led to increased adhesion of EPCs into a HUVEC monolayer and exogenous stimulation with IGFBP-2 promoted incorporation of EPCs into HUVEC tubules, giving IGFBP-2 potential as a therapeutic agent to enhance vascular repair.(236) IGFBP-2 has also been found to support haematopoietic stem cell survival and cell cycling of HSCs in the bone

marrow,(237) and therefore potentially supporting EPC contribution to vascular repair. There has not as yet been any published research examining the potential for IGFBP-2 to enhance vascular repair *in vivo*, or any examination of the effects of IGFBP-2 on resident endothelial cell recruitment in the re-endothelialization process.

5.1.3 Experimental overview

This chapter describes a series of complementary experiments designed to explore whether IGFBP-2 overexpression modulates vascular response to injury, specifically examining the action of IGFBP-2 overexpression upon neointima formation, VSMC proliferation and endothelial repair. Neointima formation was studied in response to femoral artery wire injury and carotid artery cuff placement in hIGFBP2$^{iGLOBAL\text{-}tg}$ mice. Endothelial regeneration following femoral artery wire injury was studied in hIGFBP2$^{iGLOBAL\text{-}tg}$ mice and hIGFBP2^{global} mice. Finally, the proliferative effects of exogenous hIGFBP-2 administration to VSMC were examined using an *in vitro* cell counting assay, with human VSMC derived from both saphenous vein and internal mammary artery.

5.1.4 Aims

The aim of these experiments was to ascertain the effects of increasing IGFBP-2 on vascular remodelling and vascular repair.

5.1.5 Objectives

1. Investigate the role of IGFBP-2 overexpression in the development of vascular neointima
2. Investigate the role of IGFBP-2 overexpression in vascular repair, as assessed by re-endothelialization following wire injury

3. Investigate if direct stimulation with IGFBP-2 has a proliferative effect on human VSMC

5.2 Methods

5.2.1 Neointima formation and re-endothelialization

Femoral wire injury and carotid cuff placement were performed as described in section 2.2.

5.2.1.1 Paraffin embedding and sectioning

Four weeks post procedure, mice were placed under isoflurane anaesthetic using a Vet-Tech delivery system. Ventricular puncture was performed followed by injection of 4% paraformaldehyde solution into the circulation to allow for tissue fixation followed by confirmation of permanent cessation of circulation by removal of the heart. The aortic arch and carotids were removed and placed in processing cassettes (Fisherbrand) to maintain their orientation and lumen patency, and stored in 4% paraformaldehyde. The lower abdominal aorta together with the femoral arteries were removed and stored in the same manner. Following this, samples were embedded in paraffin by Dr Natallia Makava and Dr Anna Skromna, and stored at room temperature.

Sample sectioning was performed using a rotary microtome (Leica Biosystems). Paraffin embedded samples were cut into trapezoid shapes to aid identification of injured artery versus sham. They were kept on ice to ensure clean sectioning. 10 µm sections were cut until near the point of injury, then 5 µm sections were cut in groups of ten, and placed in a water bath at 40°Celsius until the paraffin became translucent. Samples were then transferred to coated slides (Sigma-Aldrich) for storage.

5.2.1.2 Staining and analysis

Slides were first heated at 80 °Celsius for thirty minutes to melt the paraffin. Xylene was then used in three serial washes to deparaffinise the slides, followed by three serial 100% ethanol washes. Samples at this point and after each subsequent step were washed with

distilled water, with excess water carefully removed each time. Samples were then stained with 0.5% potassium permanganate for ten minutes, before 2% Oxalic acid was used to remove excess stain. Samples were then placed in Miller's Elastin Stain for 2.5 hours. Following this, samples were washed three times in 100% ethanol to remove excess stain, before being placed in Van Gieson stain for 45 minutes. Three serial Xylene and 100% ethanol washes were again performed. Samples were then mounted on glass coverslips (VWR) using DPX mounting medium and stored for imaging.

Imaging was performed using an Olympus BX41 microscope and Image Pro Plus software. Comparable points of neointima formation were identified using anatomical landmarks for each sample. For carotid cuff experiments, four images over a 100 µm section were analysed, as for some segments distortion of neointima by clot formation was evident soon after the point of initial neointima formation. For femoral wire injury experiments, four images were taken at 100 µm intervals from the point of consistent neointima formation. Image Pro Plus was used to manually measure neointima and medial volume, data were collated in Microsoft Excel, and analysis was performed using Graphpad Prism software, with unpaired Student's t-test used to compare neointima volume between groups.

5.2.2 Re-endothelialization

Femoral wire injury was carried out using hIGFBP2$^{iGLOBAL\text{-}tg}$ mice as described in section 2.2. Four weeks post femoral wire injury, mice were anaesthetised with isoflurane, and Evans blue dye (Sigma-Aldrich, 50-75 µL of a 5% solution) was injected via the inferior vena cava under direct visualisation. Evans blue dye binds to exposed albumin in the tunica media, therefore highlighting areas of artery which are not endothelialized.(303)

Immediately following injection with Evans blue dye, mice were injected with PBS via intracardiac injection followed by 4% PFA solution, to fix tissue for further cleaning and imaging. Femoral arteries were removed as described above and imaged *en-face*.

Analysis was performed using ImageJ. The femoral artery was measured from the iliac bifurcation to the site of ligation. Analysis began 5 millimetres above the bifurcation, and the next 5 millimetres of each section of femoral artery was analysed for each sample, ensuring consistent measurement that did not depend upon the length of aorta harvested. Data were collated in Microsoft Excel, and Graphpad Prism software was used for data analysis, with unpaired Student's t-test used to compare measurements.

5.2.3 VSMC cell counting assay

Human primary vascular smooth muscle cells of saphenous vein and internal mammary artery origin were cultured as previously described in section 2.2.4. Once confluent, cells were trypsinised, counted using a haemocytometer and seeded onto a 24 well plate in DMEM with 10% FBS at 10,000 cells per well, for three time points (Day 0, 4, 7) and in triplicate. The next day wells were washed with PBS and serum free media was added for 72 hours.

After this serum starving period, the media was removed. DMEM with 10% FBS was added to three wells to act as a positive control; DMEM with 0.4% FBS was added to control wells for days four and seven, and DMEM with 0.4% FBS plus 15 nM recombinant hIGFBP-2 was added to treatment wells. Three day 0 wells were trypsinised and cells were counted using a haemocytometer to provide a baseline reading. This was repeated on days four and seven, with positive control wells also counted on day seven.

Data were collated in Microsoft Excel, and Graphpad Prism software was used for data analysis, with 2-way ANOVA used to analyse the difference in cell number between groups.

5.2.4 IGFBP-2 expression after vascular injury

Femoral wire injury was performed as detailed in section 5.2.1.2. Both injured and uninjured femoral arteries were harvested at time points of zero hours, 24 hours and 72 hours. Three mice underwent injury for each time point, and two uninjured mice were also harvested at each time point. These samples were assessed by RT-qPCR for levels of murine IGFBP-2, and GAPDH was used as a housekeeper gene. Data were collected using Microsoft Excel, analysed using Graphpad Prism, and 2-way ANOVA was used to assess for change in IGFBP-2 expression at different time periods.

5.3 Results

5.3.1 Neointima formation

Injured carotid arteries all exhibited significant narrowing proximal to cuff placement, as seen in Figure 5-1. There was no significant difference in neointima area between Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice and their Cre negative controls (Figure 5-2 A-B). There was also no significant difference seen in interlaminal space, or lumen area of carotid arteries undergoing sham surgery (Figure 5-2 C-E).

Similarly, no significant difference in neointima between Cre positive and Cre negative hIGFBP2$^{iGLOBAL\text{-}tg}$ mice was observed in femoral arteries after wire injury (Figure 5-4 A-B). There was no difference in interlaminal space (Figure 5-4 C-D), but a significant difference in lumen area of femoral arteries undergoing sham surgery was observed (Figure 5-4 E).

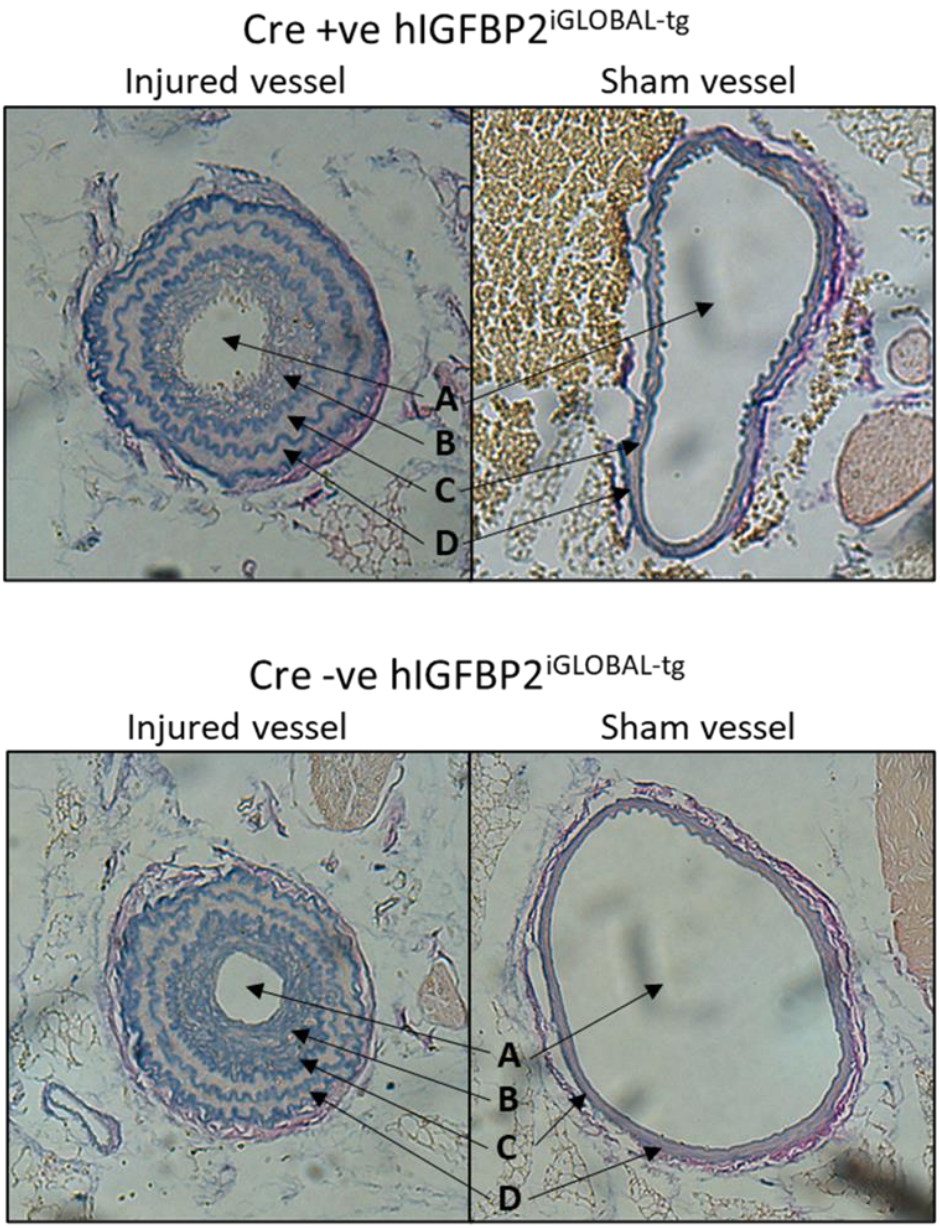


Figure 5-1. Representative images of carotid cuff injury in hIGFBP2$^{iGLOBAL-tg}$ mice. Sections were stained with Miller and Van-Gieson stains as described in section 5.2.1.4 and imaged at 100x magnification. Sections were taken above the aortic arch and below cuff placement at the carotid bifurcation, from the point of consistent neointima formation and before the point of thrombus formation. A: lumen; B: neointima, C: internal elastic lamina; D: external elastic lamina

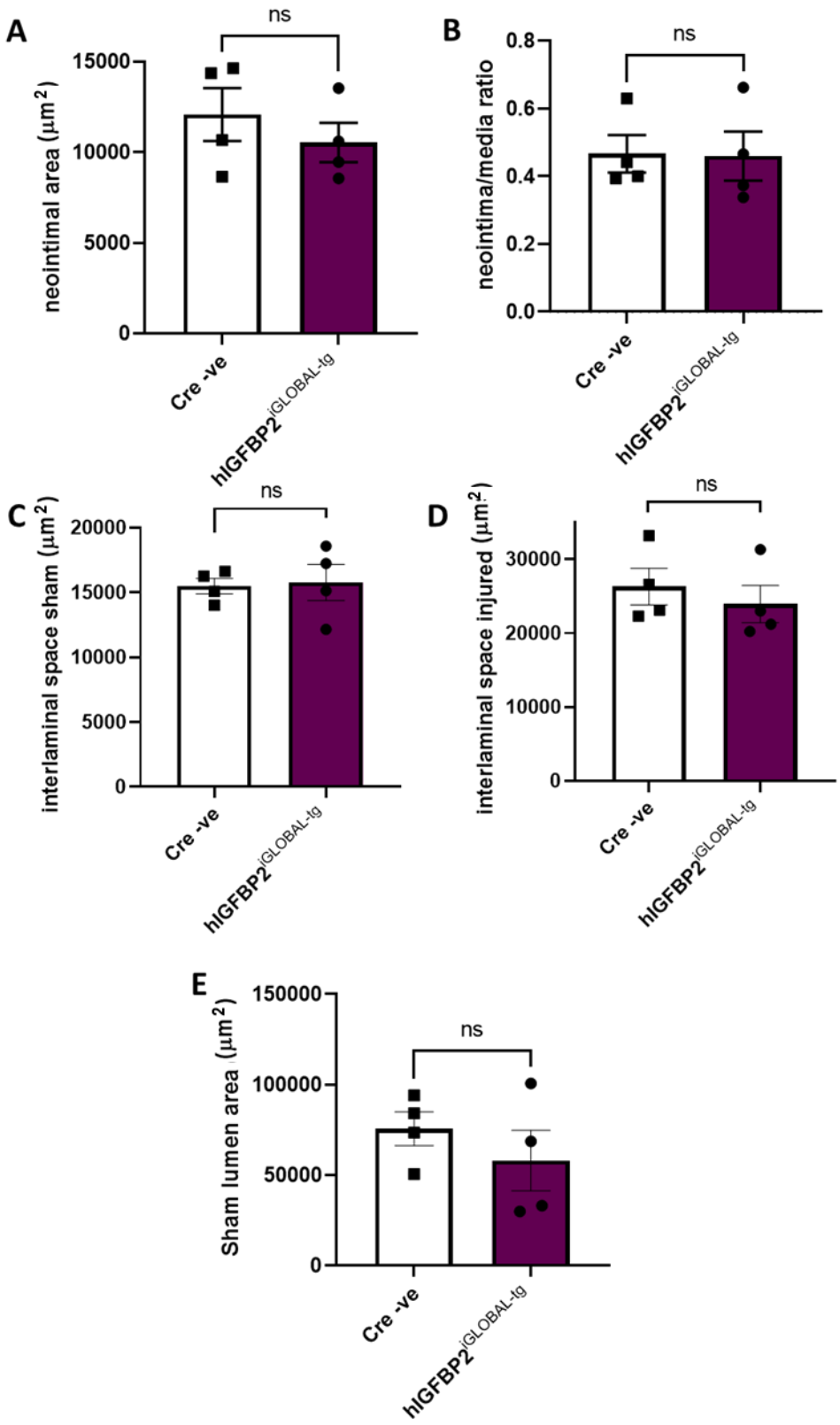


Figure 5-2. Carotid cuff placement: neointimal area; neointima/media ratio; interlaminal space; sham lumen area. No significant difference was seen in neointima formation between Cre +ve hIGFBP2$^{iGLOBAL\text{-}tg}$ and Cre -ve control mice (n=4,4)

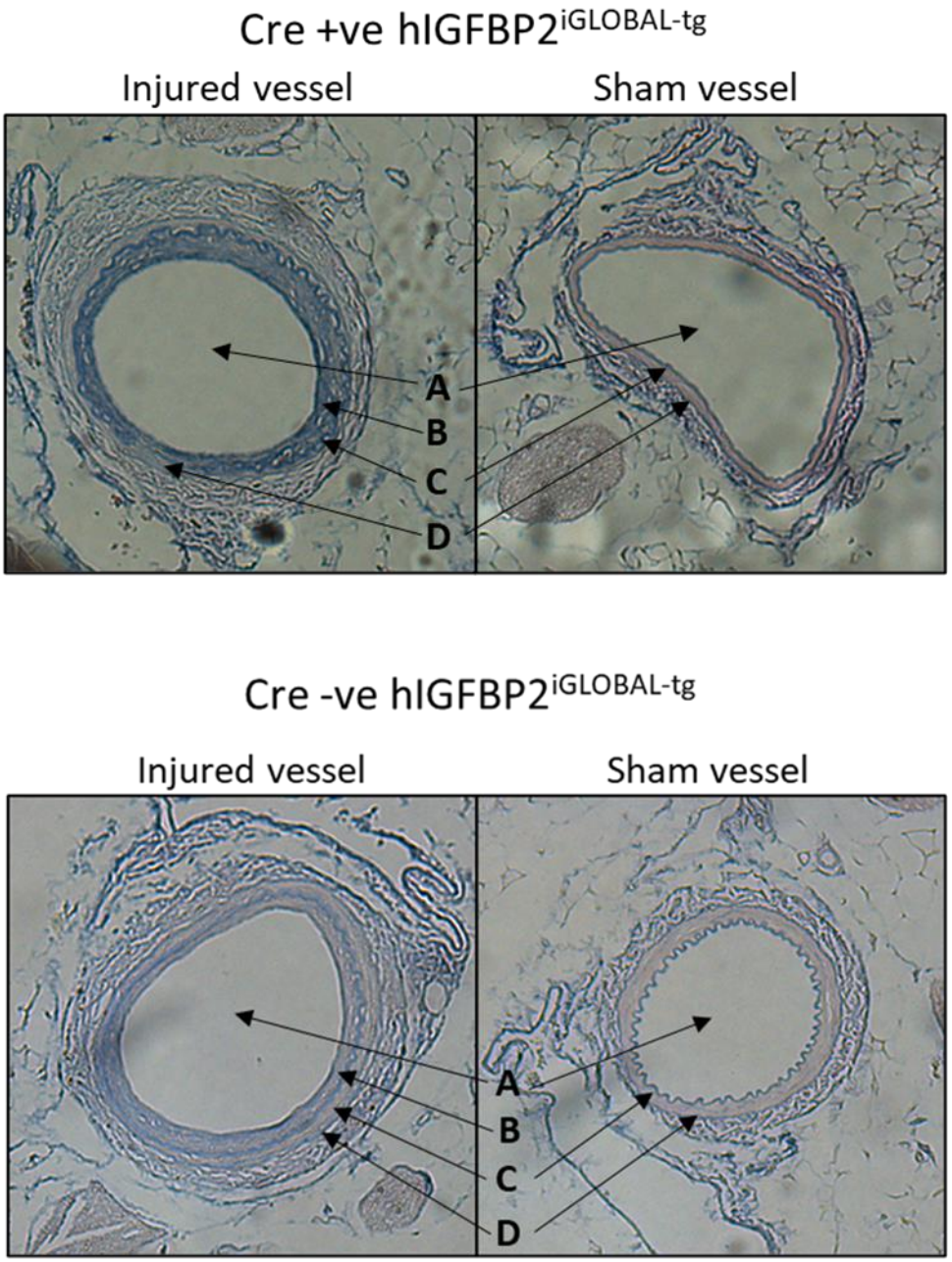


Figure 5-3. Representative images for femoral artery wire injury neointima formation in hIGFBP2$^{iGLOBAL-tg}$ mice. Sections were stained with Miller and Van-Gieson stains as described in section 5.2.1.4 and imaged at 100x magnification. Sections were taken of the CFA, with analysis of 4 sections at 100µM intervals from the point of consistent neointima formation. A: lumen; B: neointima, C: internal elastic lamina; D: external elastic lamina

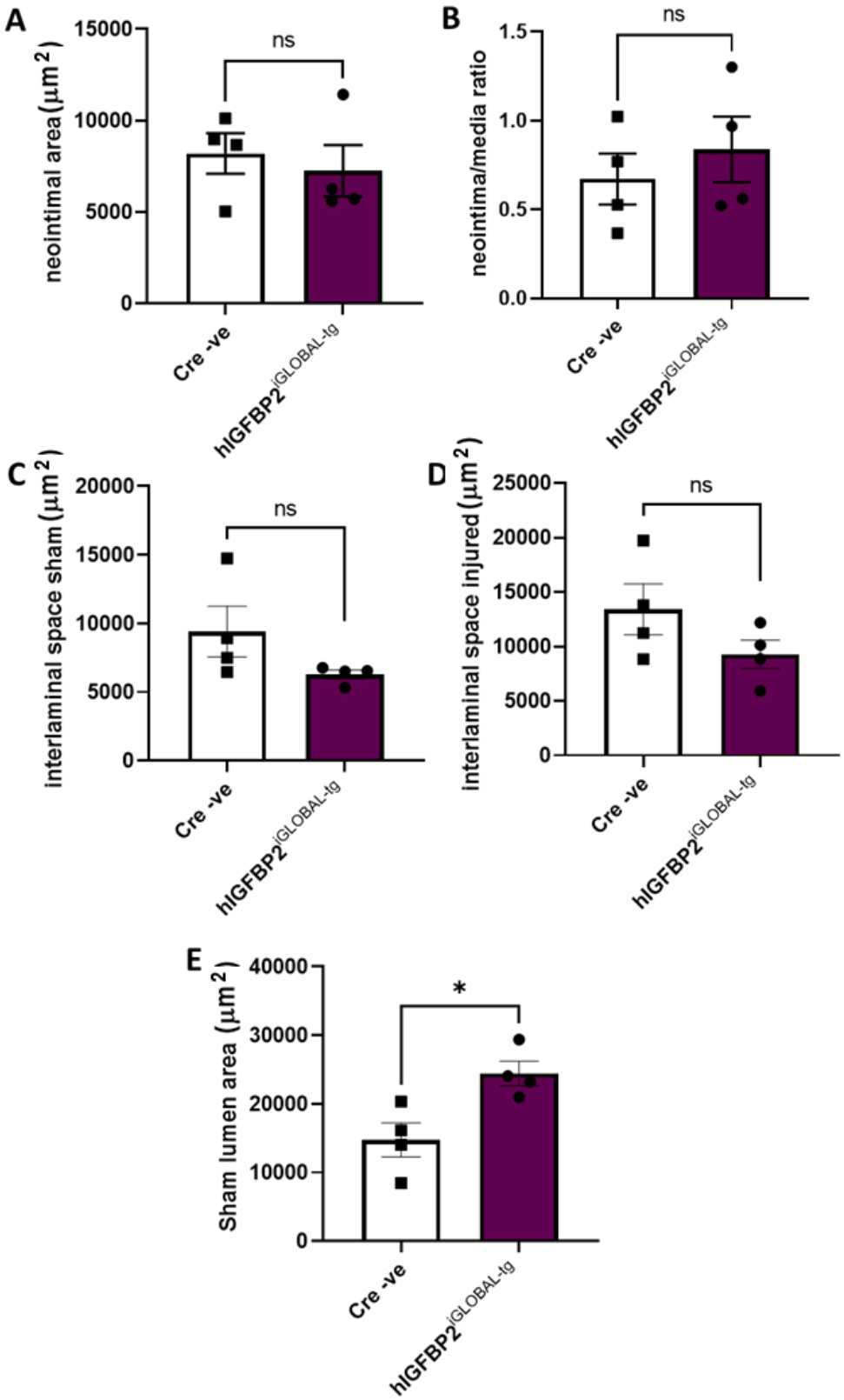


Figure 5-4. Femoral wire injury: neointimal area; neointima/media ratio; interlaminal space; sham lumen area. No significant difference was seen in neointima formation between Cre +ve hIGFBP2$^{iGLOBAL\text{-}tg}$ and Cre -ve control mice (n=4,4)

5.3.2 VSMC cell counting assay

Cell counting assays were performed as described in Section 5.2.3 on human saphenous vein (SV) VSMC and internal mammary artery (IMA) VSMC. Addition of recombinant hIGFBP-2 had no effect on SV VSMC cell number. There was a significant increase in IMA VSMC cell number when stimulated with hIGFBP-2 (Figure 5-5).

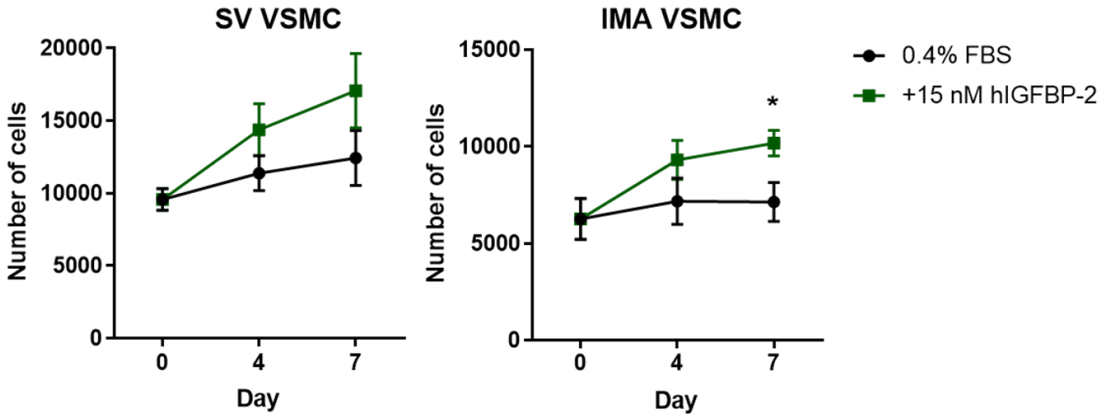


Figure 5-5. Saphenous vein and internal mammary artery VSMC cell number. 15 nM hIGFBP-2 was added to treatment cells over a seven day period. There was no significant difference in cell count between treated and untreated SV VSMC (n=7,7). There was a significant increase in IMA VSMC cell count when stimulated with hIGFBP-2 (p=0.0123) (n=5,5).

5.3.3 Re-endothelialization

Femoral wire injury was performed in hIGFBP2$^{iGLOBAL\text{-}tg}$ mice and Cre negative controls as set out in Section 5.2.2, and the arteries were harvested four weeks post procedure. Previous unpublished work by our group used the same procedure to examine re-endothelialization in the hIGFBP2^{global} mouse strain. Analysis was performed as described in section 6.2.2. Figure 5-6 A shows representative images of WT and hIGFBP2^{global} femoral arteries, with Evans Blue Dye demonstrating areas absent of endothelium. There was significantly less re-endothelialization seen with overexpression of hIGFBP-2 (Figure 5-6 B). Figure 5-6 C shows representative images of Cre -ve and hIGFBP2$^{iGLOBAL\text{-}tg}$ femoral arteries, with Evans Blue Dye demonstrating areas absent of

endothelium. Again, there was significantly less re-endothelialization seen with overexpression of hIGFBP-2 (Figure 5-6 D).

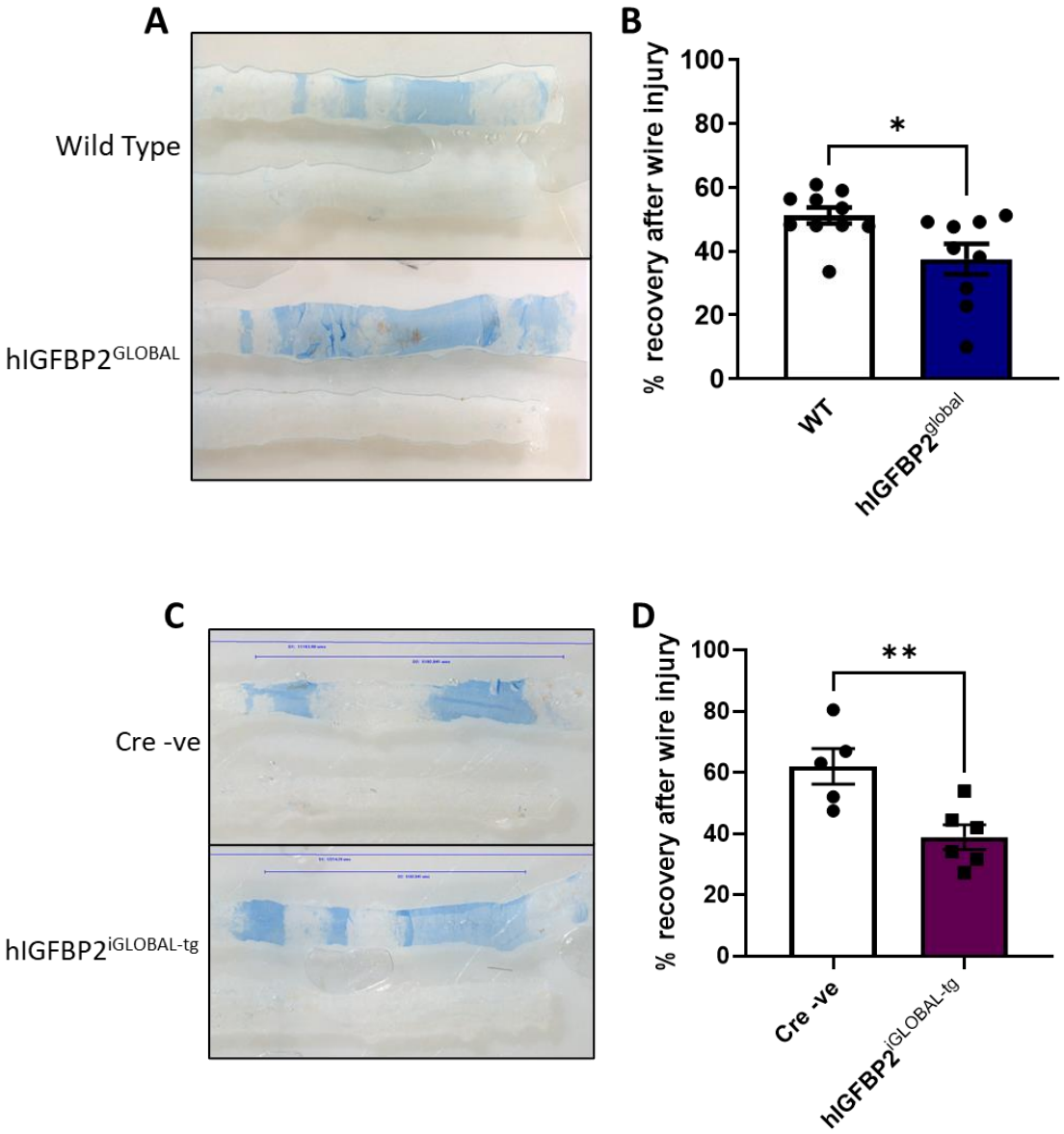


Figure 5-6. Re-endothelialization following femoral wire injury. A,B: representative images and quantitative analysis of WT and hIGFBP2^{global} femoral arteries four weeks after wire injury, with Evans Blue Dye demonstrating areas absent of endothelium. There was significantly less re-endothelialization seen with overexpression of hIGFBP-2 (p=0.02)(n=10,9). **C,D:** representative images and quantitative analysis of Cre -ve and hIGFBP2$^{iGLOBAL-tg}$ femoral arteries four weeks after wire injury, with Evans Blue Dye demonstrating areas absent of endothelium. There was significantly less re-endothelialization seen with overexpression of hIGFBP-2 (p=0.008)(n=5,6).

5.3.4 IGFBP2 gene expression after vascular injury

Femoral wire injury was performed in wild type mice to investigate the effects of injury on endogenous gene expression of IGFBP2. Femoral arteries were harvested at time points of 24 hours, 72 hours and seven days after injury. Three mice underwent injury for each time point. These samples were assessed by RT-PCR for levels of murine IGFBP2, and GAPDH was used as a housekeeper gene. No difference in IGFBP2 expression was identified in injured versus non-injured femoral artery at any of the assessed time points (Figure 5-7).

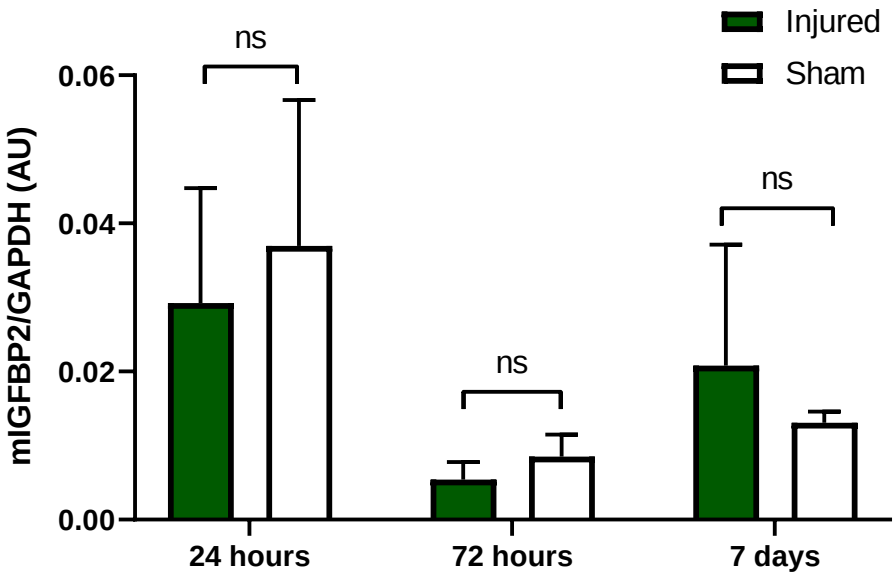


Figure 5-7. Murine IGFBP-2 expression following arterial injury. No significant difference in IGFBP2 expression was identified in injured versus non-injured femoral arteries at each assessed time point, and no significant difference in IGFBP2 expression was seen between injured femoral arteries at different time points (n=3,3).

5.4 Discussion

5.4.1 Neointima formation and VSMC cell number

Neointima formation was assessed in two different experimental models, carotid cuff placement and femoral wire injury, with Miller-Van Gieson staining of paraffin embedded samples used to quantify neointima area (Figure 5-1 and 5-3). Both of these models were used in the same mice, to minimise the number required for experiments, as per the 3R guidelines.

We did not find any difference in neointima quantity between Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice and their Cre negative controls, in either carotid cuff neointima formation or neointima following femoral wire injury (Figure 5-2 and 5-4). This was assessed in two different ways, as absolute area of neointima, and as a ratio compared to media area, with no significant difference seen for either. Additionally, there was no significant difference between groups in the tunica media area of injured arteries, which demonstrated a fixed vasoconstricted phenotype, particularly in the carotid cuff experiments (Figure 5-2 D and 5-4 D). This is a feature particularly associated with this model of neointima formation, and one thought to relate to increased angiotensin-1 receptor expression within VSMC and subsequent Rho-kinase activation induced by the adventitial injury caused by cuff placement.(304)(305)

Further work is required to assess if there are differences in the overall composition of neointima, prove what the contributions of different cell types are, and if these are affected by differential IGFBP-2 expression. *In vivo* overexpression of IGFBP-2 may actually increases VSMC apoptosis, rather than enhancing VSMC proliferation, This has also been postulated in a previous study by Patel *et al*, who found that several IGFBPs including IGFBP-2 were upregulated in atherosclerotic plaque, and may contribute to plaque instability due to increased VSMC apoptosis, a feature hypothesised to be the

result of impaired IGF-I survival signalling related in part to increased IGFBP expression.(233)

There was no significant difference between genotypes in luminal area of sham-operated carotid arteries, but there was a demonstrated significant difference between genotypes in femoral artery luminal area from sham-operated limbs, although this demonstrated hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice actually had a larger luminal area than Cre negative controls (Figure 5-4 E). This was the opposite of what may have been expected, given the reduction in bodyweight and aorta size in Cre positive mice that I have previously described, and so is likely a spurious result related to small sample size and the inaccuracy of histology to assess vessel lumen size. These findings do however demonstrate that luminal size of both carotid and femoral arteries in Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice was not significantly less than Cre negative controls, and therefore should not have had a mechanical impact on either neointima or re-endothelialization experiments.

Conversely to these findings, human internal mammary artery VSMCs treated with 15 nM hIGFBP-2 *in vitro* demonstrated increased cell number over a seven day period (Figure 5-5). Addition of recombinant hIGFBP-2 had no effect on SV VSMC cell number. This may relate to the higher proportion of synthetic VSMC in cells derived from human vein samples, although this is difficult to assess given the considerable plasticity demonstrated by VSMC grown in culture. Given this was a simple cell counting assay, it may be that exposure to hIGFBP-2 conferred protection from apoptosis and increased cell survival, rather than purely stimulating cellular proliferation. This would be at odds with our *in vivo* neointima findings and those hypothesised by Patel *et al* regarding an increase in VSMC apoptosis through inhibition of IGF-I action,(233) and so future investigation employing cell survival assays with both exogenous stimulation and endogenous overexpression of IGFBP-2 would be of interest.

These experiments had several limitations. The primary limitation in this chapter is the small sample size for each experiment, which predominantly relates to restrictions and delays caused by the Covid-19 pandemic. Our neointima experiments stained to examine elastin content, but did not identify VSMC specifically, or other components of the ECM. We had therefore also intended to perform immunohistochemistry assays after carotid cuff and femoral arterial wire injury to examine the content and position of VSMC within neointimal layers and the tunica media. We had also planned to perform EdU proliferation assays using IMA VSMC lentivirally transduced to over-express hIGFBP-2, to more accurately mirror our *in vivo* experiments, and more accurately ascertain whether exposure to hIGFBP-2 promotes cellular proliferation. Unfortunately it was not possible to perform these experiments due to severely restricted lab time related to the Covid-19 pandemic. There are plans to perform the immunohistochemistry staining in the near future in collaboration with other groups within our lab, to complement our current findings.

5.4.2 Re-endothelialization and IGFBP-2 expression after arterial injury

Re-endothelialization was assessed by femoral artery wire injury, as described in section 5.2.2. Previous unpublished work by our group had demonstrated reduced re-endothelialization in hIGFBP2^{global} mice, and this was confirmed with reanalysis of the data obtained from these experiments (Figure 5-6 B). Although IGFBP-2 levels were not significantly increased in hIGFBP2^{global} mice bred for this current project, these arterial injury experiments were performed several years ago in mice from a previous cohort in which IGFBP-2 levels were increased, prior to the possible resultant genetic drift in this mouse line.

We therefore repeated these experiments in the hIGFBP2$^{iGLOBAL\text{-}tg}$ line, and again found a significant reduction in endothelial regeneration in mice with global overexpression of IGFBP-2 (Figure 5-6 D), consistent with our previous findings. Others have shown that HUVECs overexpressing IGFBP-2 *in vitro* demonstrated increased adhesion of EPCs to an endothelial monolayer, and IGFBP-2 stimulation increased uptake of EPCs in HUVEC microtubules, both of which would lead to expectations for IGFBP-2 to increase re-endothelialization *in vivo*.(236) That these findings contrast with ours emphasises the possible differences in IGFBP-2 action between *in vivo* and *in vitro* environments, as well as potential differences seen in endothelial cells of different species origin. We also found no evidence of altered endothelial function, or differences in NO bioavailability, when the endothelium was intact in our aortic vasomotor studies. It is therefore not yet clear why reduced endothelial regeneration was seen following injury, and further investigation is required.

Endothelial regeneration is an important beneficial response to arterial injury, as it is thought to inhibit neointimal hyperplasia. Conversely, endothelial disruption and reduced re-endothelialization is associated with increased neointimal formation, demonstrating an reciprocal, inverse relationship between the two.(306)(307) We would therefore expect to see increased neointima formation following wire injury in the hIGFBP2$^{iGLOBAL\text{-}tg}$ Cre positive mice, given the witnessed reduction in endothelial generation with hIGFBP-2 overexpression. We did not see any evidence of this in our neointima studies however, with no difference in neointimal quantity between groups (Figure 5-2 A-B and 5-4 A-B). One potential explanation for this is the possibility of IGF inhibition by IGFBP-2 leading to increased VSMC apoptosis and reduced VSMC proliferation, emphasising the importance of our planned immunohistochemistry assays to accurately delineate the individual neointima components. Future experiments could also be performed to measure free IGF levels in these mice, to determine if increased IGF binding is contributing to IGF inhibition.

Several questions remain regarding these findings. It is not clear which components of neointima are affected by hIGFBP-2, and further experiments should be performed to ascertain if collagen and VSMC content of neointima is affected. Additionally, it is not clear if the variable effects of IGFBP-2 seen relate to cellular environment, experiment design, or degree of IGFBP-2 exposure. Ideally, further experiments should be performed to expand on these findings.

5.4.3 Summary

In this chapter, we have ascertained that hIGFBP-2 stimulation modulates IMA VSMC number *in vitro*, but that overexpression of hIGFBP-2 does not lead to altered neointima area *in vivo* after arterial injury. Further investigation is required to ascertain whether IGFBP-2 has qualitative effects on neointima formation and to confirm whether the composition and maturation of neointima is altered by IGFBP-2. Surprisingly, overexpression of hIGFBP-2 in transgenic murine models reduces endothelial regeneration after arterial injury, and the reasons for this observation require further scrutiny. Arterial expression of murine IGFBP-2 is not altered after wire-injury, suggesting that IGFBP-2 is unlikely to play a major role in pathological arterial remodelling in mice.

Chapter 6 IGFBP-2 actions on vascular regeneration

6.1 Background

Vascular regeneration describes the formation of a new vascular network to provide blood supply to tissue that has been subject to ischaemic insult, through occlusion or insufficiency of the pre-existing vasculature.(82) The formation of new blood vessels from pre-existing vasculature is termed ‘angiogenesis’, and the process by which this occurs is described in detail in Section 1.6.1. Angiogenesis can occur as a physiological, reparative response to ischaemic insult, but can also have pathophysiological consequences, usually related to malignancy and accelerated tumour growth, or when vascular regeneration in response to ischaemia goes awry, such as that seen in ocular proliferative retinopathy.(308)

Frequently, physiological vascular regeneration fails to adequately restore circulation to perfused tissue, with ongoing ischaemia as a result, especially when surgical or percutaneous methods of revascularisation are not possible.(103) Augmented vascular regeneration has therefore been a source of research interest for some time, with multiple positive pre-clinical studies but with limited translational success in larger clinical trials thus far.(309)

The insulin-like growth factors and their binding proteins have been implicated in angiogenic processes in a wide array of cellular environments and through multiple signalling pathways.(100) IGFBP-2 in particular has been implicated in angiogenesis through several different mechanisms, both IGF-dependent and independent, and is discussed in detail in Section 1.10.5. These findings have predominantly been in the context of tumour angiogenesis, although recent work has shown IGFBP-2 has

proangiogenic effects *in vivo* in zebrafish embryos, as well as *in vitro* HUVEC angiogenesis assays.(310) In addition, unpublished work by our group has shown that exogeneous stimulation of HUVECs with IGFBP-2 can augment sprouting angiogenesis, primarily through induction of ERK 1/2 phosphorylation, an established important signalling pathway for endothelial cell proliferation and migration during angiogenesis.(311)

Using the hIGFBP2$^{iEC\text{-}TG}$ mouse line, our laboratory has demonstrated in as yet unpublished data that endothelial overexpression of hIGFBP-2 increases neonatal retinal angiogenesis and enhances vascular recovery after hind limb ischaemia. These data provide evidence that IGFBP-2 can enhance angiogenesis *in vivo*, and when endogenously overexpressed, but does not answer if these proangiogenic effects can be targeted to areas of ischaemic insult.

Endothelial colony forming cells have the ability to participate in re-endothelialization and to form neovasculature, demonstrating strong potential for use in therapeutic angiogenesis, and various mechanisms to enhance the proangiogenic actions of ECFCs have been explored.(312) Our discovery that IGFBP-2 exerts direct proangiogenic effects in endothelial cells raises the possibility that IGFBP-2 may also augment the angiogenic actions of ECFCs. Should it be confirmed that ECFCs could be transduced to overexpress IGFBP-2, and that this overexpression augmented their ability to stimulate angiogenesis, then it may be that these augmented ECFCs could be used as a cell-based therapy to stimulate angiogenesis in ischaemic tissue that cannot otherwise be revascularized.

6.1.1 Experimental overview

This chapter describes a series of complementary experiments designed to explore the effects of IGFBP-2 within endothelial cells, specifically examining the action of both IGFBP-2 overexpression and exogeneous stimulation upon endothelial sprouting angiogenesis and wound closure. Endothelial colony forming cells were derived from human subjects, human umbilical vein endothelial cells were from pooled donors from a commercial supplier, and pulmonary endothelial cells were derived from the hIGFBP2^{iEC-tg} mouse line. These cells were used in a series of cytodex bead sprouting angiogenesis assays, with ECFCs and HUVECs transduced using a lentivirus method to overexpress hIGFBP-2. Additionally, ECFCs were stimulated with exogenous hIGFBP-2, and the effects of this on both sprouting angiogenesis and wound closure were examined. Finally, immunoblotting for kinase signalling pathways was performed in transduced ECFCs and HUVECs, to determine if upregulation of phosphorylation was responsible for augmentation of sprouting angiogenesis.

6.1.2 Aim

The overall aim of this set of experiments was to ascertain the effects of both overexpression of IGFBP-2 and stimulation with exogenous IGFBP-2 on the angiogenic potential of endothelial cells, and the pathways through which augmented angiogenesis may occur.

6.1.3 Objectives

1. Investigate the effects of hIGFBP-2 stimulation on wound closure and angiogenesis in endothelial colony forming cells.
2. Investigate if endothelial cells could be transduced to overexpress hIGFBP-2.
3. If endothelial cells could overexpress IGFBP-2, examine the effects of this on their potential for angiogenesis, and the pathways by which this occurs.

6.2 Methods

6.2.1 Lentiviral transduction

Lentiviral transduction has been established as an effective and reproducible method of gene delivery into cells, allowing for continued transgene expression. As well as providing reliable continued expression of a transgene of interest, it has also been used in gene therapy and immunotherapy, and so its translational impact has already been established.(313)

Dr Alex Bruns kindly created the human IGFBP2 lentivirus particles used for doxycycline-dependent expression of this gene of interest in all experiments. To achieve this, hIGFBP-2 was cloned into the plasmid pLVX Tet-One (TaKaRa) resulting in the plasmid pLVX-hIGFBP2. In this plasmid, expression of the gene of interest is under the control of the inducible promoter P_{TRE3GS}, whereas expression of a transactivator protein (Tet-One 3G) is driven by the constitutive promoter human phosphoglycerate kinase (P_{hPGK}). Upon addition of doxycycline, the transactivator protein undergoes a conformational change allowing it to bind to the TRE3GS promoter thus initiating expression of hIGFBP2.

For production of lentivirus particles, the Lenti-X Packaging Single Shot system (TaKaRa) comprising VSV-G packaging plasmids and transfection reagent was used. Briefly, the plasmid pLVX-hIGFBP2 was mixed with the packaging system as per manufacturer's instructions and added to HEK-LentiX cells (TaKaRa) (Figure 6-1). Cell culture supernatant was harvested after 48 hours, replaced with fresh medium, harvested again 72 hours after transfection, concentrated using the Lenti-X Concentrator (TaKaRa), and subsequently quantified by lentivirus-associated p24 ELISA (QuickTiter Lentivirus Titer Kit; Cell Biolabs).

Transduction of cells was achieved by the addition of hIGFBP-2 lentiviral particles to cell media at a Multiplicity of Infection (MOI) ratio of 10. Endothelial Cell Growth Medium MV 2 (ECGM-2) (Promocell C-22022) was used and cells were incubated for eighteen hours, before the lentiviral particles were removed and media containing two µg/mL doxycycline was added. Media was changed to media containing fresh doxycycline after 48 hours. Experiments were performed 48-72 hours after doxycycline induction to allow for maximal transgene expression. All cells used in experiments were transduced, but controls had H_2O added instead of doxycycline and so hIGFBP2 gene induction did not occur in these cells.

Successful transduction was assessed using immunoblotting. Data were collated in Microsoft Excel and analysed using GraphPad Prism. Statistical analysis was performed using paired Student's T-test.

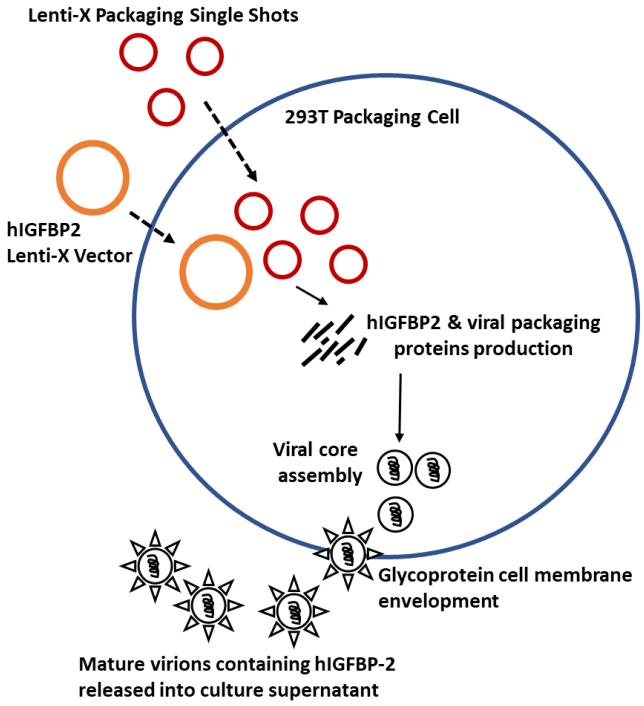


Figure 6-1. Production of hIGFBP2 lentivirus using Lenti-X transduction system. The hIGFBP2 Lenti-X vector and Lenti-X Packaging Single Shots were introduced into HEK 293T LentiX cells with resultant RNA transcription of viral packaging proteins and hIGFBP-2; viral core assembly and budding of mature hIGFBP-2 containing virions into the culture supernatant.

6.2.2 Cytodex bead sprouting angiogenesis assay

ECFCs were cultured as described in Section 2.2.1. The cytodex bead assay was adapted from a technique first described by Nakatsu *et al*.(314) Cytodex beads (Sigma-Aldrich) were used at a concentration of 400 cells per each bead. Beads were suspended in one mL EGM-2 (Lonza). Following culture, cells were trypsinised and counted using a haemocytometer. The desired volume of cells were resuspended in 1.5 mL of EGM-2, before being added to the cytodex beads. The cells and beads were then transferred into a covered, autoclaved fluorescence-activated cell sorting (FACS) tube, and incubated in 37°C and 5% carbon dioxide. Beads were agitated every 20 minutes for four hours to ensure even coverage of the beads by cells. After four hours the solution was carefully transferred from the FACs tube into a T25 flask, and incubated overnight.

The next day, the coated beads were carefully dislodged from the T25 flask by careful tapping. They were then aspirated into a 15 mL Falcon tube and the beads were allowed to settle before being moved into a 1.5 mL Eppendorf, and carefully washed three times with three mL of EGM-2 media, allowing a minimum of five minutes between washes to allow the beads to properly settle and ensuring no beads were accidentally removed with each wash.

During these washes, a fibrinogen/aprotinin solution was prepared: two mg/mL of fibrinogen (Sigma-Aldrich) in PBS and 0.15 units/mL of aprotinin (Sigma-Aldrich). A 24 well plate was also prepared by pipetting 12.5 µL thrombin (Sigma-Aldrich) into the centre of each well that would be used. After the third media wash, cell coated beads were transferred to the fibrinogen/aprotinin solution. If applicable, 15 nM recombinant hIGFBP-2 (Novus Biologicals) was added to the solution at this stage. 15 nM IGFBP-2 was used as this has previously been demonstrated by our group to increase angiogenesis in a bead assay using HUVECs.(219)

The bead/fibrinogen suspension was well mixed then 500 µL was carefully added to each well directly into the thrombin. Once all the wells were treated, the plate was carefully transferred to an incubator for 10-15 minutes for the clot to solidify. One mL of EGM-2 was added to each well drop-wise with a pipette, to avoid clot disruption. Fifteen nM recombinant hIGFBP-2 was also added to the media for relevant experiments.

After 24-72 hours, depending on cell type assessed, beads were imaged using an Olympus Fluorescence microscope at 10x magnification. Images were assessed for sprout number per bead using ImageJ. Data were collated using Microsoft Excel and analysed using GraphPad Prism, with paired Student's t-test used to assess for statistical difference between groups.

6.2.3 Wound closure assay

ECFCs were cultured as previously described in Section 2.2.1. Twenty-four hours prior to experiment, media was changed from EGM-2 supplemented with 20% FBS to EGM-2 supplemented with 2% FBS, to avoid over-exposure to IGFBP-2 prior to the assay. Confluent cells were trypsinised, counted using a haemocytometer and plated onto a 96 well Essen Image Lock plate, coated with 2% gelatin. Forty thousand cells per well were plated, with five to six replicates used for each condition, and wells were made up to 100 µL of EGM-2 with 2% FBS. Empty wells were filled with 100 µL of PBS. The 96 well plate was then incubated for 48 hours to ensure confluency prior to the scratch assay.

The Incucyte WoundMaker (Essen Bioscience) was then used to create equal scratches in all cells. If scratches were not complete then a second attempt would be made. The wells were then cleaned twice with 100 µL PBS, before 100 µL 2% EGM-2 was then added back to the relevant wells, with the addition of 15 nM recombinant hIGFBP-2 if

required. The Incucyte Zoom system (Essen Bioscience) was then used to take images of the scratched wells at one hour intervals for a total of 24 hours, and analysis performed using Incucyte Zoom Software (Essen Bioscience) to assess for extent of wound closure (Figure 6-2). Data were collated using Microsoft Excel and analysed using GraphPad Prism, with paired Student's t-test used to assess for statistical difference between groups.

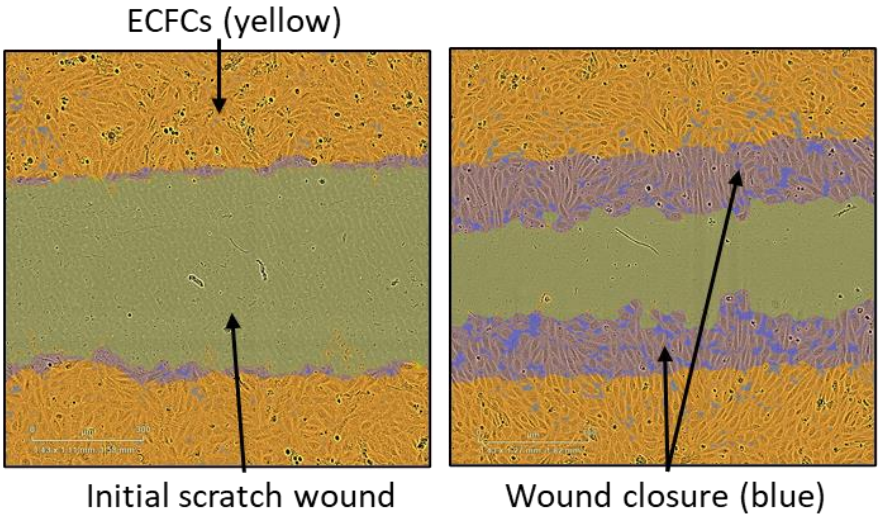


Figure 6-2. Representative images of ECFC wound closure after visual analysis using Incucyte Zoom Software. ECFCs are highlighted in yellow, with the area of wound closure after 12 hours highlighted in blue.

6.3 Results

6.3.1 ECFC culture and characterisation

ECFCs were cultured as described in Section 2.2.1. Our group has previously published and characterised ECFCs using flow cytometry to demonstrate these cells exhibit universal expression of the endothelial markers CD31, CD144, CD146, and CD309, with absent expression of the monocyte marker CD14 and the leukocyte marker CD45 (139). As the same protocol was used, and typical endothelial cell colonies were demonstrated at the described time period of 14-21 days after initial culture (Figure 6-3), it was felt adequate to characterise ECFCs based on appearance alone.

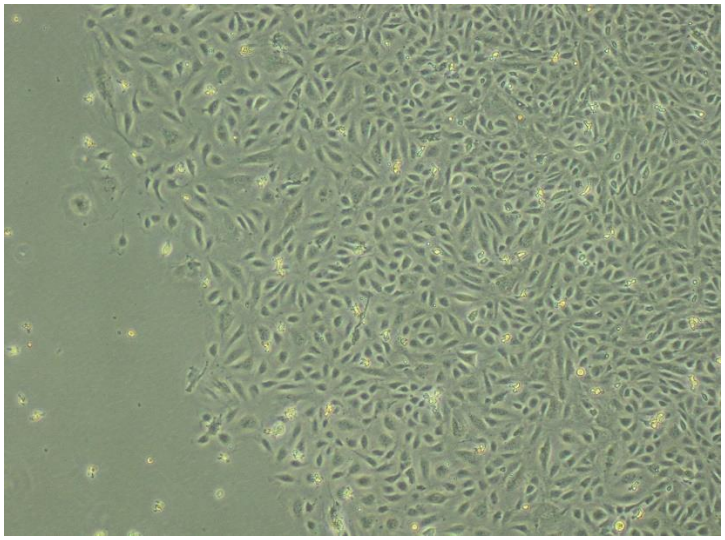


Figure 6-3. Representative image of endothelial colony forming cell colony. These cells demonstrate cobblestone appearance consistent with a mature endothelial cell phenotype. They became evident 14-21 days after initial culture, and once present were markedly proliferative and readily passaged.

6.3.2 ECFC signalling pathway expression

ECFCs from several individual healthy donors were assessed for baseline IGFBP-2 expression, as well as common signalling pathways involved in angiogenesis and cell proliferation. IGFBP-2 was shown to be expressed by ECFCs, with a wide variety in expression evidenced between cells from individual donors. There was no clear

relationship between IGFBP-2 expression and baseline activation of angiogenic and proliferative cell signalling pathways (Figure 6-4).

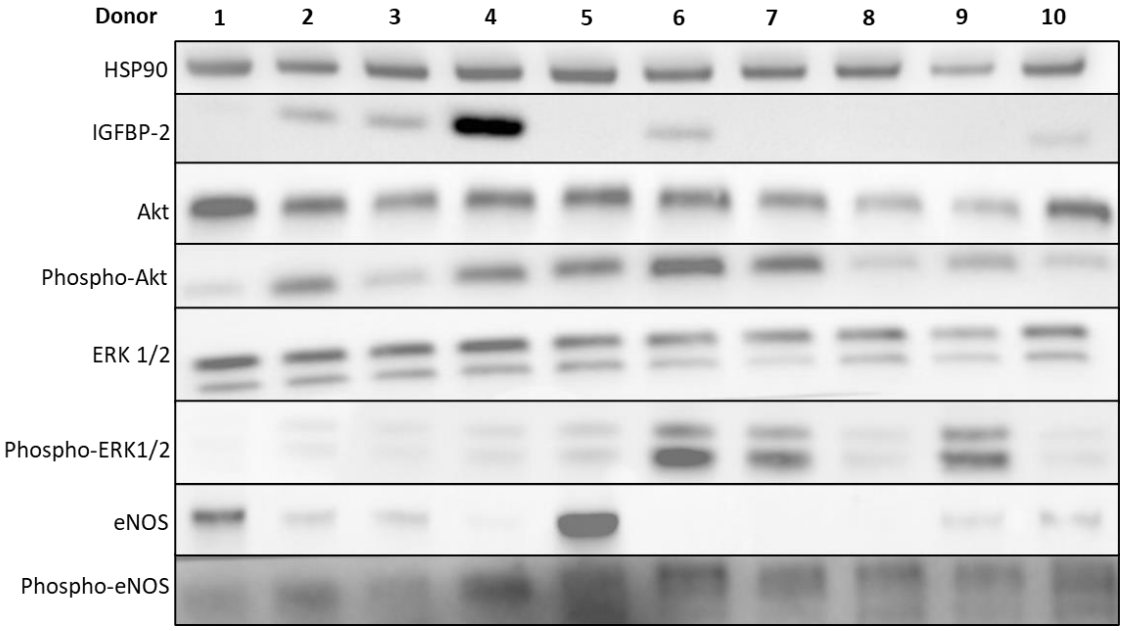


Figure 6-4. ECFC baseline IGFBP-2 expression and associated protein signalling pathways. IGFBP-2 from individual healthy donors was variably expressed by ECFCs, with visual inspection revealing no clear relationship between IGFBP-2 expression and signalling pathway phosphorylation (n=10).

6.3.3 hIGFBP-2 stimulation

Recombinant hIGFBP-2 was used to stimulate ECFCs in models of wound closure and sprouting angiogenesis. Fifteen nM hIGFBP-2 was used as this has previously been demonstrated by our group to significantly enhance sprouting angiogenesis in HUVECs.(219)

6.3.3.1 ECFC wound closure

Wound closure assays were performed as previously described in Section 6.2.3. Images were taken at one hour intervals over a 24 hour period. Images were reviewed and a twelve hour cut off was used, to avoid complete wound closure and allow for accurate

assessment between conditions. No significant increase In wound closure was seen in ECFCs stimulated with 15 nM hIGFBP-2 over this 12 hour period (Figure 6-5).

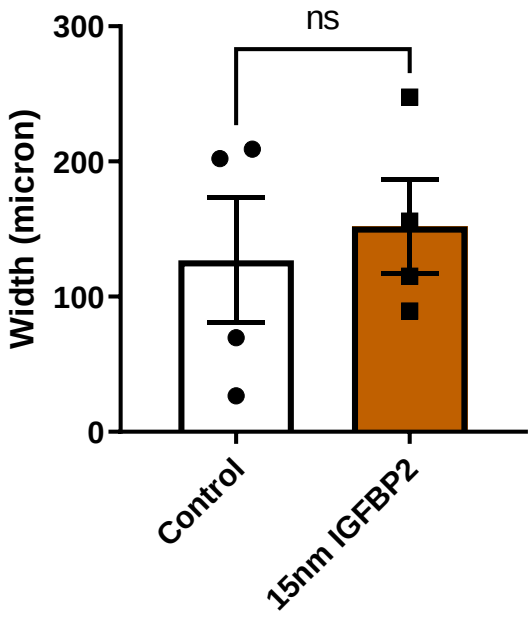


Figure 6-5. Wound closure of ECFCs stimulated with hIGFBP-2. No significant difference was seen in wound closure between ECFCs stimulated with hIGFBP-2 and control cells (n=4,4).

6.3.3.2 ECFC Cytodex bead sprouting angiogenesis assay

Cytodex bead sprouting angiogenesis assays were performed as previously described in Section 6.2.2, with imaging of beads performed at 72 hours. A significant increase In sprouting angiogenesis was seen in ECFCs stimulated with 15 nM hIGFBP-2 over a 72 hour period (Figure 6-6 C).

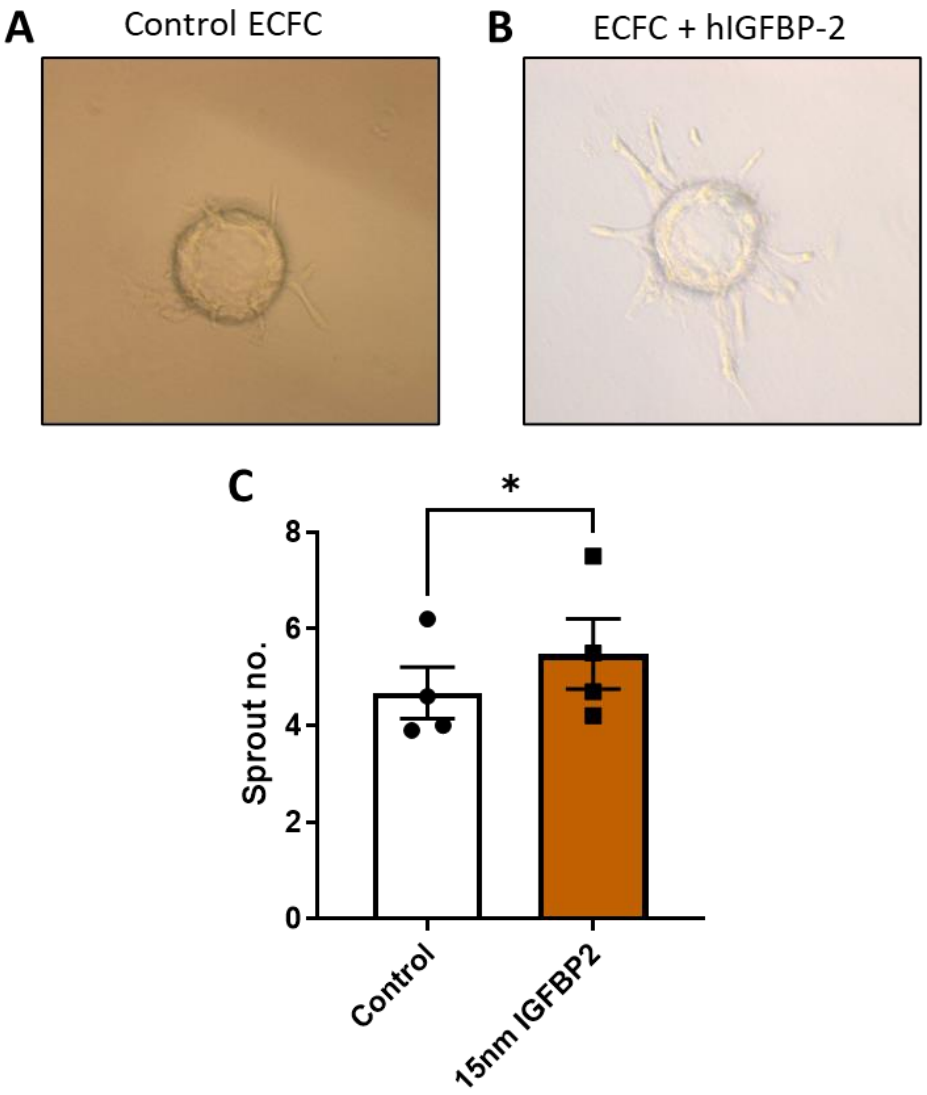


Figure 6-6. Cytodex bead sprouting angiogenesis in ECFCs stimulated with hIGFBP-2. A significant increase in sprouting angiogenesis was seen in ECFCs stimulated with 15 nM hIGFBP-2 over a 72 hour period (p=0.031, n=4,4)

6.3.4 hIGFBP-2 overexpression

6.3.4.1 hIGFBP2$^{iEC\text{-}tg}$ PEC cytodex bead assay

hIGFBP2$^{iEC\text{-}tg}$ mice were injected with tamoxifen at 3-4 weeks of age, and lungs were harvested at 5-6 weeks. Pulmonary endothelial cells were then cultured from harvested lungs as previously described in Section 2.2.2. Cytodex bead sprouting angiogenesis assays were performed in the pulmonary endothelial cells as previously described in Section 6.2.2, with imaging of beads occurring at 24 hours (Figure 6-7 A-B show representative images). At 24 hours, sprout number was significantly greater with PECs

derived from Cre positive hIGFBP2$^{iEC\text{-}tg}$ mice than those from Cre negative controls (Figure 6-7 C).

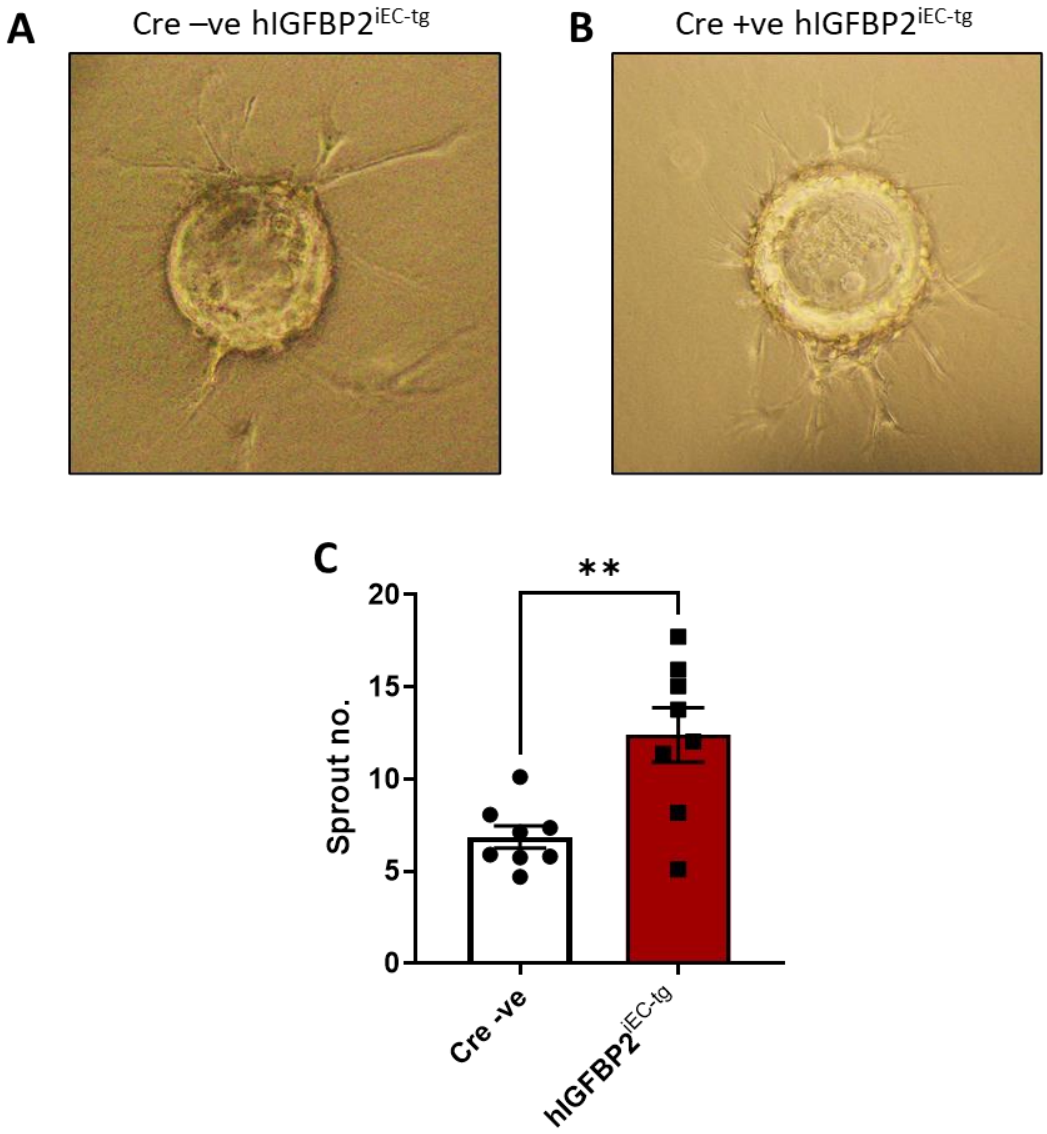


Figure 6-7. hIGFBP2$^{iEC\text{-}tg}$ PEC Cytodex bead sprouting angiogenesis. At 24 hours, sprout number was significantly greater with PECs derived from Cre +ve mice than those from Cre -ve mice (p=0.001, n=8,8).

6.3.4.2 HUVEC and ECFC transduction

HUVECs and ECFCs were cultured as described in sections 2.2.3 and 2.2.1, and transduced as described in section 6.2.1. As described previously, all cells were transduced, but control cells were not treated with doxycycline and therefore were not

expected to overexpress hIGFBP-2. In tandem with performed bead assays, cells were treated in the same manner in a 6 well plate, and lysed for immunoblotting on the day beads were imaged. Overexpression of hIGFBP-2 as assessed by immunoblotting is shown below, with significant overexpression of hIGFBP-2 demonstrated in doxycycline treated ECFCs, but with no significant change in doxycycline treated HUVECs (Figure 6-8).

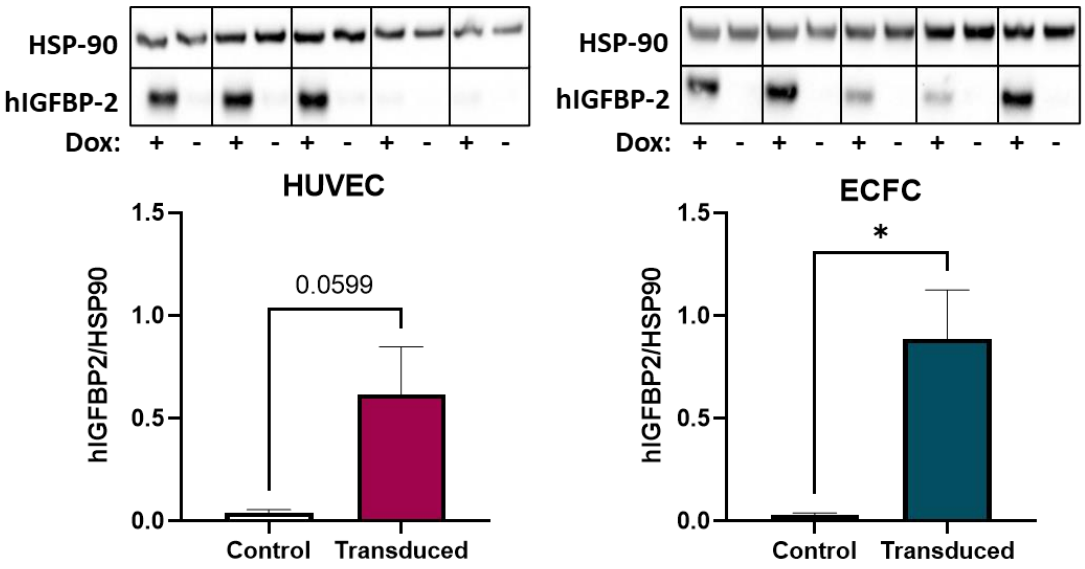


Figure 6-8. Lentiviral transduction of HUVEC and ECFC. All cells were transduced with lentivirus. Overexpression of hIGFBP-2 occurred following treatment with doxycycline (represented by +). Control cells were not treated with doxycycline and so hIGFBP-2 overexpression did not occur (represented by -). Significant overexpression of hIGFBP-2 was demonstrated in transduced ECFCs treated with doxycycline (p=0.02) but no significant change was seen in transduced HUVECs treated with doxycycline (p=0.059) (n=5,5).

6.3.4.3 HUVEC cytodex bead assay

Cytodex bead assays were performed as described in Section 6.2.2, and beads were imaged at 48 hours (representative images Figure 6-9 A-B). HUVECs transduced with an hIGFBP-2 lentiviral supernatant and then treated with doxycycline demonstrated increased sprouting angiogenesis compared to controls treated with lentivirus but not transduced with doxycycline (Figure 6-9 C).

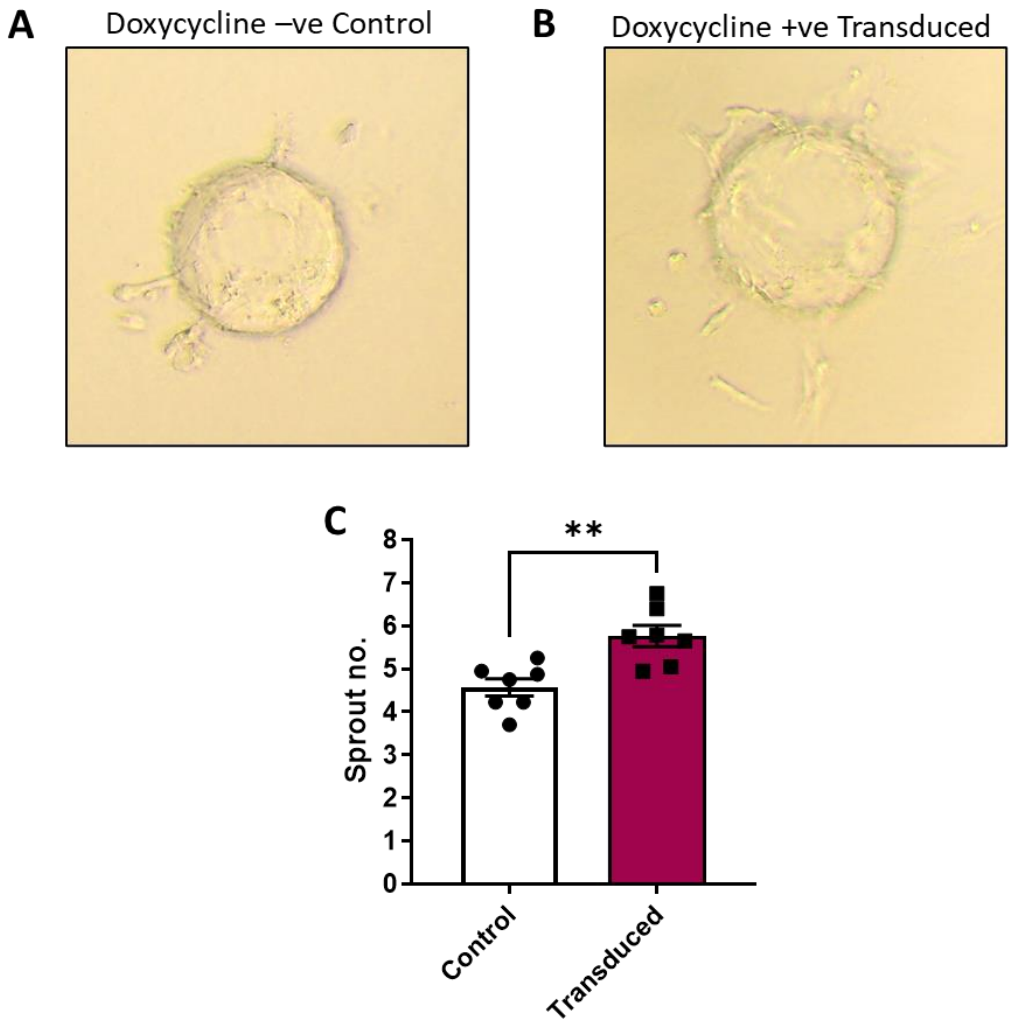


Figure 6-9. Transduced HUVEC Cytodex bead sprouting angiogenesis. HUVECs transduced to overexpress IGFBP-2 demonstrated increased sprouting angiogenesis compared to controls treated with lentivirus but not transduced with doxycycline (p=0.0028, n=7,7).

6.3.4.4 ECFC cytodex bead assay

Cytodex bead assays were performed as described in Section 6.2.2, and beads were imaged at 48 hours (representative images Figure 6-10 A-B). ECFCs transduced with an hIGFBP-2 lentiviral supernatant and then treated with doxycycline demonstrated increased sprouting angiogenesis compared to controls treated with lentivirus but not transduced with doxycycline (Figure 6-10 C).

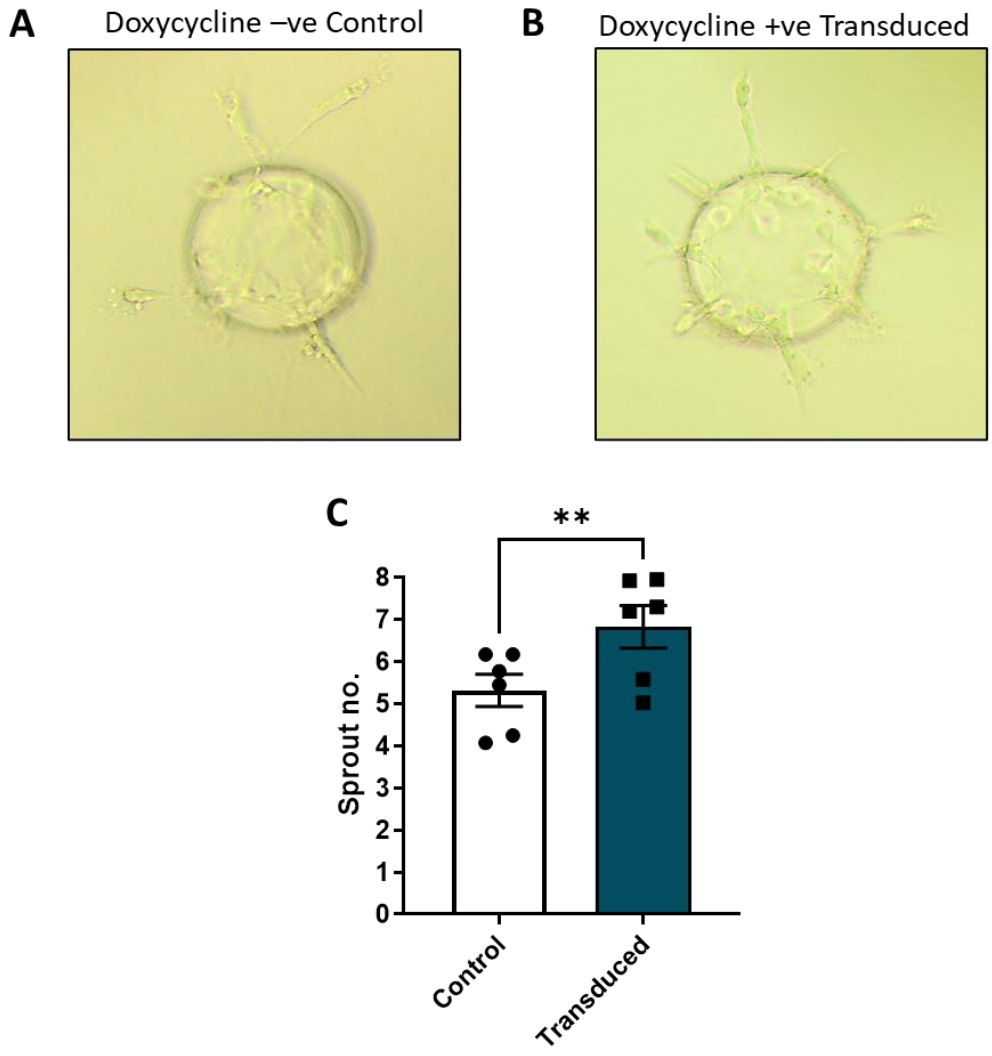


Figure 6-10. Transduced ECFC Cytodex bead sprouting angiogenesis. ECFCs transduced to overexpress IGFBP-2 demonstrated increased sprouting angiogenesis compared to controls treated with lentivirus but not transduced with doxycycline (p=0.0011, n=6,6).

6.3.5 hIGFBP-2 overexpression and kinase signalling pathways

Immunoblotting was performed to assess for phosphorylation of Akt and ERK1/2 signalling pathways following successful transduction of IGFBP-2 overexpression in both HUVECs and ECFCs. No significant difference was seen in phosphorylation ratios for either Akt or ERK signalling in either HUVECs (Figure 6-11 A) or ECFCs (Figure 6-11 B).

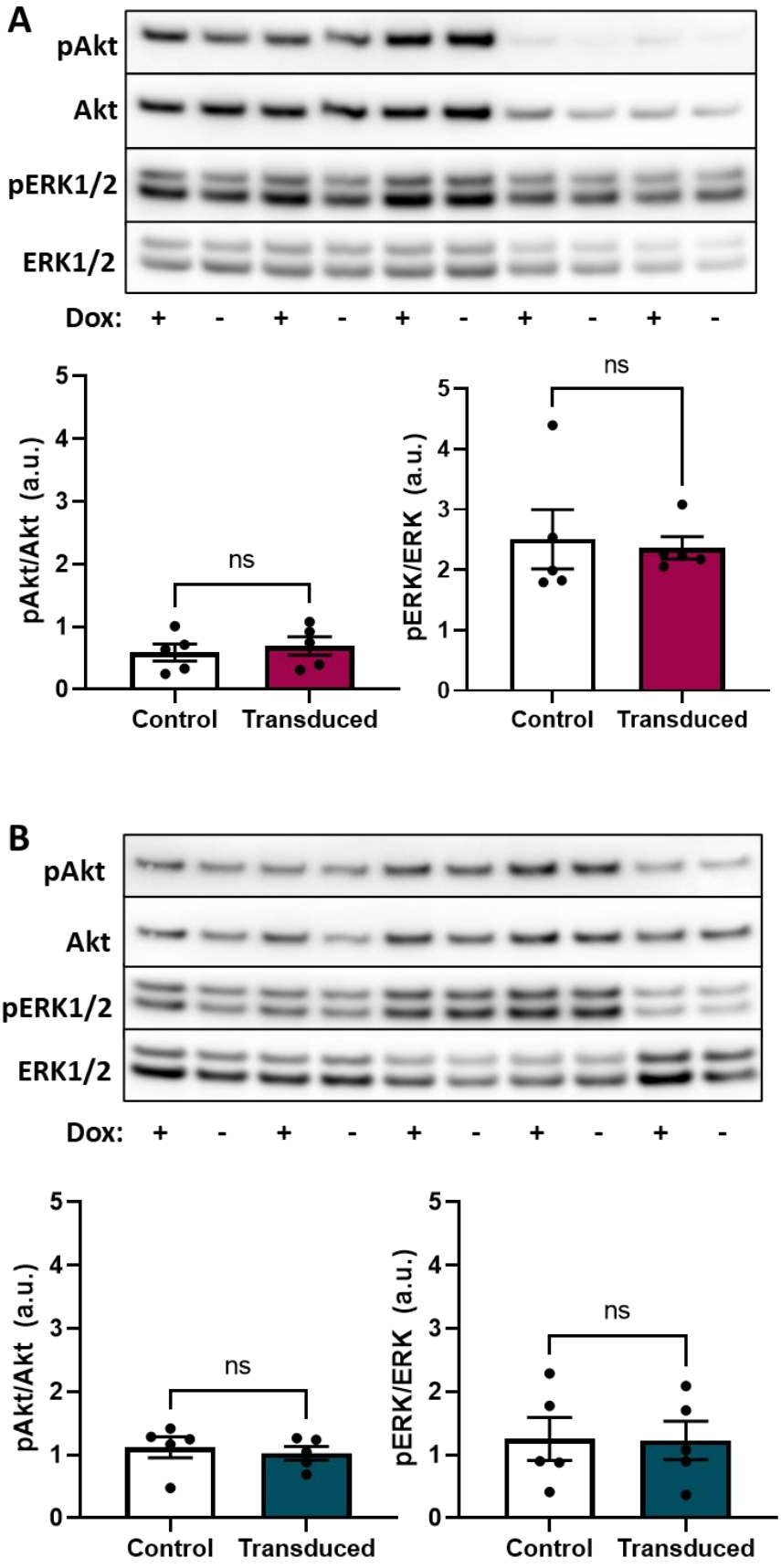


Figure 6-11. Phosphorylation of Akt and ERK signalling pathways in transduced HUVECs (A) and ECFCs (B). No significant difference was observed in phosphorylation of signalling pathways in either group (n=5,5)

6.4 Discussion

Vascular regeneration, and the effects of IGFBP-2 within angiogenesis and vascular regeneration, were the focus of this chapter. Angiogenesis is a complex process, and can occur in response to several signalling pathways. ECFCs have been proven to aid in vascular regeneration processes, and have been touted as having potential as a cell based therapy to augment vascular regeneration after ischaemic injury. Our initial intention was to prove the continual overexpression of hIGFBP-2 in transduced ECFCs, examine *in vitro* effects on angiogenesis, and subsequently infuse transduced ECFCs into recipient mice following hind limb ischaemia, a method which has previously been employed by our group to demonstrate treated ECFCs aid in vascular perfusion recovery after ischaemia (139). Unfortunately restrictions due to the COVID pandemic precluded these planned *in vivo* experiments, and limited sample size for our *in vitro* experiments, but these will be considered for future experiments.

IGFBP-2 expression has not been examined closely in ECFCs, although its expression in ECFCs has been identified in a transcriptome-wide study of ECFCs using RNA sequencing, as well as in HUVECs and human coronary artery endothelial cells.(315) Here, we have demonstrated with immunoblotting that ECFCs express IGFBP-2, although this expression is variable and does not relate to baseline ERK and Akt phosphorylation (Figure 6-4). Exogenous hIGFBP-2 stimulation, using a concentration of hIGFBP-2 that has been demonstrated by our group to upregulate angiogenesis in HUVECs, also demonstrated a small but significant increase in sprouting angiogenesis in ECFCs (Figure 6-6).

No significant change in wound closure was seen following IGFBP-2 stimulation in our experiments (Figure 6-5). Wound closure relies upon both cell proliferation and cell migration, and it may be that these processes in endothelial cells are not influenced by

IGFBP-2 in the same manner that sprouting angiogenesis appears to be. However, the angiogenic process is one of tip cell migration and endothelial cell proliferation,(74) and therefore it does not seem likely that IGFBP-2 could stimulate angiogenesis without having any effect on endothelial cell proliferation or migration. IGFBP-2 stimulation has also been demonstrated to positively influence VSMC proliferation, in this thesis and by others,(223) and has been demonstrated to positively influence tumour cell proliferation and migration in a number of other studies.(228)(279)(293) The lack of differential response seen here may therefore relate to experimental technique and low numbers examined, rather than a true lack of response. Wound closure assays were performed early in the course of this thesis, when 20% FCS culture media was used routinely to maintain ECFCs, rather than just during initial ECFC culture. This was removed 24 hours prior to wound closure assays and replaced by 2% FCS, as used continually in subsequent assays, but it may be that both control and treated cells had received maximal exposure to IGFBP-2 by this stage, and so treating cells with exogenous IGFBP-2 had no additional effect. Further experiments individually examining endothelial cell proliferation and migration, using techniques such as EdU proliferation assays and Boyden chamber migration assays respectively, are warranted. These could be performed using both increased concentrations of hIGFBP-2 to exogenously stimulate cells, as well as transducing ECFCs to constitutively overexpress hIGFBP-2, as described below.

We demonstrated it was possible to transduce ECFCs and HUVECs to overexpress IGFBP-2 through the delivery of a lentiviral transgene vector (Figure 6-8). Although hIGFBP-2 overexpression did not reach statistical significance when assessed by immunoblotting of transduced HUVECs, the experiment was performed over several weeks with protein lysates frozen for analysis on one blot. The doxycycline treated samples which demonstrated less hIGFBP-2 expression were the samples collected first, and so may have been subject to protein degradation over time.

Overexpression of hIGFBP-2 did lead to an increase in sprouting angiogenesis in both ECFCs and HUVECs, although this increase was modest (Figures 6-9 C and 6-10 C). A modest increase of intact vessels may be preferable however to a more potent angiogenic effect but with dysfunctional, leaky vasculature, as can be seen with unregulated VEGF induced angiogenesis.(316) This merits further study, possibly through *in vitro* assessment of transendothelial electrical conductance, or using *in vivo* models of ischaemia to assess effective reperfusion, such as murine hind limb ischaemia models.

Immunoblotting was performed to identify possibly signalling pathways through which this increase in sprouting angiogenesis may occur. No increase in phosphorylation of Akt or ERK was seen in either transduced HUVECs or ECFCs (Figure 6-11). This contrasts with previous findings from our group that stimulation of HUVECs with hIGFBP-2 acutely increases both ERK and Akt phosphorylation.(219) Nevertheless, the finding that overexpression of hIGFBP-2 in transduced ECFCs and HUVECs increased sprouting angiogenesis, but without clearly increasing activity in the commonly associated signalling pathways, is intriguing. It may be that changes in signalling pathway phosphorylation are acute rather than persistent, and require assessment immediately following increased exposure to IGFBP-2, as was the case for the positive findings in HUVECs that our group have previously made.(219) Signalling pathway phosphorylation may therefore change temporally despite continued, unchanged exposure to IGFBP-2, and this merits further investigation, through the assessment of phosphorylation ratios at multiple time points following IGFBP-2 exposure.

Other proangiogenic signalling pathways that have been linked to IGFBP-2 activity such as HIF-1α, FAK and NFκ-B could also be assessed, although prior studies which have

seen increased activation of these pathways have described upregulation in Akt phosphorylation as the outcome of pathway activation.(219)(293) Other studies have described IGFBP-2 influencing angiogenesis through VEGF upregulation after Akt pathway activation,(217) and this could be explored further, although our group did not find an associated increase in VEGF expression with increased Akt or ERK phosphorylation after acute stimulation with hIGFBP-2.(219) Clarification is also required as to whether the effects observed occur through intracellular mechanisms, nuclear translocation and transcriptional upregulation, or are autocrine/paracrine in nature, as all these mechanisms have been described in relation to IGFBP-2 function.(202)(205) Transcriptional regulation could be investigated with RNA sequencing assays examining genes implicated in angiogenesis, and autocrine/paracrine influence could be assessed using sprouting angiogenesis assays in cells treated with conditioned media from hIGFBP2$^{iEC\text{-}tg}$ PECs and lentiviral transduced endothelial cells.

There was a larger increase in sprouting angiogenesis seen in PECs derived from Cre positive hIGFBP2$^{iEC\text{-}tg}$ mice, than was seen in either exogenous stimulation with hIGFBP-2, or lentiviral transduced overexpression of hIGFBP-2 (Figure 6-7). This larger effect size may relate to greater endothelial expression of IGFBP-2 in the transgenic mice when compared to lentiviral transduction, but it was not possible to directly compare IGFBP-2 expression between these, so this interpretation is speculative. It may also relate to differences in endothelial cell type and alterations in signalling pathways between the groups. Recent unpublished work in our group has also confirmed upregulation of ERK phosphorylation in aortas isolated from these mice, suggesting activation of the ERK signalling cascade may be responsible for the increased propensity to sprouting angiogenesis seen in endothelial cells derived from these mice, compared to a lack of difference in signalling pathway activity seen in the transduced endothelial cells used here. These inconsistent findings mean it remains unclear how IGFBP-2 overexpression increases sprouting angiogenesis, and responsible signalling mechanisms are likely to

depend on individual cell types and environments. Accurately identifying these mechanisms are important prior to *in vivo* assessment of IGFBP-2 enhanced ECFCs as a vehicle to augment vascular regeneration or repair, and approaches such as the use of RNA sequencing to analyse the transcriptome of IGFBP-2 overexpressing ECs compared to controls, or mass spectrometry to examine the proteome in a similar fashion, would potentially provide unbiased answers to help focus investigation.

6.4.1 Summary

IGFBP-2 has been described as a putative proangiogenic factor in multiple cellular environments and through diverse molecular mechanisms. Here, we have confirmed that constitutive overexpression of hIGFBP-2 in pulmonary endothelial cells derived from a transgenic mouse model leads to significantly enhanced sprouting angiogenesis (Figure 6-7). It was not possible to identify signalling pathways in these specific cells, due to the difficulty in passaging PECs beyond the primary culture, although increased ERK phosphorylation has been confirmed in harvested aorta from the same transgenic line.

Endothelial colony forming cells are known to promote vascular repair and regeneration, and have been touted as a potential cell based therapy to augment tissue perfusion after ischaemic insult. Enhancing the ability of ECFCs to promote vascular regeneration would therefore be of significant value. Furthermore, ECFCs harvested from patients with vascular disease or cardiovascular risk factors may be dysfunctional, and so ECFC augmentation may be a pre-requisite for their autologous use in patients with cardiovascular disease (312). We successfully demonstrated hIGFBP-2 overexpression in both HUVECs and ECFCs following lentiviral transduction (Figure 6-8), and this IGFBP-2 overexpression increased sprouting angiogenesis compared to control cells (Figure 6-9 C and 6-10 C). Exogeneous stimulation of ECFCs by IGFBP-2 also led to a small but significant increase in sprouting angiogenesis (Figure 6-6), but did not significantly increase wound closure (Figure 6-5). Furthermore, there was no significant

increase in the ratio of phosphorylated Akt or ERK in either transduced ECFCs or HUVECs (Figure 6-11). Further work is therefore required, to establish if overexpression of IGFBP-2 in ECFCs will truly enhance their reparative properties, the pathways through which this may occur, and if transfusion of enhanced ECFCs can be demonstrated to enhance vascular repair and regeneration *in vivo* using mouse models of vascular injury and ischaemia.

Chapter 7 Conclusions and Summary

7.1 Background

The formation of new blood vessels; physiological function of quiescent vasculature; and remodelling of vasculature after injury, acute or chronic, is managed through a wide variety of complex and overlapping cellular and molecular mechanisms. Blood vessels consist of layers of different cell types, including endothelial cells, vascular smooth muscle cells and adventitial tissue.(4) These do not exist in isolation, and each tissue type will exert effects on the other; especially in the context of angiogenesis, arteriogenesis, and in the pathophysiological response to vascular injury.(105)

A potentially important protein which may influence these mechanisms is insulin-like growth factor binding protein-2. IGFBP-2, conventionally regarded as a binding protein for the insulin-like growth factors, has now been demonstrated to have a plethora of effects in different cell types beyond the simple binding and transportation of the IGFs.(210) These effects can be dependent or independent of the IGFs, and have predominantly been found to relate to increased angiogenesis and proliferation, with the majority of research thus far focussed on tumour angiogenesis, tumour cell proliferation and migration.(202) IGFBP-2 has also been demonstrated to have IGF independent metabolic actions in the reduction of propensity to obesity in the presence of diabetes, as well as increasing insulin sensitivity.(213) However, little is known about the general effects of IGFBP-2 in vascular physiological function, or pathophysiological response to vascular injury.

We have previously demonstrated that overexpression of the structurally similar binding protein, IGFBP-1, in a transgenic mouse model has positive effects on vascular

physiological function, reducing blood pressure and reducing aortic tissue response to vasoconstricting agents. These effects were demonstrated to occur through an increase in nitric oxide availability in the endothelial monolayer following upregulation of the PI3K/Akt pathway, in an IGF-independent manner.(195) In the same study, elevated IGFBP-1 levels also improved insulin sensitivity and glucose tolerance, and a later study by our group demonstrated that the beneficial metabolic effects of IGFBP-1 overexpression were mediated through binding of its RGD domain to integrin receptors,(273) leading to the hypothesis that the witnessed increase in NO bioavailability and subsequent beneficial vascular physiology effects may occur through the same integrin binding pathways.

As IGFBP-2 contains a similar RGD domain, as well as two HBD domains also demonstrated to exert IGF dependent and independent effects,(202) it was hypothesised that overexpression of IGFBP-2 may exert similar vascular physiological effects. Stimulation with exogeneous IGFBP-2 has also been shown to increase porcine VSMC proliferation through IGF-I co-dependent upregulation of PI3K/Akt signalling,(223) and IGFBP2 gene expression is upregulated in human VSMC derived from atherosclerotic plaque and in stent neointima.(232) IGFBP-2 therefore also may potentially contribute to the development of pathophysiology in the setting of acute or chronic vascular injury, and so may have either beneficial or deleterious effects depending on the cellular environment.

We therefore set out to examine the effects of IGFBP-2 in vascular biology, in both physiological and pathophysiological settings. This was achieved through the use of several transgenic mouse models of IGFBP-2 overexpression to explore *ex vivo* aorta response to vasodilatory and vasoconstrictory stimuli, as well as *in vivo* effects on blood pressure and aortic distensibility. We then used established femoral artery wire injury and carotid cuff placement models to examine the effects of IGFBP-2 overexpression on

vascular injury and neointima formation. Finally we examined whether lentivirus transduced continued overexpression of IGFBP-2 by endothelial cells could enhance angiogenesis, and therefore potentially enhance the ability of endothelial colony forming cells to augment vascular repair and regeneration.

7.2 Findings

We set out to establish the degree of hIGFBP-2 expression within several murine models. The first of these was a long established model of unconditional global overexpression, termed hIGFBP2^{global} for the purposes of this thesis. IGFBP-2 overexpression in this mouse line was driven by the endogenous promoter, and had previously been shown in our laboratory to lead to a 2.2 fold increase in total circulating IGFBP-2.(213) We found that there was no longer a significant secretion of IGFBP-2 in plasma from these transgenic mice compared to wild type controls (Figure 3-2 A), which was felt likely related to genetic drift, as this transgenic line had been in use for a number of years, with multiple rounds of replacement of breeding animals with offspring. This correlated with a lack of differential response seen when we performed aortic relaxation assays with this mouse model, with both transgenic and wild type control mice showing no difference in aortic response to acetylcholine, phenylephrine or sodium nitroprusside (Figure 4-3).

The second transgenic murine model we examined was one of conditional hIGFBP-2 overexpression selectively in the endothelium. Briefly, this was achieved through the creation of a transgenic mouse with an hIGFBP-2 gene inserted at the Rosa26 locus, which was flanked by a floxed STOP codon (Figure 3-1). These mice were then bred with Cdh5-CreERT2 mice, which contained Cre/ERT2 cDNA within the VE-Cadherin gene, and termed hIGFBP2$^{iEC\text{-}tg}$ mice. Activation of Cre-recombinase, via an intraperitoneal tamoxifen injection regime, caused overexpression of hIGFBP-2 within

the vascular endothelium, and a significant increase in secretion of hIGFBP-2 was confirmed by serum ELISA (Figure 3-2 B). Despite this marked upregulation in hIGFBP-2 expression and secretion, no differences in *ex vivo* endothelial function, NO bioavailability or VSMC function were seen in aortic vasomotor assays when compared to mice without the Cre gene encoded (Figure 4-4). This suggested that even a high degree of IGFBP-2 endothelial expression does not influence aortic physiological function, in contrast to the previously seen effects of IGFBP-1 overexpression, which influenced aortic tissue response to phenylephrine through an upregulation in NO bioavailability.(195)(272)

Assays examining *in vitro* sprouting angiogenesis were performed using pulmonary endothelial cells derived from hIGFBP2$^{iEC\text{-}tg}$ mice (Figure 6-7). These assays demonstrated a significant upregulation in sprouting angiogenesis associated with hIGFBP-2 overexpression, indicating that IGFBP-2 may have an effect on certain aspects of endothelial function, depending on cellular environment. Unfortunately due to the difficulties in culturing these cells through more than one passage, it was not possible to analyse signalling pathway protein expression in these cells, although other work by our group has established that harvested aortic tissue demonstrate upregulated ERK1/2 phosphorylation, and so IGFBP-2 may lead to enhanced angiogenesis through this signalling mechanism.

The final transgenic mouse model used was one of conditional overexpression of hIGFBP-2 in all tissues, dubbed hIGFBP2$^{iGLOBAL\text{-}tg}$. This model was created using the same floxed hIGFBP-2 mouse, but bred with a transgenic mouse with the Cre gene expressed in all tissues and driven by a cytomegalovirus promotor. Serum ELISA demonstrated a very substantial increase in hIGFBP-2 expression compared with Cre negative counterparts. It was however discovered that Cre positive mice exhibited significant hIGFBP-2 secretion before tamoxifen injection, although secretion was

enhanced further after tamoxifen injection (Figure 3-2 C). Although all mice bred were morphologically normal, bred and acted normally, this unintentional expression of hIGFBP-2 prior to tamoxifen injection did lead to a significant reduction in mouse bodyweight soon after weaning with a corresponding reduction in organ weight (Figures 3-4 and 3-5). This was felt most likely relate to unintentional low level Cre expression prior to tamoxifen stimulation, which was an established possibility when creating the mouse model.

Although this meant that IGFBP-2 overexpression may have been present from the point of embryogenesis, which was not the initial intended outcome, it was still felt appropriate to examine the effects of marked IGFBP-2 overexpression in all tissues when compared to no hIGFBP-2 expression, especially considering our original mouse model of global overexpression no longer demonstrated significant IGFBP-2 differential expression.

Using this mouse model, we discovered that the vasoconstrictor response in isolated segments of aorta to increasing concentrations of phenylephrine was significantly attenuated with global overexpression of hIGFBP-2. This differential effect was not lost when eNOS was inhibited with L-NMMA, and there was no difference in vasodilatory response to acetylcholine (Figure 4-5). This suggested that, as with endothelial specific overexpression, a marked increase in hIGFBP-2 expression has no effect on *ex vivo* endothelial function, and the reduction in vasoconstriction observed was independent of endothelial action. There was also a significant reduction in vasoconstriction in response to KCl, which acts directly on VSMC calcium signalling without ligand-receptor interaction (Figure 4-5 E).(288) These findings indicated that the effects seen may relate to alterations in contractile VSMC function, and potentially structure.

We examined the expression of the contractile VSMC genes ASMA and MYH11 by RT-PCR, both of which were significantly down-regulated in aortic tissue overexpressing hIGFBP-2 (Figure 4-9). This would be consistent with a potential phenotypic switch from contractile to synthetic VSMC, an acknowledged phenomenon which can happen in response to both ERK1/2 and PI3K/Akt upregulation, as well as many other influences.(290)(291) To look into this further we examined phosphorylation ratios of several signalling pathways using immunoblotting of aortic tissue, and found that at 6 weeks post tamoxifen injection PI3K/Akt phosphorylation was upregulated, whilst no change in ERK1/2 phosphorylation or p38MAPK phosphorylation was seen (Figure 4-10). PI3K/Akt phosphorylation has been linked to VSMC phenotypic switching, but has also been found to be protective of the contractile phenotype,(291) so it was unclear if upregulation of this pathway provides further evidence of a phenotypic shift.

Further experiments were performed using this transgenic murine model to examine the *in vivo* physiological effects of global overexpression of hIGFBP-2 on aortic distensibility and blood pressure. If a phenotypic switch to a proliferative phenotype had occurred, it may be expected that aortic distensibility would be reduced as the aorta would be stiffer.(56) We in fact saw the opposite, and aortic distensibility was significantly greater in mice overexpressing hIGFBP-2 (Figure 4-8). The aortic diameter of Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice was marginally smaller than their Cre negative counterparts, consistent with their reduced bodyweight (Figure 4-7). It may well therefore be that this difference in distensibility relates to LaPlace's law of wall tension, that increasing lumen radius leads to greater wall tension, which would in turn be expected to reduced distensibility.(299) This would explain the observed reduction in distensibility in both groups between two and six weeks, as aortic diameter increased.

We did not see any significant difference in tail-cuff blood pressure with overexpression of hIGFBP-2 at two or six weeks post tamoxifen injection, despite this difference in aortic

distensibility (Figure 4-6). Blood pressure regulation is multi-faceted, and includes systemic vascular resistance and cardiac output, in combination with aortic elasticity.(294) It would therefore be interesting to examine if IGFBP-2 overexpression has any effect on cardiac stroke volume or murine microvasculature in future experiments.

Our next step was to investigate the effects of IGFBP-2 overexpression in vascular injury and remodelling. We examined the effect of global IGFBP-2 overexpression on re-endothelialization, and, using a femoral artery wire injury model, demonstrated significantly reduced re-endothelialization associated with IGFBP-2 overexpression (Figure 5-6). This is in contrast to our previous findings of improved vascular repair with IGFBP-1 overexpression in the context of insulin resistance.(196) Other studies have demonstrated increased endothelial cell proliferation and recruitment in response to IGFBP-2 stimulation,(217)(238) and Feng *et al* described *in vitro* enhancement of EPC incorporation and adhesion into HUVEC tubules with IGFBP-2 treatment, suggesting in certain contexts IGFBP-2 may augment vascular repair.(236)

Our findings demonstrate that this augmentation of vascular repair may be dependent on cellular environment, and complement our hypothesis that IGFBP-2 overexpression may induce a VSMC phenotypic switch, as an increase in VSMC proliferation and neointima formation would reduce the capacity for vascular repair and endothelial proliferation.(307) These findings were supplemented by *in vitro* VSMC cell counting assays. In these assays we stimulated both arterial and venous derived human VSMC with IGFBP-2, and found that arterial VSMC cell count was significantly increased compared to non-stimulated control cells, but there was no differential cell count in venous derived samples (Figure 5-5), potentially reflecting a greater change in proliferative capability or improved cell survival in arterial samples, compared to the already proliferative venous samples.

Given these findings, we therefore used the same femoral wire injury experiments, as well as established carotid cuff techniques, to examine neointima formation, using paraffin sectioning and Miller-Van Gieson elastin staining. Interestingly, we did not find an increase in neointima volume in either femoral wire injury or carotid cuff experiments when comparing Cre positive hIGFBP2$^{iGLOBAL\text{-}tg}$ mice and their Cre negative counterparts (Figures 5-2 and 5-4). This raised the possibility that, rather than inducing a phenotypic switch in VSMCs, IGFBP-2 overexpression may induce VSMC apoptosis through IGF-I inhibition, an action which has previously been attributed to IGFBP-2 and proposed to be a contributory factor to atherosclerotic plaque development.(233) Ours and others' findings of increased VSMC proliferation with IGFBP-2 stimulation would argue against this theory,(223) but our findings here emphasise that the effects of IGFBP-2 are likely to be environment and cell type dependent. Although unfortunately precluded in this thesis due to the COVID-19 pandemic, further experiments are planned using immunohistochemistry to accurately delineate the neointimal components in the same models of femoral injury and carotid cuff formation, and these will be very useful to further our understanding of the effects of IGFBP-2 on VSMC and adventitial proliferation and migration in vascular injury.

Finally, we examined the angiogenic effects of IGFBP-2 stimulation and overexpression in endothelial cells, specifically HUVECs and endothelial colony forming cells. We found that both HUVECs and ECFCs could be transduced by lentiviral vector to overexpress hIGFBP-2 (Figure 6-8). This correlated in both HUVECs and ECFCs with a significant upregulation in sprouting angiogenesis, although this difference was small (Figures 6-9 and 6-10). We examined phosphorylation of common signalling pathways in these transduced cells, and did not find increased phosphorylation ratios in either PI3K/Akt or ERK1/2 signalling pathways (Figure 6-11), contrasting with previous findings by our group in which both Akt and ERK phosphorylation was acutely and robustly upregulated

in HUVECs following stimulation with exogenous IGFBP-2.(219) Further experiments are therefore required to clarify the exact effects of IGFBP-2 on angiogenesis, and the pathways through which these actions occur.

7.3 General conclusions

IGFBP-2 is known to affect angiogenesis in certain cell types, but little was known about its role in other aspects of vascular biology. We have demonstrated, using murine models of conditional hIGFBP-2 overexpression, that IGFBP-2 does not affect endothelial vasomotor function, but does inhibit the vasoconstrictor response to phenylephrine when IGFBP-2 is overexpressed globally (Figure 4-5). This occurs independently of NO bioavailability and with unchanged endothelial responses, and therefore we postulated that alterations in VSMC function may be responsible for the observed inhibition of vasoconstrictor response.

We demonstrated a reduction of contractile vascular smooth muscle cell markers in aortic tissue harvested from mice with global upregulation of IGFBP-2 expression (Figure 4-9), and reduced endothelial regeneration after wire injury in these same mice (Figure 5-6). We did not however demonstrate increased neointima formation following femoral wire insertion or in carotid cuff models of vascular injury, and so targeting IGFBP-2 in this respect may not prove useful to prevent restenosis.

Furthermore, we witnessed no significant change in blood pressure between groups (Figure 4-6), and although there was a witnessed difference in aortic distensibility (Figure 4-8), it was felt this most likely represented the acknowledged effects of blood flow within smaller and larger vessels, as there was a significant difference in aortic lumen diameter between control mice and mice globally overexpressing IGFBP-2 (Figure 4-7).

Despite the detrimental effects of IGFBP-2 on endothelial regeneration *in vivo*, constitutive overexpression of IGFBP-2 and exogenous stimulation with IGFBP-2 was associated with increased sprouting angiogenesis *in vitro* (Figures 6-9 and 6-10), and so IGFBP-2 may prove useful to enhance vascular regeneration after ischaemic injury, and merits further investigation to this effect.

7.4 Limitations

I have described in preceding chapters several limitations when interpreting these data. A major limitation is a current lack of understanding of how the effects of IGF-I may have influenced our findings. The changes seen related to a marked increase in IGFBP-2 expression and secretion, which may be expected to cause a reduction in IGF-I bioavailability, and even if this is not the case, we also do not know whether the effects seen were dependent or independent of IGF-I action. We additionally do not know what effect this marked upregulation in IGFBP-2 had on the production of the other murine IGFBPs, and further work is required to identify these effects.

Another key consideration is that the effects seen when using the transgenic murine models corresponded to levels of IGFBP-2 that were extremely supra-physiological. Although this is useful when considering IGFBP-2 in a pharmacological sense as a putative therapeutic, similar effects may not have been witnessed at lower concentrations. We therefore can only comment on the effects of IGFBP-2 in this context, but cannot describe if IGFBP-2 plays a role in these same functions when expressed at a physiological level. Similarly, although no adverse effects were seen in mouse behaviour or breeding, a reduction in growth was evident in mice globally overexpressing

IGFBP-2, and the long term effects of marked upregulation was not examined, but may be deleterious given the association between IGFBP-2 and cancer progression.(202)

7.5 Future directions

While our findings are thought provoking and novel, there are several areas which remain under-explored and which would benefit from further investigation in the future, which I will outline below.

Our neointima injury models demonstrated no difference in neointima volume. However, as staining of the neointima was only for elastin, it was not possible to delineate the other neointimal components, and there may be potential alterations in neointima phenotype. Further experiments using cryosectioning and immunohistochemistry to delineate individual components of neointima were planned, and samples harvested, but unfortunately limitations in place due to the Covid-19 pandemic meant that it was not possible to perform these experiments. Understanding the contribution of VSMC and adventitial components to the development of this neointima is of utmost importance, and is a direction our group plan to take in the near future.

As described above, we did not explore the effects of marked overexpression of IGFBP-2 on IGF-I action specifically, or IGF binding. It may be that the effects seen reflected a general inhibition of IGF action rather than any specific effects of IGFBP-2. This is important to clarify, and future work could examine this interaction in more detail, potentially through the development of a transgenic mouse line expressing a mutated form of hIGFBP-2 which cannot bind IGF-I. It would be important to see if my findings are replicated using mice which overexpress IGFBP-2, but not to such a significant degree. This would potentially allow a clearer comparison between control and

transgenic mice, if a lesser degree of IGFBP-2 overexpression led to less growth restriction in these mice. Homozygous mice were used for all experiments, as heterozygous mice had been found to have low expression of IGFBP-2 prior to the start of my study. I feel this should be revisited, as the use of heterozygous mice with less overexpression of hIGFBP-2 would allow for dose-dependent effects of IGFBP-2 to be assessed.

Finally, I feel it would be important to assess the effects of IGFBP-2 overexpression on cardiac tissue and heart function. IGFBP-2 is known to be highly expressed in cardiac tissue, upregulation of PI3K/Akt and MAPK pathways have also been linked to myocardial hypertrophy, and circulating IGFBP-2 levels can be used as a predictor of mortality for heart failure in humans.(317)(318)(319) Having a better understanding of the cardiac effects of IGFBP-2 will help further our understanding of the influence IGFBP-2 can have within the cardiovascular system, and how these effects could potentially be harnessed therapeutically.

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