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The control of blood vessel formation and function through the Von Willebrand Factor – Angiopoietin 2 pathway: *in vitro*, *in vivo* and patient studies

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Statement of originality

I Koval Elrado Smith confirm that the work presented in this thesis is my own. Where work has been derived from other sources I have indicated at the necessary places in this thesis.

“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

-Albert Einstein

For my family

Abstract

Von Willebrand Factor (VWF) is a large glycoprotein synthesised by endothelial cells (EC) and megakaryocytes. Defects in VWF cause the most common inherited bleeding disorder in humans, Von Willebrand disease (VWD). Some patients with VWD present with vascular malformations in the gastrointestinal (GI) tract (angiodysplasia), which can lead to severe intractable bleeding. Angiodysplasia may be linked to the ability of VWF to regulate blood vessel formation. VWF drives the formation of Weibel Palade Bodies (WPB) which store a key regulator of angiogenesis and permeability, Angiopoietin 2 (Ang-2).

In this study, I investigate how VWF controls vascular formation and function through Ang-2. Inhibition of VWF expression in ECs caused enhanced synthesis and release of Ang-2. In addition, ECs isolated from a patient with a severe quantitative defect in VWF (type 3 VWD) showed enhanced synthesis and release of Ang-2, confirming that VWF regulates Ang-2 storage and synthesis in ECs. I investigate whether VWF regulates Ang-2 *in vivo*, and find that Ang-2 expression is increased in the hearts but not lungs of VWF-deficient mice, indicating a tissue specific regulation. I then investigate the pathway though which VWF may regulate Ang-2 expression. I show that VWF controls Ang-2 synthesis through a pathway involving Akt phosphorylation and the transcription factor FOXO1. I demonstrate a role for atorvastatin in pharmacological manipulation of this pathway, which may be relevant for patients with angiodysplasia and therapeutic manipulation of Ang-2 levels.

Inhibition of Ang-2 was able to normalise the VWF-dependent increase in *in vitro* vascular sprouting. *In vivo*, increased expression of Ang-2 in the heart of VWF-deficient mice correlates with enhanced vascularisation. Furthermore, cardiac vessels from VWF-deficient mice showed enhanced permeability compared to controls assessed by perfusion of low (4.4×10^3) molecular weight dextran. The results of this thesis provides evidence for the role of VWF in controlling angiogenesis via an Ang-2 dependent pathway. Thus may suggest a novel molecular mechanism which may contribute to VWD associated vascular malformations.

Table of Contents

Publications & Presentations

Manuscripts arising from this thesis:

- **Smith, K.,** Starke, RD., Dufton, NP., McKinnon, TAJ., Paschalaki, KE., Millar, CM.,Laffan, MA., Randi, AM., Endothelial Von Willebrand Factor controls blood vessel formation and function via the regulation of the growth factor Angiopoietin 2, *manuscript in preparation*

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- **Smith, K.,** Starke, RD., Dufton, NP., McKinnon, TAJ., Millar, CM.,Laffan, MA., Randi, AM., Von Willebrand Factor controls blood vessel formation and function via the Angiopoietin 2, *28th* UK Cell Adhesion Society Meeting , September 2016, London, UK (Oral)

- **Smith, K.,** Starke, RD.,Ribezzo, F., McKinnon, TAJ., Millar, CM.,Laffan, MA., Randi, AM., Von Willebrand Factor controls the expression of Angiopoietin 2, *Joint European Vascular Biology Organisation and European Microcirculation Society* Meeting , June 2015, Pisa, Italy (Poster)

Manuscripts not arising from this thesis:

- Mckinnon, TAJ., Starke, RD., **Smith, K.,** Randi, AM., Laffan, MA Von Willebrand Factor Binds to the Endothelial Growth Factor Angiopoietin-2 within Endothelial Cells and after Release from Weibel Palade Bodies, (*under revision)*, Thrombosis and Haemostasis

- Starke, RD., Ishihara, J., **Smith, K,** Mckinnon, TAJ., Kubala, E., Sudiwala, S., Mazi, S., Shima, D., Laffan, MA., Hubbell, J., Randi, AM., Von Willebrand Factor binds to and regulates the function of Vascular Endothelial Growth Factor, *manuscript in preparation*.

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Abbreviations

aa: amino acid

ADAMTS13: a disintegrin and metalloproteinase with thrombospondin motifs 13

AdenoO: Adenovirus empty

Ang-1: Angiopoietin 1

Ang-2: Angiopoietin 2

Ang-3: Angiopoietin 3

Ang-4: Angiopoietin 4

BAEC: bovine aortic endothelial cells

BBB: blood brain barrier

BOEC: blood outgrowth endothelial cells

BrdU: 5-bromo-2'-dexyuridine

CA-Akt: constitutively active Akt adenovirus

cDNA: complementary DNA

CLP: cecal ligation and puncture

COMP: cartilage oligomeric matrix protein

COPD: chronic obstructive pulmonary disease

COX: cyclooxygenase

cVWF: VWF concentrate

DDAVP: 1-deamino-8-D-arginine vasopressin

DLL4: Delta like ligand 4

DMSO: dimethyl sulfoxide

EC: endothelial cell

ECGF: endothelial cell growth factor

ECM: extracellular matrix

ECM: extracellular matrix

EGM-2: endothelial growth factor 2

ELISA: enzyme linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

EPC: endothelial progenitor cell

ER: endoplasmic reticulum

ERK: extracellular signal regulated kinase

ETS: E26 transformation specific

FAK: focal adhesion kinase

FAT: focal adhesion target

FCS: fetal calf serum

FITC: fluorescein isothiocyanate

FLD: fibrinogen like domain

FOXO1: forkhead box O1

FVIII: Factor VIII

g: gravity

Gal-3: galectin 3

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GI: gastrointestinal

HBSS: hanks balanced salt solution

HHT: hereditary haemorrhagic telangiectasia

HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A

HMWD: high molecular weight dextran

HMWM: high molecular weight multimer

hr: hour

HRP: horse radish peroxidase

HUVEC: human umbilical vein endothelial cells

hVWF: in house purified VWF

ICAM1: intercellular adhesion molecule 1

Ig: Immunoglobulin

IL-8: interleukin 8

ip: intra peritoneal

IQR: interquartile range

IU: international units

iv: intravenous

kDa: kilo dalton

KLF: krupple like factor

LMWD: low molecular weight dextran

LMWM: low molecular weight multimer

LPS: lipopolysaccharide

MAPK: mitogen activated protein kinase

MIDAS: metal ion dependent adhesion site

min: min

MMPs: matrix metalloproteinases

MOI: multiplicity of infection

NRP-1: neuropilin 1

OPG: osteoprotegerin

PAH: pulmonary arterial hypertension

PBS: phosphate buffered saline

PI3K: phosphoinositide 3-kinase

PLCγ: phospholipase C gamma

PlGF: placental growth factor

PMA: phorbol 12-myristate 13-acetate

qRT-PCR: quantitative real time polymerase chain reaction

RGD: arginine, glycine, aspartic acid

RIPA: ristocein induced platelet aggregation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of mean

SEM: standard error of mean

siRNA: short interfering RNA

SMC: smooth muscle cell

SSC: superclustering

TF: tissue factor

TGFβ: transforming growth factor beta

Tie-2: Tyrosine Kinase with Immunoglobulin Epidermal growth factor like domains

TNF: tumour necrosis factor alpha

TRITC: tetramethylrhodamine

TTP: thrombocytopenia purpura

VCAM1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

VEGFR2: vascular endothelial growth factor receptor 2

VWD: Von Willebrand Disease

VWF: Von Willebrand Factor

WPB: Weibel palade body

1 Chapter One: Introduction

1.1 The vasculature

One of the first organ systems to appear during embryogenesis is the cardiovascular system; which, driven by the heart, moves blood and its constituents through an intricate network of blood vessels (Risau, 1997). These vessels provide a route for immunological surveillance, nutrient and gaseous exchange and removal of toxic materials from tissues. This closed loop network consists of the arterial system, carrying blood away from the heart at high pressures and the venous system providing a return route; the exception to this being the pulmonary artery and vein. The capillary system provides not only the connection between the arterial and venous system, but is required for nutrient and metabolite exchange with the tissue.

Capillaries are the smallest blood vessels, consisting of endothelial cells (ECs) which line the lumen of all blood vessels. The EC layer provides a direct interface between blood and the underlying vessel wall. Perivascular cells such as pericytes surround capillaries, but larger vessels are supported by vascular smooth muscle cells (VSMC). These two cell types interact and modulate each other's activity in a paracrine fashion through growth factor signals (discussed in detail below). ECs play an important role in blood vessel formation and vascular maintenance, through their ability to regulate vessel wall permeability and respond to changes a plethora of mechanical and chemical signals (discussed in detail below).

Formation of blood vessels occurs through three main processes; vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis describes the formation of the first primitive vascular plexus during development by angioblasts. These angioblasts aggregate to form blood islands which subsequently differentiate into ECs which assemble to form the vascular plexus (figure 1.1). This process does not require the presence of an established vasculature. Formation of subsequent blood vessels from the pre-existing vasculature occurs through the process of angiogenesis. Distinct from angiogenesis, the process of arteriogenesis occurs as a result of the growth of collateral arterioles into larger collateral arteries. Arteriogenesis occurs from mechanical expansion of arterioles due to increased blood pressure caused by an arterial occlusion (reviewed in van Royen et al 2001).

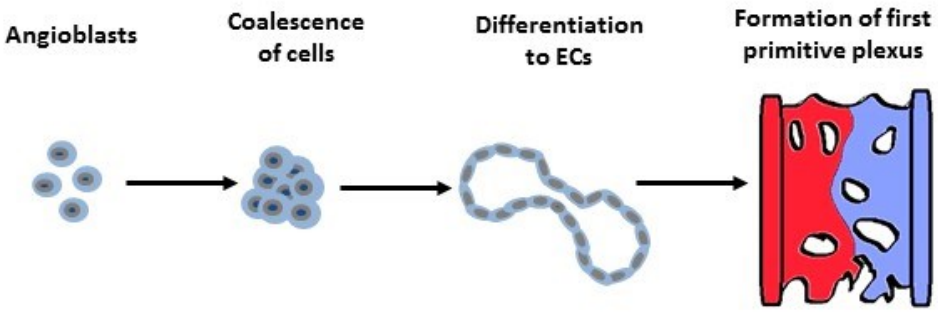

Figure 1.1 Formation of the first primitive vascular plexus through vasculogenesis. During development hemangioblasts develop into endothelial progenitor cells known as angioblasts. These cells coalescence to form blood islands and differentiate into endothelial cells where they start to aggregate, align and begin to form the first primitive plexus in the embryo. Modified from Carmeliet 2003.

1.2 Overview of Angiogenesis

Most of our understanding of the cellular basis of angiogenesis has come from elegant *in vivo* studies using the developing zebrafish embryo and *ex vivo* analysis of the vasculature in the retina of new-born mice. This is because in the adult, physiological angiogenesis is relatively low except during the menstrual cycle, pregnancy and wound healing. The process of angiogenesis involves a large number of molecular players (Carmeliet, 2003, Carmeliet and Jain, 2011). For the purpose of this project, I will focus on three categories: growth factor signalling pathways, adhesion mechanisms and transcriptional regulators. Before describing in detail some of these mechanisms relevant to this thesis, I will first provide an overall sequence of events during angiogenesis.

1.2.1 Sprouting angiogenesis: basic events

Under normal physiological conditions EC turnover is relatively low, which may in part be explained by their plasticity and ability to respond to stimuli in their environment. One of the most potent and well characterised growth factor signals to initiate angiogenesis is vascular endothelial growth factor (VEGF). VEGF is released from hypoxic tissues, tumour or inflammatory cells. In the retina, VEGF released from astrocytes is retained within the local environment (Stone et al., 1995, Provis et al., 1997). VEGF splice variants expressing a heparin binding domain have been shown to be important for VEGF binding to the extracellular matrix (ECM). Splice variants that do not bind the ECM readily diffuse further away from the area of production, thereby establishing a VEGF gradient (Ruhrberg et al., 2002). VEGF activates a single EC, which takes on a more motile/invasive phenotype and extends filopodia (Figure 1.2B). This “tip cell” guides this EC sprout towards the stimuli (Gerhardt et al., 2003, Lamalice et al., 2007). In the quiescent endothelium (figure 1.2A), cell-cell contacts maintain barriers. During angiogenesis, VEGF also causes phosphorylation and internalisation of junctional molecules such as VE-Cadherin (Dejana, 2004, Esser et al., 1998), causing junctions to loosen (figure 1.2C) to allow migration. If all the EC migrated, the integrity of the vessel would be compromised, therefore trailing stalk cells proliferate behind tip cells to maintain the integrity of the vessel (figure 1.2D). Stalk cells proliferate and elongate the stalk and driving directional movement towards the angiogenic stimuli. To establish a new anastomosis (figure 1.2E), tip

cell behaviour must be suppressed. This occurs upon repulsion signals after establishing contact with another sprout or re-establishing a balance between pro and anti angiogenic signals (reviewed in Adams and Alitalo, 2007).

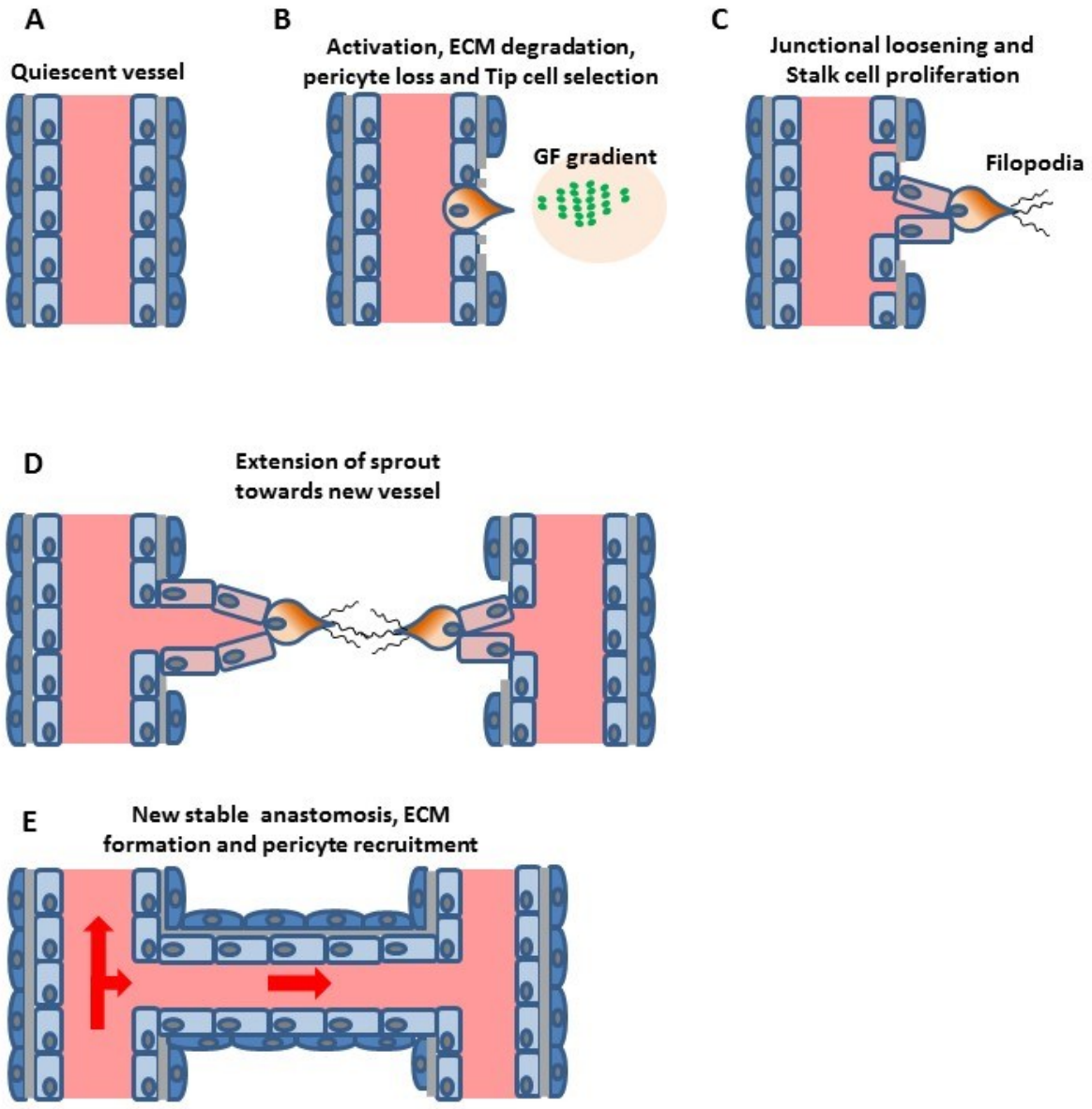

Figure 1.2 Formation of blood vessels from the pre-existing vasculature by angiogenesis. (A) A quiescient blood vessel with endothelial cells, matrix and supporting mural cells. (B) The presence of growth factors leads to selection of one endothelial cell known as a tip-cell which changes morphology and migrates towards the angiogenic region. Basement matrix of the angiogenic vessel degrades to facilitate angiogenesis and support cells such as pericytes loose attachment. (C) Tip cells extend filopodia and migrate towards the angiogenic stimulus with trailing stalk cells proliferating behind the tip cell to maintain the the integrity of the new sprout. EC-EC junctions loosen in the vasculature a facilitate EC proliferation and movement. (D) Cessation of angiogenesis usually occurs when a tip cell meets a new vessel, another tip cell or growth factor stimulus. (E) Formation of a lumen after fusion of the newly formed sprout allows establishment of a new sprout and blood flow. Mural cell recruitment and ECM formation provides the final stages in the formation of a stable plexus.

1.2.2 Maturation

Recruitment of mural cells such as pericytes plays an important role in maintaining vascular integrity (Lindblom et al., 2003, Hellström et al., 2001). Pericytes not only provide growth factor signals critical for vascular maintenance, but also facilitate matrix deposition (Gerhardt and Betsholtz, 2003). As mentioned above (section 1.1), EC-mural cell cross talk plays an important role in vascular homeostasis. This is demonstrated from the release of platelet-derived growth factor (PDGF)-B from ECs which causes pericyte recruitment (Lindblom et al., 2003). In particular, pericytes secrete angiopoietin 1 (Ang-1) which provides vital stabilisation signals to the endothelium (discussed in detail in section 1.3.2). The production of transforming growth factor beta 1 (TGFβ-1) is another important growth factor signal which promotes the production of ECM, helping to promote a stable vessel (Gaengel et al., 2009). Collectively, these signals prevent the EC from proliferating and recruit pericytes and VSMC, resulting in a mature vessel able to withstand blood flow pressures.

1.2.3 Pathological angiogenesis

Since blood vessels are present in all tissues, it is easy to understand their contribution to disease when their formation is impaired. Dysregulated angiogenesis has been implicated in a number of diseases, such as rheumatoid arthritis, diabetic retinopathy, vascular malformations and metastasis of tumours (reviewed in Carmeliet 2003, Carmeliet and Jain, 2011, Junquera et al., 1999). Both physiological and pathological angiogenesis share a similar cascade of events. In the tumour microenvironment, hypoxia and oncogenic production of growth factors stimulates the same cascade described above. This angiogenic response although beneficial for the tumour, by providing the necessary nutrients associated with a good blood supply, causes continuous production of growth factors leading to defective vascular function (Ziyad and Iruela-Arispe, 2011). In particular, these vessels often have a characteristic leaky and tortuous phenotype (Weis and Cheresh, 2011). In addition, the leaky nature of these vessels provide a route where tumour cells can enter the blood supply to the host facilitating metastasis.

Despite potent efficacy of anti-angiogenic therapies in pre-clinical models, most notably anti-VEGF treatment, translation to clinical use have been not very promising (Perren et al., 2011).

The exact mechanism for the discrepancies between pre-clinical and clinical outcomes remain unknown. In part, it is thought that drug delivery in patients is hindered due to the defective tumour vasculature. In addition, resistance to these drugs has been a major limiting factor in establishing effective treatment regimes. New focus of research has been to take both dual growth factor inhibition approaches as well as improving vascular function to increase drug delivery and efficacy (Cao, 2016).

1.3 Signalling pathways regulating angiogenesis

1.3.1 VEGF-VEGFR2 signalling in angiogenesis

It is noteworthy that, despite the complexity of angiogenesis, one growth factor, namely VEGFA has been implicated in most aspects of the process (Ruhrberg, 2003). The VEGF family comprises seven members: VEGFs A-F and placental growth factor (PlGF), and three receptors: VEGFR1, also known as Flt-1, VEGFR2, also known as Flk-1 and VEGFR3, also known as Flk-4 (Hoeben et al., 2004, Koch and Claesson-Welsh, 2012). It is well recognised that the VEGF family members have different functions; however this section will focus mainly on the biology of $VEGFA_{165}$ and its interplay with VEGFR2 because this is considered the main VEGF receptor involved in angiogenesis.

VEGFR2 is the most intensely studied of the VEGF receptors because of its restriction to ECs. Global deletion of VEGFR2 in mice is lethal resulting in death at E8.5 (Shalaby et al., 1995). These mice failed to form “blood islands”, the initial step of angioblasts coalescence during vasculogenesis. Later studies found that complete VEGFA-null mice demonstrated abnormal vascular formation resulting in embryonic lethality similar to VEGFR2-null animals at E8.5 (Carmeliet et al., 1996, Ferrara et al., 1996). These studies provided some of the first evidence for the accepted view that VEGFR2 is the main signal transducer of VEGFA during vascular development.

VEGFA binding to VEGFR2 causes dimerization of the receptor resulting in downstream phosphorylation of tyrosine resides on the intracellular tail of receptor. The best characterised of these residues are Y951, Y1059, Y1175 and Y1214 (Koch and Claesson-Welsh, 2012, Simons et al., 2016). For the purposes of this thesis, I will focus on Y1175 residue which

has been described to be crucial for embryonic development because inactivation of this residue *in vivo* results in a similar phenotype to the VEGFR2 null mouse (Sakurai et al., 2005, Shalaby et al., 1995). $VEGFA_{165}$ activation of VEGFR2 through Y1175 has been shown to lead to activation of ERK through activation of phospholipase C gamma (PLCγ) (Takahashi et al., 2001). This supports previous work from this group which suggested that VEGFR2 activation of PLCγ-protein kinase C (PKC)-ERK led to activation of a mitotic programme in ECs which was independent of PI3K-Akt signalling (Takahashi et al., 1999). Additionally, the adaptor protein Shb binds to Y1175 and is activated by phosphorylation of this residue on VEGFR2. Shb can directly interact with Focal Adhesion Kinase (FAK) which is tyrosine phosphorylated (Holmqvist et al., 2003, Holmqvist et al., 2004). FAK is a key mediator of cell migration through its interaction with integrins (discussed in section 1.3.3) and actin rearrangements (reviewed in Parsons, 2003). This pathway is thought to be one manner in which VEGF can promote cell migration. Activation of PI3K in VEGF-stimulated cells triggers activation of Akt promoting pathways involved in cell survival (Abid et al., 2004, Gerber et al., 1998).

Akt activation by VEGF is known to activate endothelial nitric oxide synthase (eNOS) and stimulate internalisation of the junctional molecule VE-Cadherin (Koch and Claesson-Welsh, 2012, Dejana, 2004, Esser et al., 1998). A decrease in junctional localisation of VE-Cadherin stimulated by VEGF is one mechanism through which VEGF can induce permeability. VE-Cadherin associates with β-catenin which can translocate to the nucleus and induce transcription (Corada et al., 2010). The cadherin family can also associate with growth factors and modulate signalling. It is thought that this is at least one potential mechanism through which EC in a confluent (quiescent) monolayer can respond in the presence of pro-angiogenic growth factors like VEGF (Dejana, 2004).

At least two mechanisms exist for fine tuning of VEGFR2 signalling. Neuropillin-1 (NRP-1) is a single transmembrane protein originally thought to lack catalytic function. This co-receptor binds to VEGFR2 to enhance signalling (Koch et al., 2011, Mamluk et al., 2002). Inhibition of NRP-1 expression reduced $VEGFA_{165}$-induced phosphorylation of both Y1175 on VEGFR2, and S473 on Akt. However, inhibition of NRP-1 in the presence of $VEGFA_{165}$ did not affect ERK1/2-phosphorylation (Raimondi et al., 2014). Additionally, NRP-1 deficiency resulted in severe deficiency in cardiac, neural and yolk sac development (Kawasaki et al., 1999), suggesting the

importance of NRP-1 for embryonic vascular development. A second mechanism of regulating VEGFR2 signalling is through integrins such αvβ3. αvβ3 appears to limit the contribution of NRP-1 to VEGFR2 signalling (Reynolds et al., 2002, Robinson et al., 2009). Mice deficient in integrin β3 show enhanced angiogenesis and increased VEGFR2 signalling (Reynolds et al., 2002). In addition, inhibition of NRP-1 in β3 null cells significantly reduced the enhanced VEGFR2 signalling (Robinson et al., 2009). These findings highlight the complex regulation of this signalling pathway which is so vital to angiogenesis.

1.3.2 Angiopoietin-Tie-2 signalling in angiogenesis

1.3.2.1 Angiopoietin system receptors: Tie-1 and Tie-2

Tyrosine kinase with immunoglobulin-like and epidermal growth factor (EGF) like domain 1 and 2 (Tie-1 and Tie-2) were originally discovered in the early 1990s in a search for endothelial protein tyrosine kinases contributing to cardiogenesis and haematopoiesis (Partanen et al., 1992, Fagiani and Christofori, 2013). Tie-2 is the predominate receptor on endothelial cells for the transduction of angiopoietin signalling. Although the extracellular domains of these receptors share only 30% amino acid (aa) homology, the overall protein structure is very similar (Yu et al., 2013). These tyrosine kinases as the name suggests, consist of immunoglobulin and EGF-like repeats with three fibronectin type III domains (arrangement shown in figure 1.3A). The angiopoietin ligands interact with Tie-2 via the C-terminal fibronectin-like domain. While most receptor tyrosine kinases undergo conformational change induced by ligand binding, Tie-2 is unique in that binding and signal transduction occurs instead through a lock and key mechanism (Barton et al., 2006). Intracellular signals are conducted by a tyrosine kinase region.

Using mice deficient in Tie-2, Dumon et al., demonstrated that Tie-2 is required for vascular development, since its deletion caused embryonic lethality with vascular defects around E9.5, accompanied by haemorrhage and an underdeveloped heart (Dumont et al., 1994). Deletion of Tie-1 in murine embryos also produced haemorrhage similar to that seen in the Tie-2-deficient mouse. However, the vascular defects appeared to be less pronounced,

suggesting that while Tie-2 was required for early cardiac development and vascular proliferation,Tie-1 might be dispensable (Puri et al., 1995).

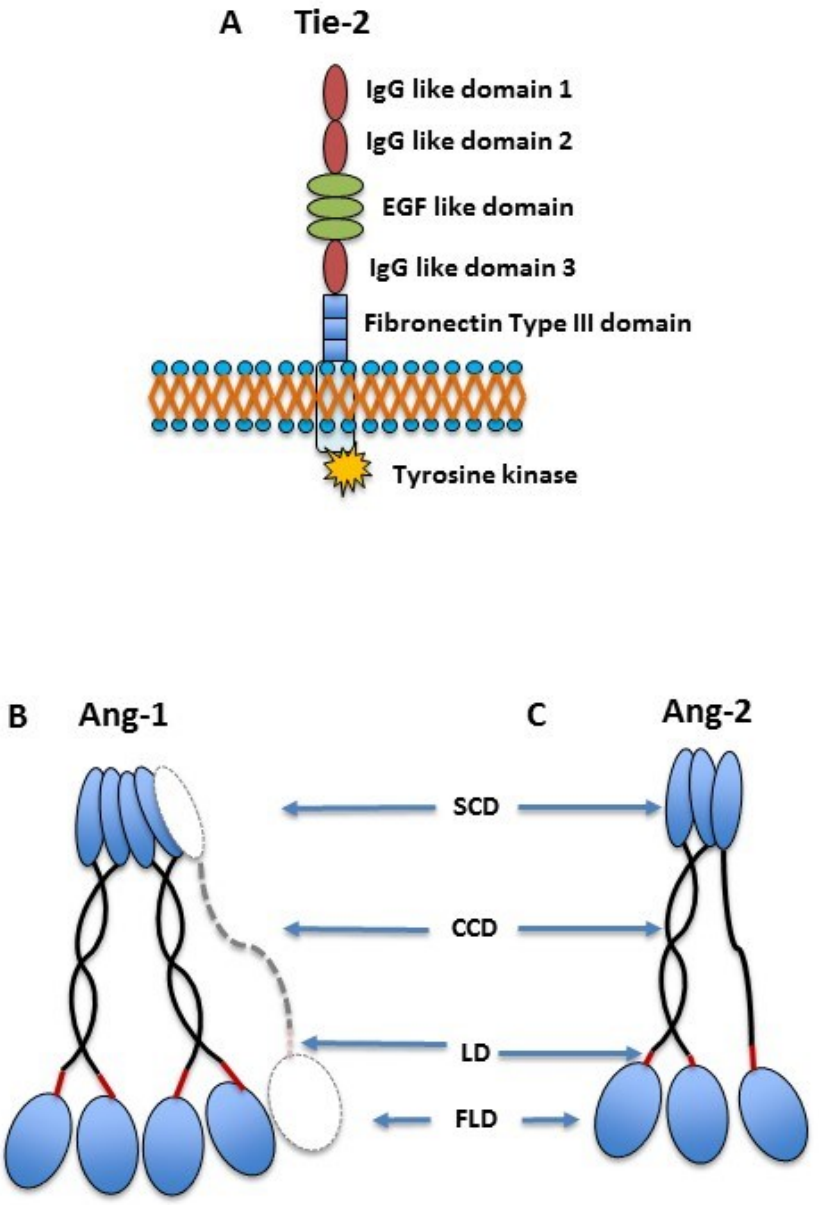

Figure 1.3 Angiopoietins and Tie-2 structure. (A) Schematic representation of Tie-2 receptor. Immunoglobulin (IgG)-like domains (Red), epidermal growth factor (EGF)-like domains (green) and three fibronectin type III domain repeats (blue box).Intracellular signals are conducted by a tyrosine kinase domain. Schematic representation of Angiopoietin 1 (Ang-1) (B) and Angiopoietin 2 (Ang-2) (C). Structurally these ligands for the Tie-2 receptor are very similar comprising a super clustering region (SCD), coiled-coiled domain (CCD), linker domain (LD) and fibrinogen like domain responsible for Tie-2 binding (FLD). Ang-1 exists primarily as higher order oligomers while Ang-2 primarily exists as trimers (Barton et al., 2005; Barton et al., 2006).

1.3.2.2 Angiopoietin system ligands: focus on Ang-1 and Ang-2

Early cloning studies revealed that there are four members of the angiopoietin family. The least studied members of this family are Ang-3 and Ang-4. Ang-3 is found in mouse and Ang-4 has been identified as the human orthologue. Ang-4 stimulates Tie-2 activity similarly to Ang-1, but Ang-3 (the mouse counterpart) does not. (Valenzuela et al., 1999). All angiopoietins share a similar structure. They consist of a super clustering domain at the N-terminus, followed by a coiled-coil domain and a fibrinogen-related domain at the C-terminus through which they bind to the Tie-2 receptor (figure 1.3B). Proteins of the angiopoietin family are known to form complex multimeric structures. While Ang-1 forms mainly higher order oligomers, Ang-2 is classically thought to exist as dimers, trimers and tetramers (Barton et al., 2005, Barton et al., 2006) (figure 1.3B and C).

Ang-1, is produced in mural cells such as pericytes and VSMC rather than the endothelium like Ang-2 (Augustin et al., 2009). Identification of Ang-1 and Ang-2 expression profiles revealed that in the adult Ang-1 was present in most tissues, whereas Ang-2 was only detected in the ovaries, uterus and placenta, suggesting a preference for tissues where active remodelling is occurring (Maisonpierre et al., 1997). Global deletion of Ang-1 revealed that these mice phenocopied the Tie-2 deleted mouse, with embryonic lethality at day E12.5-13.5 (Suri et al., 1996, Dumont et al., 1994). Although Ang-1 deficient mice do form a vascular network prior to death, this vasculature is less complex, has less association of endothelium and the ECM and has dilated vessels. Global deletion of Ang-2 in mice results in a normal Mendelian number of births, however, mice die later due to chylus ascities on 129/J background at around 12 weeks (Gale et al., 2002). This study also revealed that the defects in lymphatic vessels was rescued by Ang-1 administration. Despite normal blood vessels in the adult, analysis of postnatal retinal vascularisation revealed a decrease vascularisation in Ang-2-deficient mice compared to controls. This demonstrated that while Ang-2 was dispensable for embryonic blood vessel development, it was required for postnatal angiogenesis. Together these studies demonstrated that both Ang-1 and Ang-2 signal transduction via the Tie-2 receptor are important for vascular maintenance but only Ang-1 is required for vascular formation during development.

1.3.2.3 Angiopoietin signalling in the endothelium

1.3.2.3.1 Angiopoietin signalling in vascular quiescence

Overexpression of Ang-1 has been shown to synergise with VEGF in angiogenesis (Thurston et al., 2000, Thurston et al., 1999). In mice treated with mustard oil to induce permeability, both adeno overexpression and genetic overexpression of Ang-1 rendered the vasculature of skin and ear resistant to potent permeability and inflammatory agent-induced effects. These studies proposed that Ang-1 signalling via Tie-2 promoted vascular stability signals. Recently it has been suggested that Ang-1 may not be required for quiescence (Jeansson et al., 2011). In this study, genetic deletion of Ang-1 before E13.5 resulted in death as expected with the global Ang-1 deficient mouse. Surprisingly however, deletion of Ang-1 after E13.5 resulted in mice being born normally with no apparent vascular defects. Additionally, post-natal vascularisation of the retina was normal compared to controls. In a model of wound healing this study also found that deletion of Ang-1 after E13.5 resulted in enhanced would closure and angiogenesis, suggesting the Ang-1 acts to limit angiogenesis.

In vitro studies demonstrated that stimulation of endothelial cells with Ang-1 leads to clustering and phosphorylation of Tie-2 (figure 1.4) (Davis et al., 1996, Daly et al., 2004). Ang-2, on the other hand, was shown not to produce efficient clustering and therefore did not increase Tie-2 activity (Davis et al., 2003). However, recently it has been proposed that high concentrations or prolonged exposure of EC cells to Ang-2 can in fact induce Tie-2 phosphorylation (Yuan et al., 2009). This supports the accepted view that Ang-2 is a context-dependent antagonist. To delineate Tie-2 signalling in the proliferating vs the quiescent endothelium, two elegant studies reported that Ang-1 stimulation of a confluent monolayer of EC (quiescent) induced potent Tie-2 phosphorylation (Fukuhara et al., 2008, Saharinen et al., 2008). Importantly, they also revealed that Tie-2 activation at the EC-EC junction resulted in robust activation of PI3K-Akt activity. This led to downstream phosphorylation of the transcription factor FOXO1 (discussed in section 1.3.4) and increased expression of genes such as eNOS, which promote EC quiescence (Fukuhara et al., 2008, Saharinen et al., 2008). Additional studies demonstrated increased expression of the Notch ligand Delta like ligand 4 DLL4 which is an important driver of homeostasis after Ang-1 activation of ECs. The resultant increase in Akt signalling led to phosphorylation and deactivation of glycogen synthase kinase

3β (GS3Kβ), which acts to degrade β-catenin (Zhang et al., 2011). This stabilisation of β-catenin is important for the regulation of cell-cell junctions as well as transcriptional activation of notch signalling, thereby promoting EC quiescence (Zhang et al 2011; Birdsey et al 2015).

In addition, a study revealed that Tie-1 can act as an inhibitor of Tie-2 signalling (Seegar et al., 2010). This suggested that the interaction of angiopoietins with Tie-2 activates distinct downstream signalling pathways which are determined by the degree of Tie-1 signal activation. This presented a more complex regulatory mechanism of signalling via the angiopoietins. A previous report suggested that Tie-1 could signal in an Akt-dependent manner (Kontos et al., 2002); however, these findings could not be explained because Ang-1 does not bind Tie-1. Interestingly, kinase dead mutations of Tie-2 inhibit Tie-1 activity (Yuan et al., 2007). This suggested that what was originally thought of as Tie-1 signalling could be due to the activity of Tie-2. Collectively, these data suggest a model where during quiescence Ang-1 mediates vascular integrity through Tie-2, which may be fine-tuned through Tie-1 dependent mechanisms.

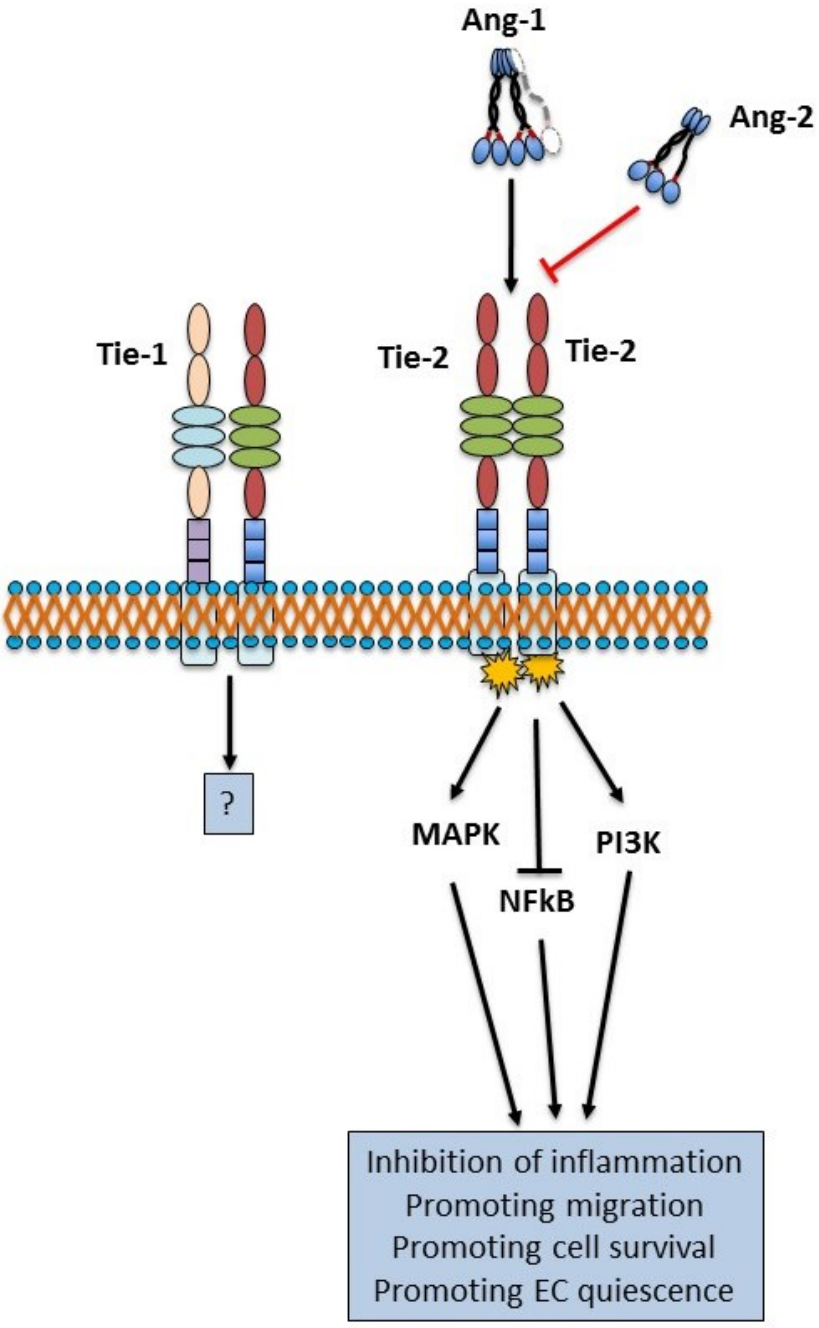

Figure 1.4 Model of Angiopoietin-Tie-2 signalling in endothelial cells. Angiopoietin 1 (Ang-1) binds to Tie-2 receptor causing Tie-2 dimerisation and autophosphorylation. Once activated, Tie-2 phosphorylation leads to activation of phosphoinositide 3- kinase (PI3K; Daly et al., 2004), Mitogen-activated protein kinases (MAPK) and inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB; reviewed in Augustin et al., 2009). Subsequently this leads to inhibition of inflammation, promoting of cell survival and quiescence. In some instances activation of Tie-2 can cause cell migration and proliferation. Ang-2 on the other hand acts as an antagonist of Tie-2 signalling. In addition, Tie-1 appears to limit Tie-2 signalling however the exact molecular mechanisms remain unclear.

1.3.2.3.2 Angiopoietin signalling during endothelial activation

In line with its homeostatic role, Ang-1 has been shown to decrease expression of pro-inflammatory molecules such as ICAM-1 and VCAM-1 in the endothelium (Kim et al., 2001). Expression of these proteins are crucial for the firm adhesion of leukocytes prior to diapedesis during the inflammatory response. In ECs, a stored pool of the Tie-2 antagonist Ang-2 is present in WPB, allowing it to be rapidly released upon endothelial activation (Fiedler et al., 2004). Moreover, Ang-2 expression is upregulated by inflammatory signals such as LPS and TNF-α (Fiedler et al., 2006, Mofarrahi et al., 2008, Fiedler et al., 2004). This causes a decrease in the ratio of Ang-1 to Ang-2, resulting in antagonism of Tie-2 signalling and increased proinflammatory molecule expression. Consistent with this proinflammatory function of Ang-2, inhibition of Ang-2 using neutralising antibodies was shown to reduce cellular infiltrates in a model of airway inflammation, (Tabruyn et al., 2010). In support of these findings, mice deficient in Ang-2 show decreased leukocyte recruitment in response to intraperitoneal injection of *Staphylococcus aureus* (Fiedler et al., 2006). In this study the authors demonstrated that 2 μg/ml Ang-2 alone had no effect on leukocyte adhesion to ECs. In addition, 0.005 ng/ml tumour necrosis factor alpha (TNF-α) did not affect leukocyte adhesion to EC. However, together Ang-2 and TNF- α appeared to induce strong adhesion of leukocytes to ECs. This suggested that Ang-2 sensitises ECs to TNF-α stimulation. (Fiedler et al., 2006).

In vitro, Ang-1 stimulation of ECs reduced basal, VEGF and thrombin induced permeability (Gamble et al., 2000). Moreover, this study showed that inhibition of the PI3K pathway did not ablate the effects of Ang-1, suggesting an alternative pathway. While this study did not characterise further which pathway was involved, it showed that Ang-1 stimulation of EC decreased phosphorylation of VE-Cadherin and suggested that this may be one way of protecting EC junctional integrity. A subsequent study revealed that thrombin induced activity of small GTPase RhoA was significantly reduced by Ang-1; in addition, this was unchanged by Ang-2 (van der Heijden et al., 2011). Given the established role of enhanced RhoA activity in regulating permeability (Amerongen et al., 2000), this suggested a potential pathway through which Ang-1-Tie-2 signalling inhibits permeability. While these cellular studies described how Ang-1-Tie-2 signalling inhibits permeability, later studies discussed in section 1.3.3 revealed that cross talk between angiopoietins and integrins may also play a role.

Decrease in Ang-1/Ang-2 ratio in plasma of patients with inflammatory diseases have been reported. A few studies have identified higher Ang-2 levels to be a predictor of more worse outcomes in inflammatory diseases such as sepsis, where currently there are limited treatment options (Orfanos et al., 2007, van der Heijden et al., 2009b). These studies showed that, during sepsis, enhanced plasma Ang-2 levels are associated with increased vascular leakage and worse outcomes. Given the conflicting *in vitro* reports which suggests an agonist nature of prolonged Ang-2 exposure (Yuan et al., 2009), two recent studies tried to delineate the potential agonist nature of Ang-2 *in vivo.* These demonstrated for the first time *in vivo* that Ang-2, under baseline conditions, activates Tie-2, which was ablated in the presence of Tie-1 (Kim et al., 2016, Korhonen et al., 2016). Under inflammatory conditions, Tie-1 is shed and this facilitates the antagonist nature of Ang-2 (Korhonen et al., 2016). Collectively, these data demonstrate that during endothelial activation, Ang-2 is predominately a pro-inflammatory molecule and causes endothelial leakage.

1.3.2.3.3 Angiopoietin 2 in postnatal vascularisation and maturation

Ang-2 expression is increased by angiogenic growth factors such as VEGF and bFGF, as well as environmental changes such as hypoxia (Hegen et al., 2004, Pichiule et al., 2004, Simon et al., 2008). Increased expression of Ang-2 at sites of vascular remodelling provided the first evidence for a role for Ang-2 in angiogenesis *in vivo* (Maisonpierre et al., 1997). *In vivo*, while embryonic vascular development appeared normal, post-natal retinal vascular development was reduced in the global Ang-2 KO mouse (Gale et al 2002). Decreased Ang-2 was also found to have profound effects on lymphangiogenesis, causing chylous ascites and post-natal death in most mice at around 12 weeks. Surprisingly, while rescue with Ang-1 corrected lymphatic defects, defects in vascularisation were not corrected. The authors proposed that in lymphangiogenesis Ang-2 acts as a classical antagonist, but during post-natal angiogenesis Ang-2 behaves like an agonist. It is noteworthy that while characterising the Ang-2 KO mouse, the Augustin lab found that backcrossing onto a C57BL/6 background reduces the effects of the chylous ascites (Fiedler et al., 2006).

At the angiogenic front of the retina, Felcht and colleagues observed decreased Tie-2 expression with enhanced Ang-2 (Felcht et al., 2012). Since angiopoietins have been shown to bind integrins (Carlson et al., 2001), Felcht and colleagues investigated whether integrins act as an alternative receptor for Ang-2 in the absence of Tie-2 (Felcht et al., 2012). The authors revealed that, during retinal angiogenesis, Ang-2 signalled via integrin $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha v\beta 5$ to activate FAK Y397 and drive sprouting. Supported by their previous work (Thomas et al., 2010), they proposed a new model whereby Ang-2, in the presence of Tie-2, binds to integrin $\alpha v\beta 3$, inducing signalling via FAK S910 causing internalisation of this integrin and therefore a destabilised phenotype (Thomas et al 2010). Since there was no evidence for Ang-2 containing an arg-gly-asp (RGD) integrin binding site, it has been proposed that Ang-2 interacts with integrins through a metal-ion-dependent adhesion (MIDAS) region. This region is present in a number of other molecules across varying cell types and has been shown to be another essential site for integrin-ligand binding(Takagi, 2007). Central to this binding is the presence of a magnesium ion (Mg^{2+}) (Bergelson and Hemler, 1995). In the 2012 study, the Augustin lab demonstrated an increase in HUVEC adhesion to plate bound Ang-2 in the presence of Mg^{2+} (Felcht et al., 2012). This provided the first suggestion for the interaction of Ang-2 with integrins. Collectively these data suggest that Ang-2 regulates postnatal vascularisation and this can occur via an Ang-2 integrin interaction.

In a mouse model of streptozotocin (STZ) induced diabetic retinopathy, pericyte coverage of vasculature was decreased (Park et al., 2014). In addition, Ang-2 levels were significantly increased in retina of mice treated with STZ. Since pericytes do not contain receptors of Ang-2, this study investigated whether integrins were involved. They found that high glucose increased the expression of $\beta 1$ and $\alpha 3$ integrin expression in pericytes. Importantly, blockage of these integrins were able to significantly reduce the Ang-2 dependent pericyte apoptosis. This suggested that Ang-2-integrin interaction may be important in regulating pathology. Analysis of Ang-2 through mutational studies revealed that a Gln residue not involved in Tie-2 binding was crucial for Ang-2 interacting with $\alpha 5\beta 1$, in particular the alpha subunit (Lee et al., 2014). This study proposed that blockade of Ang-2-integrin interaction play a key role in reducing tumour metastasis. Interestingly, in the presence of inflammatory stimuli such LPS both *in vitro* and *in vivo*, Tie-2 levels have been shown to be reduced (Ghosh et al., 2016,

Kurniati et al., 2013, Zeng et al., 2016). These studies proposed that potentially the Ang-2-integrin interaction may play a more important role in vascular pathology than initially thought.

1.3.3 Integrin and extracellular matrix in angiogenesis

As mentioned previously, ECs provide a crucial interface between the vascular lumen and the vessel wall. Their plasticity and ability to respond to various stimuli is exemplified by the high expression of both growth factor receptors and integrins on the cell surface. Integrins not only provide cell attachment to the ECM, but also convey signals from the ECM to inside the cell (outside in) and *vice versa* (inside out). These heterodimeric glycoproteins consist of an α and β subunit linked non-covalently. These associate to form twenty four individual $\alpha\beta$ combinations (reviewed in Avraamides et al.,, 2008). In ECs, the most intensely studied integrins are $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$. First identified to bind vitronectin, fibronectin and laminin respectively, the list of ligands for these cell surface receptors is increasing. In particular, the integrin $\alpha v\beta 3$ has been shown to bind von Willebrand factor (VWF) (Cheresh and Spiro, 1987).

Recognition of ligands by most integrins occurs predominately via an RGD site (Ruoslahti and Pierschbacher, 1986), however the MIDAS region has been shown to mediate ligand-integrin interaction (discussed above in section 1.3.2.3.3). In contrast to growth factor receptors, the cytoplasmic tails of integrins have no activity. Signal transduction occurs via complexing with adaptor proteins at focal adhesion complexes. These complexes comprise integrins, protein kinases, adaptor proteins and cytoskeletal proteins which bind actin. The best characterised of these protein kinases is FAK (mentioned previously in section 1.3.2.3.3). Classical activation of FAK in its kinase domain occurs at Y397, the best characterised tyrosine residue on FAK. This facilitates binding sites for other kinases such as Src which promotes further FAK residue phosphorylation at the focal adhesion targeting (FAT) domain of the FAK molecule (Lechertier and Hodivala-Dilke, 2012). This in turn promotes binding of p130Cas and the cytoskeletal protein, paxillin and resulting in cellular migration. Additionally, the less well-characterised signalling at S910 on the FAT domain of FAK is thought to promote disassociation of FAK from focal adhesions. Surprisingly, ERK2 is thought to activate this process while also promoting

paxillin-FAK association (Mitra et al., 2005). This suggests some recycling of FAK at sites of focal adhesions.

As described above, integrins such as αvβ3 have been shown to regulate signalling of growth factor receptors such as VEGFR2 (Soldi et al., 1999). In addition, the integrin αvβ3 and α5β1 have been shown to associate with the tyrosine kinase receptor Tie-2 (Cascone et al., 2005, Thomas et al., 2010). These complexes are activated by both ligands for this receptor: Ang-1 and Ang-2. In addition, different functional outcomes of angiopoietin signalling have been observed through integrin interaction. While the agonist of this receptor, Ang-1, appears to induce tyrosine FAK phosphorylation and promote cell motility (Cascone et al., 2005), the classic antagonist, Ang-2, promotes integrin internalisation and degradation and activation via S910 (Thomas et al., 2010). The later study may provide the first evidence for the function of S910 residue on integrins.

1.3.4 Transcriptional regulation of angiogenesis: FOXO1

Both growth factor and integrin signalling activate several signalling pathways which consequently are responsible for distinct transcriptional programmes. For example, distinct molecular programmes between tip and stalk cells allow differential responses during the angiogenic process. In particular, tip cells express VEGF receptor 2 (VEGFR2) which mediates the response to VEGF. Additionally, tip cells have increased expression of Delta Serrate LAG-2 (DSL) ligands like Dll4, which bind to Notch receptors on stalk cells (Phng and Gerhardt, 2009). The signalling provided by notch, suppresses expression of VEGFR2 on stalk cells and promotes expression of Jagged 1, which suppresses notch signalling in tip cells (reviewed in Adams and Alitalo, 2007). Decreased notch signalling decreased expression of the transcription factor hairy enhancer of split (HES1) which drives notch ligands (Blanco and Gerhardt, 2013). The number of transcription factors involved in angiogenesis and EC homeostasis continues to expand. For the purposes of this thesis I will highlight the role of one particular factor: Forkhead Box transcription factor (FOXO1).

FOXO1 belongs to a large family of transcription factors characterised by the DNA binding sequence termed “Forkhead box” identified in *Drosophila* (Kaestner et al., 2000). In particular,

FOXOs have been implicated in regulation of cell apoptosis, cell cycle transition, longevity, metabolism and growth (Calnan and Brunet, 2008). Additionally, mice deficient in FOXO1 die around E10.5-11 due to defective vascularisation (Hosaka et al., 2004, Furuyama et al., 2004). Interestingly, staining of the vascular plexus in the embryo at E9.5 reveal an immature but present plexus (Hosaka et al., 2004). These data suggested that FOXO1 was not required for vasculogenesis but rather for angiogenesis. Genetic deletion of FOXO1 from ECs *in vivo* caused enhanced vascular density with a blunting of the angiogenic front in the developing mouse retina in P5 pups (Wilhelm et al., 2016). Constitutive activation of FOXO1 in ECs resulted in death at E10.5 with decreased vascular complexity in the mouse retina at P5 (Wilhelm et al., 2016).

During development it seems clear that deletion of FOXO1 results in embryonic death, however, dissecting its involvement in angiogenesis has proved challenging. In line with their recent study *in vivo* (Wilhelm et al., 2016), the Potente lab showed in an *in vitro* assay, that FOXO1 siRNA caused increased EC sprouting (Potente et al., 2005). In the *ex vivo* aortic ring sprouting assay however, no effect on sprouting was observed in mice overexpressing FOXO1 (Dharaneeswaran et al., 2014). Given the differences in sprouting angiogenesis approach, no definitive conclusion can be drawn based on these findings. However, further evidence *in vitro* showed that inhibition of FOXO1 increases proliferation in the Potente lab (Potente et al., 2003) while decreasing proliferation in the Aird lab(Dharaneeswaran et al., 2014). To reconcile these differences, both the Potente and Aird lab have proposed that FOXO1 may have a “differential role” in the developing and established endothelium. To date the exact role of FOXO1 in vascular development remains unclear.

1.4 Von Willebrand Factor

1.4.1 Erik von Willebrand and Pseudohaemophillia

In 1926 Dr. Erik Adolph von Willebrand published the first paper reporting a bleeding condition, which presented in a young girl, Hjördis S, from the Aland Islands (translated in Von Willebrand, 1999). This condition presented with normal platelet numbers and morphology, which led to von Willebrand proposing the possibility of haemophilia. Since haemophillia is X-linked recessive, Hjördis S' father would have to be affected. Moreover, mothers affected with haemophilia always passed the defect onto their sons and mothers who are carriers pass the defect onto 50% of their sons. Upon tracing the pedigree of the proband, von Willebrand found that that the pattern of inheritance seemed different from haemophilia. Of the 23 members of the girl's family which were identified as "bleeders", more females than males appeared to be affected. To clarify whether this was a new observation, von Willebrand next analysed data from 27 other families affected by similar bleeding tendencies. Most notable were a few families provided by Dr Glanzmann, who first uncovered a defect in platelet integrins leading to a bleeding disorder (translated in Von Willebrand, 1999). To assist him with his analysis, von Willebrand consulted Federley, a colleague and geneticist who postulated a dominant sex-linked inheritance pattern, whereby inheritance of one dominant copy (heterozygous) in males or females is sufficient to cause a "mild sickness" while two copies (homozygous), as is possible in females, will cause "very sick" phenotypes. It would later be discovered that this hypothesis was in fact incorrect.

In the Hjördis' family, most of the deaths due to bleeding were from mild injury, such as teeth penetrating the tongue due to falls, intestinal bleeding, mouth or nasal bleeding. In some cases, von Willebrand noted bleeding to death from childbirth. In the case of Hjördis S, bleeding to death on her fourth menstrual period caused her death. The exact disease was unknown and von Willebrand called it "hereditary pseudohaemophilia."

1.4.2 Identifying Von Willebrand Factor

Extraordinarily, it was uncovered in the 1950s that infusion of plasma from patients with severe haemophilia could correct the bleeding in patients with pseudohaemophillia. (Nilsson et al., 1957). It was then discovered that patients with pseudohaemophilia showed decreased Factor VIII (FVIII) and that infusion of purified FVIII could prevent skin bleeding in some of these patients (reviewed in Sadler, 1998, Peake et al., 1974). This suggested that the plasma factor responsible complexed with FVIII and was present in patients with haemophilia. Later it was uncovered that purification of FVIII also isolated a larger protein in complex with FVIII. In the early 70s, Ted Zimmerman and colleagues purified an antibody against anti haemophilic factor. Additionally, they provided the seminal studies to show normal circulating levels of this factor in patients with haemophilia compared to von Willebrand Disease (VWD); named after the pioneering work of Erik von Willebrand (Zimmerman et al., 1971). These observations led to the discovery of Von Willebrand Factor (VWF). In addition, VWF appeared to act as a carrier for FVIII since decrease of VWF led to decreased FVIII levels(Meyer et al., 1991).

1.4.3 Molecular Biology of Von Willebrand Factor

1.4.3.1 Biosynthesis and structure of VWF

It was not until the 1970s that VWF was found to be synthesised in endothelial cells (ECs) and the platelet precursor cells: megakaryocytes (Nachman and Jaffe, 1975, Nachman et al., 1977, Jaffe et al., 1974). VWF cDNA was isolated by four separate groups simultaneously in 1985 (Sadler et al., 1985, Verweij et al., 1985, Ginsburg et al., 1985, Lynch et al., 1985). The VWF gene occupies 178 kb of the p arm of chromosome 12, consisting of 52 exons. This would rule out the initial inheritance patter proposed by Federley as VWF gene was autosomal. Unexpectedly, VWF cDNA probes also detected a second sequence on chromosome 22. However, this was later identified as pseudogene with no functionality as it contained multiple stop codons (Mancuso et al., 1991). In this study, Manusco and colleagues reported the complete structure of VWF, which provided a novel method for detecting mutations in the VWF gene without interference from the pseudogene.

The VWF sequence on chromosome 12 codes for a large, 2813 aa polypeptide. This polypeptide, known as pre-pro-VWF, encodes a 22 aa signalling peptide, and a 741 aa propeptide, with the remaining 2050 aa forming the mature peptide. These domains repeated in a "head to tail" fashion and can be represented using the formula: NH_2-D1-D2-D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH (reviewed in Sadler, 1998) (figure 1.5A). Since these early reports, advances in bioinformatics and structural analyses have allowed more detailed studies and the following structure has now been proposed: NH_2-D1-D2-D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK (Lenting et al., 2015) (figure 1.5B). The analysis of the D domains revealed that they were highly complex, consisting of a cysteine-8 (C8), trypsin inhibitor like (TIL) structure and an E module (figure 1.5C) (Springer, 2014). These domains further contain binding sites for key proteins involved in the function of VWF, including FVIII in the D' and D3 domain, platelet receptor GP1b in the A1 domain and an integrin RGD sequence in the C4 domain.

Early in the life cycle of VWF, after synthesis and entry into the endoplasmic reticulum (ER), the 22 aa signalling peptide is cleaved off. In the ER, VWF undergoes a number of posttranslational modifications including glycosylation by oligosaccharyltransferase (Lenting et al., 2015). The ER is also the site at which pro-VWF molecules dimerise through disulphide bonds at the C-terminal end (CK domain) (Springer, 2014). Upon arrival in the Golgi apparatus, VWF dimers begin their processing for multimer formation. Key to this multimerization process is the drop in pH to 6.2 in the trans-golgi compartment and the presence of calcium ions (Ca^{2+}) (Wagner et al., 1986). These conditions facilitate dimerization of all the VWF domains between A2 to CK as well as dimerization of D1-D2 regions. This is often described as the "zipper model" (Springer, 2014) (figure 1.6A). In addition, this environment favours VWF multimerization. The enzyme Furin cleaves VWF between the D2-D' domain. Following this, the D1-D3 domains can form non-covalently linked partners. During the trans-golgi dimerization events, VWF dimers begin to assemble into helices. The formation of the zipped up conformation is not limited to 2 molecules of VWF. As shown in (figure 1.6B), pro-VWF dimers assemble at the N terminal D domains. The D1 and D2 (propeptide) is important for VWF multimerization.

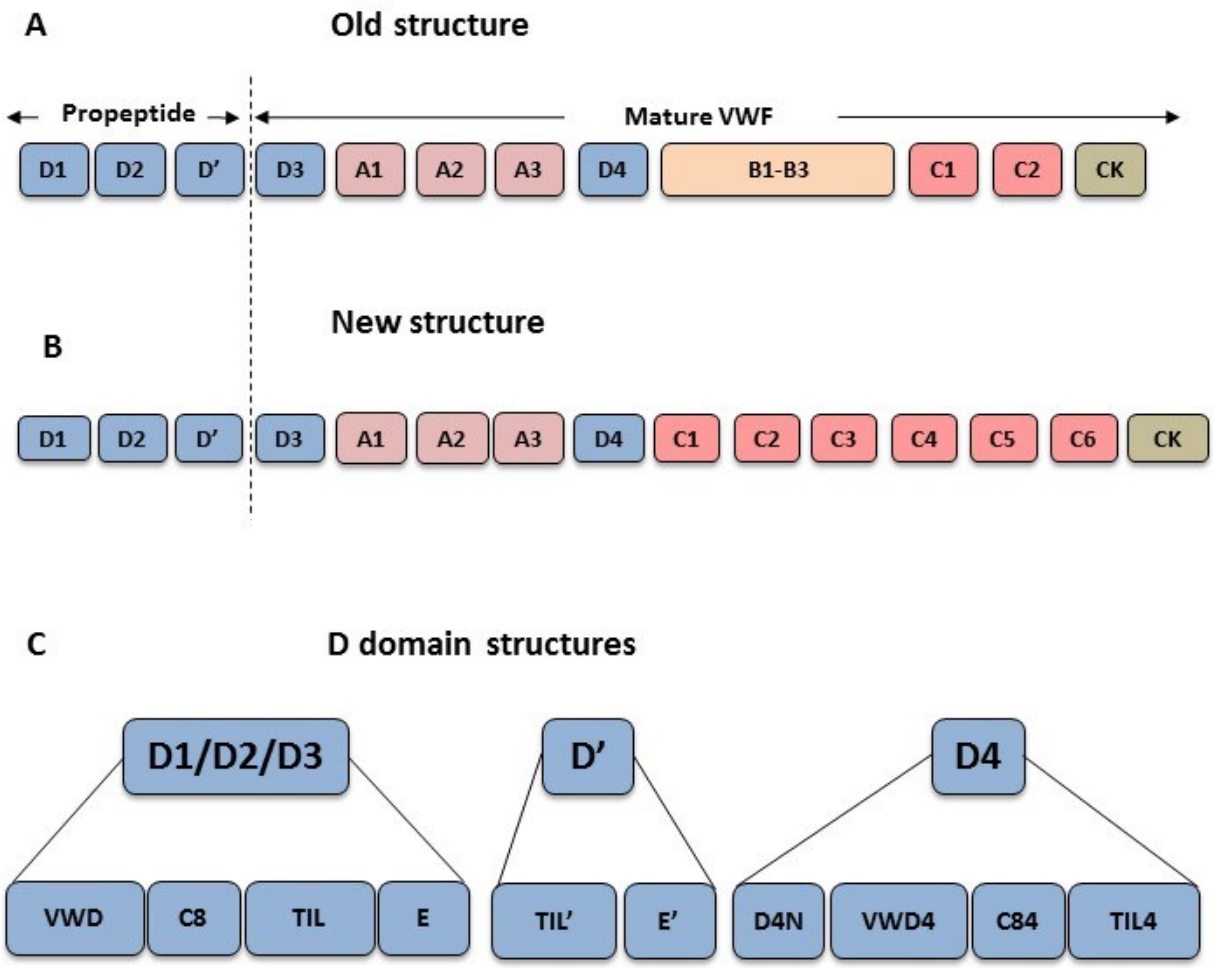

Figure 1.5 Structure of VWF domains. (A)Original structure of VWF containing VWF pro peptide plus 4 distinct domains (reviewed in Sadler 1998). (B) New VWF structure proposed by Zhou et al., 2012. Compared to the original structure, the new structure contained C1-C6, replacing the B1-B3 domains. (C) In addition, this study showed that the D domains contained a VWD module, cysteine-8 (C8) domain, trypsin-like (TIL) structure and an E module. The D' domain does not contain the VWD and C8 modules. In addition, the D4 domain has a unique sequence designated D4N.

1.4.3.2 Storage of VWF

VWF is stored in α-granules in platelets and in cigar-shaped organelles in ECs known as Weibel Palade Bodies (WPB) (Metcalf et al., 2008). α-granules do not require VWF for their formation while VWF is essential for the formation of WPB (Lenting et al., 2015). Interestingly, transfection of the pro-peptide of VWF and recombinant (rVWF) into non-endothelial cells can cause formation of WPB structures (Wagner et al., 1991, Blagoveshchenskaya et al., 2002, Michaux et al., 2003). The complex folding nature of VWF is crucial for its ability to compact such large quantities into organelles ranging in size from 0.5 to 5 µm in length and 0.1 to 0.3 µm in diameter (Valentijn et al., 2011). The higher molecular weight multimers (HMWM) of VWF are stored in WPB and released upon stimulation, while the lower molecular weight multimers (LMWM) are released constitutively (Lopes da Silva and Cutler, 2016). Since HMWM of VWF are known to be more haemostatic, the observation by Lopes and Cutler may explain the prothrombotic nature of the endothelium after activation and release of WPB. Immunofluorescence and EM imaging studies show that WPB have a distinct rod-like shape with regular striations (illustrated in figure 1.6C-D).

VWF is the main constituents of WPB, however WPB can store a number of proteins such as Osteoprotegerin (OPG), Galectin-3 (Gal-3), P-selectin, Interleukin 8 (IL-8) and Ang-2 to name just a few (Metcalf et al., 2008) (Table 1-1). These proteins regulate endothelial processes from responses to haemostasis, angiogenesis and inflammation (Rondaij et al., 2006). Work from Fiedler and colleagues showed that some of these proteins are not stored mutually exclusive (Fiedler et al., 2004). For example, Ang-2 and P-selectin do not appear to be stored in the same WPB. Moreover, WPB distribution appears heterogeneous among ECs within the same vascular bed(Gebrane-Younès et al., 1991).

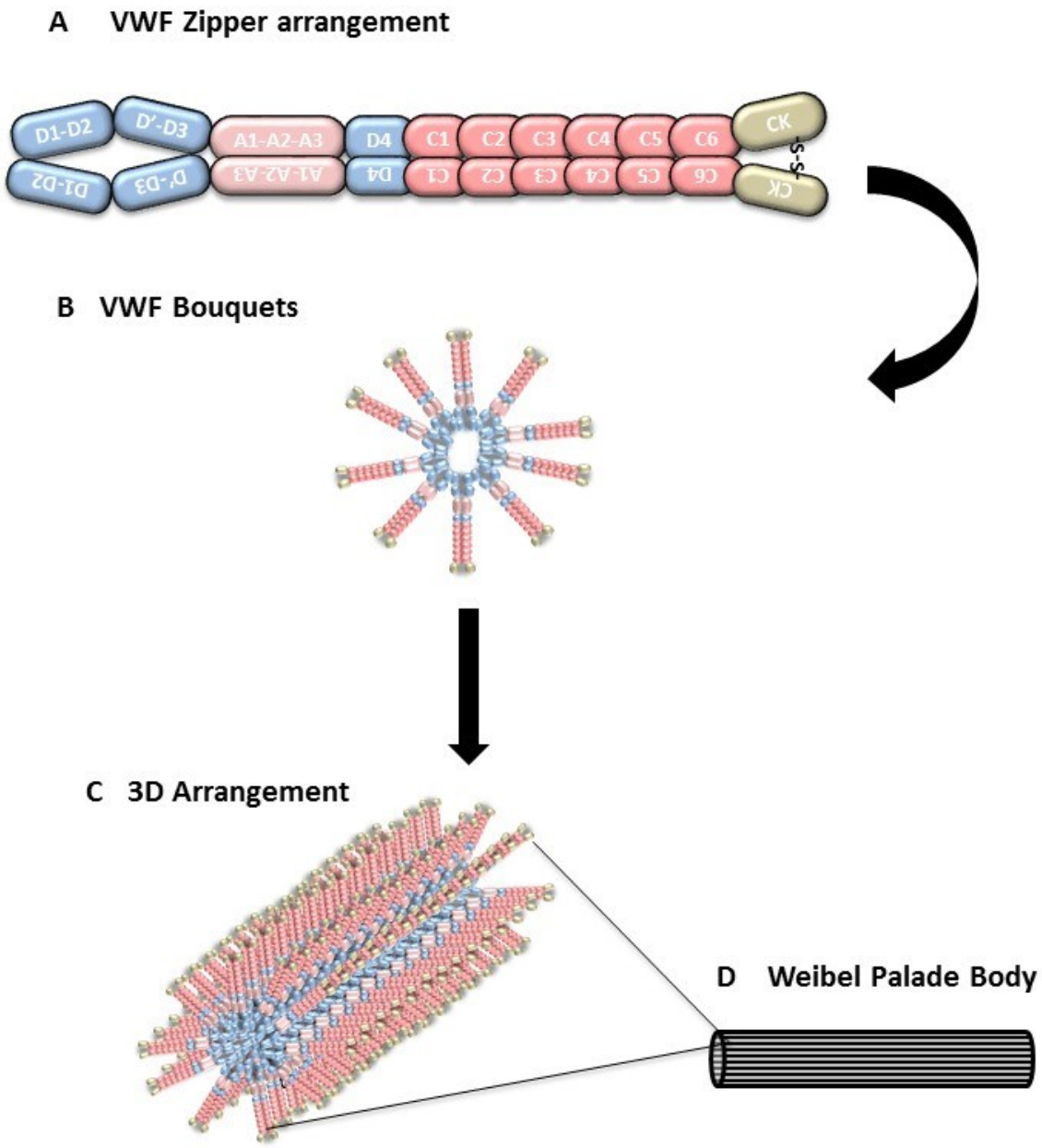

Figure 1.6 Arrangement and storage of VWF in WPBs. (A) After cleavage of the signalling peptide, proVWF leaves the ER and proceeds to the Golgi where the low pH and high calcium facilitates VWF dimerises via C terminal disulphide bonds. VWF "zips" up along the C domains, A domains up to the D domains. (B) VWF D1-D2 region help facilitate multimerization where dimers start to organise into a head to head order. (C) VWF heads start to arrange into a helical structure which is allows compaction of large amounts of VWF. This structure forms the primitive stacks of longitudinal striations observed in Weibel Palade Bodies (D).

Table 1-1 Selected list of WPB contents from Randi et al., 2013

78 kDa-regulated protein	Endoplasmin	Integrin alpha-5	Protein disulfide-isomerase A3
α1,3-Fucosyltransferase VI	Endothelial protein C receptor	Interleukin-8	Protein disulfide-isomerase A4
α-2-HS-glycoprotein	Endothelin-1	Lysozyme g-like protein 2	Protein disulfide-isomerase
Angiopoietin-2	Endothelin-converting enzyme	Matrix Gla protein	P-selectin
Biglycan	Epididymis-specific alpha-mannosidase	Nucleobindin-1	Puromycin-sensitive aminopeptidase-like protein
Calcitonin gene related peptide	Eoxtaxin-3	Osteoprotegerin	Rab3D
Calreticulin	FVIII	Pentraxin-related protein PTX3	Rab27A
CD63	FXIIIa	Plasma alpha-L-Fucosidase	Serpin H1
Cell Surface glycoprotein MUC18	Galectin-1	Plasma glutamate carboxypeptidase	Thrombospondin-1
Clusterin	Galectin-3	Plasminogen activator inhibitor 1	von Willebrand factor A domain-containing protein 5B1
Collagen alpha-1(I) chain	Insulin receptor-related protein	Platelet endothelial cell adhesion molecule	**von Willebrand factor ***
EGF-containing fibulin-like extracellular matrix protein 1	Insulin-like growth factor binding protein 7	Plexin D1	V-set and immunoglobulin domain-containing protein 8

*predominant component of WPB

1.4.3.3 Endothelial storage and release of VWF

Sporn *et al* originally used labelling studies to propose that approximately 5 – 10% of VWF is released via a regulated process in response to a secretagogue, with constitutive, unregulated release accounting for the other 90 – 95% (Sporn et al., 1987). Controversially, studies performed by Tsai *et al* based on the multimeric nature of VWF released from endothelial cells suggested the opposite: that the majority of VWF release occurs via a regulated secretory pathway (Tsai et al., 1991). To address these conflicting reports, Giblin et al., have since provided evidence for a third model of VWF release. In these studies, the authors demonstrated that proVWF followed by mature VWF was released into the media of metabolically labelled HUVEC (Giblin et al., 2008). The released proVWF was less stable and consistently disappeared from the media at a faster rate than mature VWF, which appeared much later. The later release of mature VWF appeared to occur via a pH-dependent mechanism and, unlike the proVWF, was not affected by blockage of golgi apparatus-dependent trafficking with the inhibitor, Brefeldin A. The authors concluded that at least 80% of what was thought to be constitutively-released VWF was actually released by this new "basal" mechanism, which appeared to involve post-Golgi secretory organelles.

Recent studies by Lopes da Silva and Cutler have demonstrated that most VWF release from ECs appears to be from the apical surface (Lopes da Silva and Cutler, 2016). Dissecting whether this VWF released was controlled by basal or constitutive release, this study found that most of the basolateral VWF originated from constitutive release, while apical appeared to be basal and regulated. These experiments provided key information in support of the work by Gibin et al., reporting that both the basal and regulated VWF multimeric patterns appeared to account for a much greater proportion of VWF release than constitutive release. Since VWF multimerization persists in WPB, it could be concluded that both basal and secretagogue-induced mechanisms cause the higher molecular weight multimer release of VWF. For the purposes of this thesis, constitutive is defined as release directly from trans-Golgi without storage in WPB and basal secretion as unstimulated release from WPB.

Surprisingly, two different secretagogue-induced pathways for VWF release exist in endothelial cells (reviewed in Rondaij et al, 2006). Activators of VWF release can either cause

an increase in intracellular calcium such as thrombin (de Groot et al., 1984) or an increase in cyclic AMP (cAMP) such as vasopressin or analogue 1-deamino-8-D-arginine vasopressin (DDAVP). Traditionally, conversion of phosphatidylinositol bisphosphate (PIP_2) into inositol 1,4,5 triphosphate (IP_3) causes activation of RhoA GTPase leading to endothelial permeability in a calcium dependent manner. In contrast, activation of adenylate cyclase (AC), which drives cAMP generation via activation of protein kinase A, has been linked with promoting vascular stability. Fundamental to understanding VWF release from the endothelium were studies uncovering the role of Rab proteins in WPB maturity and recruitment. Zografou and colleagues have shown that WPB recruit Rab GTPase proteins: Rab27A and Rab3D during maturation (Zografou et al., 2012). In addition, Nightingale et al., showed that inhibition of Rab27A caused enhanced release of VWF consisting of primarily low molecular weight multimers (Nightingale et al., 2009). Crucially, this study identified the effector protein, myosin and Rab27a interacting protein (MyRIP), as a vital coregulator of Rab27a-dependent WPB maturation. These studies provided key evidence to demonstrate that WPB tethering to actin structures was important for their maturation.

While previous studies on WPB exocytosis were performed in fixed cells, studies from (Romani de Wit et al., 2003) provided key evidence of random movements of WPB in resting EC. Transfection of HUVEC with GFP-tagged VWF allowed the movements of WPB to be mapped, demonstrating that these organelles were in fact not static, instead displaying more random motility which often led to membrane fusion events. In addition, (Babich et al., 2008) showed that these random events caused the rapid increase in pH (from exposure to the extracellular environment) that led to deacidification of WPB and content release. Puzzling observations from these studies were the apparent random nature of the contents released, however these observations could be in part explained by the heterogeneous nature of WPB contents. The finding that decreased autophagy leads to decreased VWF release suggests that autophagy may also play a role in this process. While these studies have greatly increased our understanding of the mechanisms controlling VWF release, they have also highlighted the complexity of this process.

1.4.3.4 Clearance of plasma VWF

1.4.3.4.1 Enzymatic cleavage of VWF

VWF released from EC have higher molecular weight multimers which have a propensity for greater interaction with the platelet receptor Glycoprotein1b (Gp1b). This can lead to the formation of spontaneous platelet aggregates which can cause vessel occlusion and detrimental consequences for the tissue. To circumvent this occurrence and preventing, unnecessary aggregate formation, VWF is cleaved into smaller forms by A distintegrin and metalloproteinase with thrombospondin type 1 motif member 13 (ADAMTS13) proteinase. On the EC cell surface VWF is tethered to the endothelium via integrin $\alpha v\beta 3$ (Huang et al., 2009, Cheresh and Spiro, 1987). The presence of flow unravels and exposes the full-length molecule (Huang et al., 2009). This allows exposure of the various domains of VWF including the A1-A2-A3 domain. This is significant because it facilitates the cleavage of VWF by ADAMTS13 in the A2 domain, which is normally hidden by the A1 and A3 domains (Crawley et al., 2011, Huang et al., 2009). Once cleaved from the EC surface, VWF forms a globular shape which may protect it from ADAMT13 degradation. However, conflicting reports have suggested that ADAMTS13 alone may not regulate the amount of VWF multimerization or levels (Casari et al., 2013, Motto et al., 2005). The ABO blood group and regulation of VWF uptake in tissues have proposed alternative mechanisms for VWF clearance and variations in levels in healthy individuals (reviewed in Lenting 2015). These studies suggested proteolysis and clearance of VWF may be separate mechanisms.

1.4.3.4.2 Non-enzymatic mediated cleavage of VWF

The half-life of plasma VWF after vasopressin treatment is about 16 hours, however, this can range from 16 to 24 hours even in healthy individuals (Lenting et al., 2015). One observation that started to shed light on VWF clearance was the finding that non-O blood groups have a longer VWF half-life than O blood groups (Gallinaro et al., 2008). This supported previous work from O'Donnell and colleagues which showed that VWF from individuals lacking h antigen, the precursor for A and B antigens, had lower circulating VWF levels, similar to individuals with type O blood group (O'Donnell et al., 2005). In order to evaluate whether

this was due to differences in synthesis of VWF, Gallinaro et al measured DDAVP-induced release of VWF in patients with O vs non-O blood groups (Gallinaro et al., 2008). No difference could be observed, indicating a role for clearance mechanisms instead.

VWF is a heavily glycosylated protein (Denis et al., 2008). Since glycoproteins are a key component of immune system recognition (Kumar et al., 2011); it is possible that glycosylation of VWF may play a role in its clearance. Stoddart et al., demonstrated enhanced clearance of VWF lacking O-linked glycosylation in a model of perfusion in rats (Stoddart et al., 1996). In addition, sialylation of VWF by ST3-GalIV transferase appears to play an important role in regulating VWF levels, since mice deficient in ST3GalIV have reduced VWF levels (Ellies et al., 2002). These studies suggest that post-translational modifications protect against the degradation of VWF.

Immunohistochemistry from van Schooten et al showed that VWF colocalises with macrophages in organs normally associated with clearance, such as the liver, further increasing the probability of an immune role in VWF clearance (van Schooten et al., 2008). Moreover, in support of these findings, mice deficient in macrophages have increased VWF survival (reviewed in Lenting et al., 2015). Despite description of the macrophage role in clearance of VWF, the particular receptor responsible was yet unclear. Work from the Lenting lab provided key evidence showing that deletion of lipoprotein receptor related protein 1 (LRP-1) in macrophages prolonged VWF half-life in the circulation (Rastegarlari et al., 2012). In addition, a number of other studies have identified other VWF receptors including Siglec-5 and Ashwell receptor (reviewed in Lenting et al., 2015). These data provided a number of receptors and at least one cell type which may be responsible for the clearance of VWF however have no addressed involvement of other cell types.

A genome wide association study (GWAS) identified single polymorphism nucleotides (SNPs) in C-type lectin member 4 family receptor (CLEC4M) which may contribute the variations in VWF levels in the circulation(Smith et al., 2010). In addition, work from the Lillicrap lab showed that CLEC4M binds VWF on the cell surface causing internalisation and degradation of VWF (Rydz et al., 2013). Additionally, CLEC4M is expressed on sinusoidal ECs which highlights one way through which ECs in the liver may be involved in clearance of VWF.

Moreover SNPs in CLEC4M have been shown to be associated with variation in VWF levels in patients with type 1 VWD (Sanders et al., 2015). These studies have expanded our knowledge of VWF clearance implicating the role for the endothelium itself at least in the liver.

1.4.4 The role of VWF in haemostasis

1.4.4.1 VWF in platelet capture: primary haemostasis

The arresting of platelets at the site of vascular injury is the primary step in repairing a vascular damage to prevent bleeding (figure 1.7). VWF is essential for platelet adhesion to the subendothelium. Basal and secretagogue-induced VWF release allows VWF deposition on the cell surface and ECM. On the cell surface, VWF is anchored by integrin $\alpha v\beta 3$ and P-selectin (Cheresh and Spiro, 1987, Padilla et al., 2004). In the presence of flow, VWF unravels to expose binding sites for platelets leading to platelet capture. In the ECM, fibrillar collagen exposed after vascular injury binds to VWF (Romijn et al., 2001, Mazzucato et al., 1999). Since VWF in the circulation does not bind to platelets, immobilisation of plasma VWF to the ECM unveils VWF A1 domain which contains the binding site for the glycoprotein receptor GbIbα on platelets (Peyvandi et al., 2011, Savage et al., 1996). This initial platelet and VWF interaction plays a key role in the platelet rolling at the site of vascular injury (figure 1.7). In addition, other ECM components such as fibronectin engage the integrin $\alpha 5\beta 1$ on platelets to help encourage firm arrest. Initial VWF-GpIbα engagement leads to an increase in intracellular Ca^{2+} levels(Kroll et al., 1991), which is vital for activation of integrin $\alpha IIb\beta 3$ (GpIIb-IIIa) on platelets. This provides the second key step in platelet activation by facilitating further platelet aggregation. Moreover, firm adhesion of platelets to the site of vascular injury induces conformational changes and α granules release leading to the release of several pro thrombotic agents such as thromboxane A2 (TxA2).

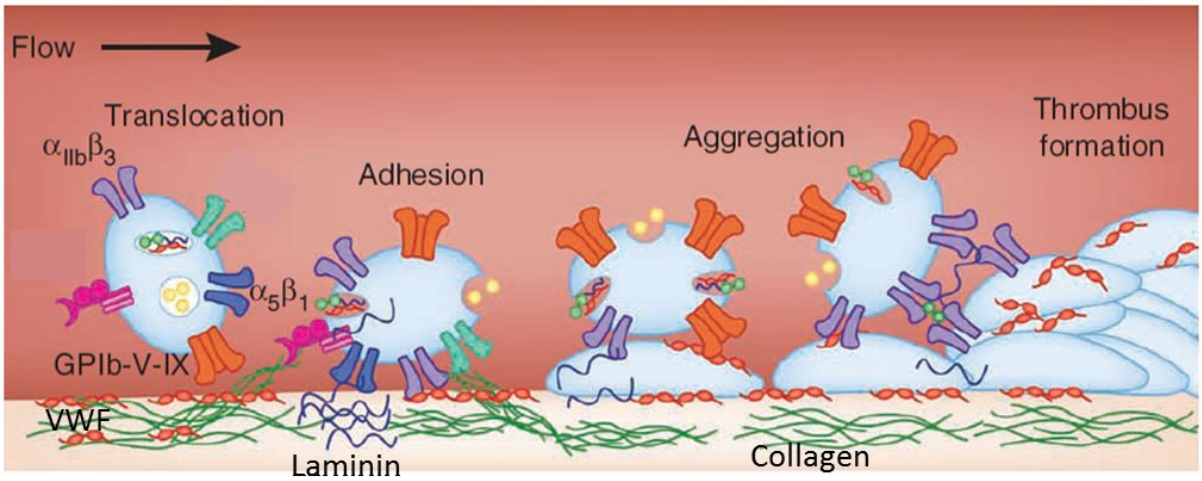

Figure 1.7 Platelet adhesion to the sub endothelium. VWF is released from the injured endothelium. In addition, collagen is exposed (green) which mediated VWF immobilisation. Platelet adhesion to the site of vascular injury is initialised by slow rolling of the platelet due to VWF-GP1B-V-IX interaction. This activates intracellular signalling pathways within the platelet to change integrin conformational engaging other matrix proteins such as laminin. This results in firm adhesion to the site of injury and increase release of platelet alpha granule, which can release several prothrombotic molecules. These initiate autocrine signalling to platelets causing conformational change and further platelet adhesion to the site of injury. Reproduced with permission from Jackson 2011.

1.4.4.2 VWF bind to and stabilises FVIII: secondary haemostasis

The formation of a stable fibrin clot is important for completion of haemostasis. Tissue factor (TF) released from damaged vessels binds to and activates coagulation factor VII (FVII). Activated FIX uses activated FVIII (FVIIIa) as a cofactor for conversion of FX, facilitating prothrombin activation and fibrinogenesis (Palta et al., 2014). Key to this final process is the availability of FVIII as a substrate. FVIII binding to VWF covalently after release from EC protects it from proteolytic degradation by protein C. Recent reports suggest that the VWF-FVIII interaction can also occur intracellularly in some endothelial beds (Turner and Moake, 2015). This demonstrates why in early reports of VWD, patients presented with haemophilia like symptoms as well as low FVIII levels. Later studies revealed that mutations in the FVIII binding domain (D'-D3) of VWF could result in a VWD subtype which is characterised by low FVIII levels and normal VWF levels.

1.5 Von Willebrand Disease

Von Willebrand disease (VWD) is the most common congenital bleeding disorder, due to decrease or dysfunction of VWF. The most recent review of population data on VWD incidence has reported the prevalence of around 0.6-1.5% (Leebeek and Eikenboom, 2016). Since VWD is inherited in an autosomal inheritance pattern, both males and females should be equally affected, however the increased prevalence among females is likely due to women presenting with more haemostatic challenges in life due to menstruation and child birth. VWD is a very heterogeneous disorder both at the genetic and phenotypic level. The clinical presentation of the disease is not only dependent on the mutation present, but also the activity of the second allele (Keeney and Cumming, 2001). This partially explains the initial observations made by von Willebrand in 1926 when he suggested that the disease inheritance may result in a mild phenotype or severe phenotype because of the second allele.

In the early 70s, Howard and Firkin described the use of the antibiotic ristocetin to cause platelet aggregation known as ristocetin induced platelet aggregation (RIPA). The use of ristocetin in VWD plasma facilitated a useful method to detect the activity of VWF regulating platelet aggregation (Koutts, 2006). Combined with VWF antigen levels and FVIII measurements this has led to the classification of more than 20 VWD subtypes in the categories discussed below (Sadler et al., 2006).

1.5.1 Congenital Von Willebrand Disease

Congenital VWD can be classified into two main categories: quantitative defects in VWF (subdivided into type 1 and type 3) and qualitative defects in VWF (type 2). Type 1 VWD accounts for about 80% of VWD cases. Type 2 VWD is further subdivided based on the defect in VWF and accounts for 15-20% of cases. The rare type 3 group account for <5% of VWD cases (Leebeek and Eikenboom, 2016). Although it is common for diseases resulting in absence of a protein to be a result of gene deletions; this is very rarely seen in VWD (Ginsburg and Bowie, 1992). The inheritance pattern of VWD is dependent on the type of VWD. In type 1 and type 2 VWD, mutations are inherited in an autosomal dominant manner, while type 3 VWD mutations are generally recessive. Key to the VWD phenotypes is understanding the link between the mutation and the functional outcome in the VWF protein.

Normal levels of VWF in plasma can range from 50 IU/dl to 150 IU/dl (Leebeek and Eikenboom, 2016). In patients with type 1 VWD there is often a partial quantitative defect of VWF. Providing a specific cut off for VWF antigen (VWF:Ag) levels for a type 1 diagnosis is difficult; however some guidelines suggest any value below 30-40 IU/dl are for consideration of a type 1 diagnosis (Ng et al., 2015). In practice, a diagnosis of type 1 has to take into account the patient's bleeding history and evaluation of family data. Unsurprisingly, with reduced VWF levels, these patients have reduced VWF rictocetin cofactor activity (VWF:RCo). With a collective approach of history, laboratory data and clinical presentation, a number of algorithms have been suggested for assessing VWD classifications. For type 1 diagnosis a history of bleeding, VWF mutations and VWF:Ag to VWF:RCo ratio of >0.6 can be considered a type 1 patient (Laffan et al., 2014). In patients where VWF:Ag are normal and VWF:RCo is increased or decreased this could suggest a defect in VWF function.

Type 2 VWD classification encompasses an umbrella of qualitative defects in VWF. Patients are broadly subclassified in the Type 2 category based on the ability of VWF to interact with platelets, multimerization pattern and, in the case of Type 2N, the lack of VWF interaction with FVIII. Type 2A and Type 2B VWD broadly differ based on the locality of the VWD mutations, VWF:RCo and platelet numbers. In type 2A, HMWM of VWF are reduced and VWF:RCo is low due to decrease platelet binding. Mutations in the A2 domain can result in a protein with increased susceptibility to cleavage by ADAMTS13. Additionally mutations in D3, A2 and CK can result in defects in multimerization. An additional scenario is a protein that has defective processing leading to ER retention of the HMWM and secretion of the smaller multimers. In type 2B, VWD is characterised by increase binding of VWF to platelets and increase clearance of VWF-platelet aggregates (Leebeek and Eikenboom, 2016). In these patients thrombocytopenia can also present due to enhanced clearance. A rare form of type 2 VWD known as type 2M results from distinct A1 domain mutations which cause decreased binding to platelets, despite normal HMW multimers (figure 1.8).

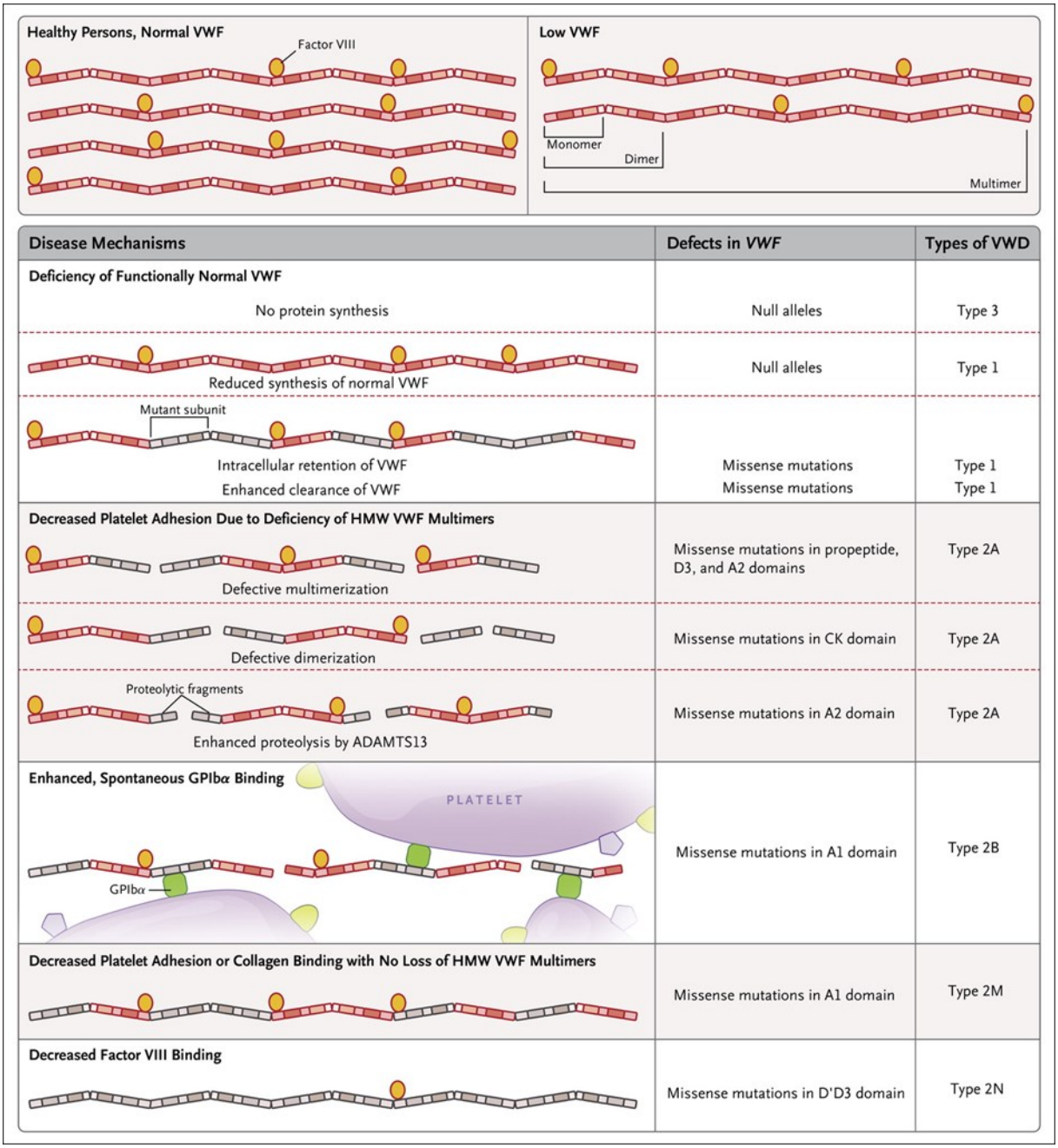

Figure 1.8 Classification of Von Willebrand Disease based on mechanism. VWF in the healthy individual circulates as high molecular weight multimers (HMWM). In addition, VWF binds to FVIII to protect it from degradation. In von Willebrand Disease (VWD) both quantitative and qualitative defects can occur. In patients with complete absence of VWF (type 3), no protein is detected in circulation. In some patients, low circulating levels of VWF (type1) could be as a result of decreased synthesis, enhanced clearance or intracellular retention of processed VWF. Qualitative defects encompass an umbrella of functional defects in VWF. Type 2A VWD patients have low circulating HMWM due to either defective multimerization, dimerization or enhanced proteolysis. Type 2B VWD patients have low HMWM due to increase clearance because of enhanced, spontaneous platelet binding. Type 2M patients have normal circulating HMWM but a decrease in platelet binding. Type 2N VWD patients have decreased FVIII binding due to mutations in the D'-D3 domain of VWF. Figure Reproduced with permission from Leebeek and Eikenboom 2016, Copyright Massachusetts Medical Society, 2016.

1.5.2 Acquired von Willebrand Syndrome

While largely this thesis will refer to congenital VWD, similarities in mechanisms will be drawn from specific examples of acquired von Willebrand syndromes (AVWS). AVWS defines loss or dysfunction of VWF which can develop in association with clinical conditions such as myeloproliferative and malignant disorders, aortic valve stenosis (Heyde's syndrome) and during use of left ventricular assist devices (LVADs) (reviewed in Tiede, 2012). According to the current hypothesis, AVWS in myeloproliferative and malignant disorders results from VWF binding to surface of cells and clearance of HMWM (Richard et al., 1990, Budde et al., 1984). In the cases of LVADS, shear stress caused by the device, which leads to elongation and unfolding of VWF. This results in exposure of the cleavage site for ADAMTS13, and hence in excessive VWF proteolysis with loss of high molecular weight (HMW) multimers, the more functionally active form of VWF. Interestingly, removal of the device results in immediate recovery of AVWS and decreased bleeding (Crow et al., 2010, Jilma-Stohlawetz et al., 2016). In patients with aortic stenosis a marked reduction in the haemostatic HMWM of VWF have been observed. Moreover in patients who underwent valve replacement, the abnormalities in VWF multimerization were reversed (Casonato et al., 2011, Solomon et al., 2011, Vincentelli et al., 2003). Higher shear has been demonstrated to unravel VWF and expose the A2 domain for cleavage by ADAMTS13 (Tsai et al., 1994, de Groot and Lane, 2008, Schneider et al., 2007), suggesting a possible mechanism through which aortic stenosis was causing AVWS.

1.5.3 Treatment of Von Willebrand Disease

VWD treatment takes two basic approaches: VWF replacement or stimulating release of existing VWF pools (Leebeek and Eikenboom, 2016). VWF replacement using plasma-derived VWF to treat severe quantitative defects remains largely unchanged over many decades. Refinement of techniques have allowed purer forms of plasma-derived VWF to be isolated, eliminating the risk of transmission of viruses or other blood borne pathogens. To enhance release, the secretagogue Desmopressin (DDAVP) is used, which induces cellular release of VWF. The approach in a Type 2 patient is often more complex. While a patient with Type 2N VWD would require FVIII infusions, patients with Type 2A and Type 2B have issues with both HMWD clearance as well as defects with platelet binding. Type 2 VWD patients are therefore treated on a case by case basis. In some cases, VWF replacement therapy may be the only

option, as the DDVAP response can be variable in this group (Tosetto and Castaman, 2015). The prophylaxis therapeutic approach provides the missing factor, in this case VWF, regularly. In VWD, this approach is relatively new, compared to conditions such as haemophilia where this has been practiced for decades (Berntorp, 2008). Refinement of this approach depends not only on patient compliance but also with the cost associated with manufacturing.

1.5.4 Blood Outgrowth Endothelial cells (BOEC) as a novel tool for understanding VWD

1.5.4.1 Origins and Characterisation of BOEC

The use of human umbilical veins to obtain ECs for the purpose of understanding their physiology heralded the birth of modern day understanding of endothelial biology (Jaffe et al., 1973). Another novel breakthrough in this area was the ability to isolate circulating endothelial progenitor cells (EPCs) from peripheral blood (Asahara et al., 1997). The first question this finding raised was the origin of these EC. To address this, Lin et al., have performed an elegant study on patients undergoing bone marrow transplants from mismatched donors (male to female or vice versa)(Lin et al., 2000). Blood derived outgrowth endothelial cells expanded in culture appeared to originate from the donor. This suggested that endothelial progenitor cells originated from bone marrow niches. Since then, a number of studies have tried to compare the expansion potential of endothelial progenitor cells (EPCs), As yet no general consensus of nomenclature has been reached (reviewed in Rafii and Lyden, 2003). Hur et al., demonstrated that isolation of early EPC (eEPC) and late EPC (now accepted as Blood Outgrowth Endothelial Cells: BOEC) is possible from the same donor (Hur et al., 2004). Compared to eEPCs, which die after 2-4 weeks in culture, BOEC arise around 12-21 days and last longer, display an endothelial morphology and are highly proliferative.

The Yoder lab has provided a generally accepted point of view that BOEC are truly endothelial cells (reviewed in Hirschi et al Hirschi et al., 2008). In support, Medina *et al* showed in 2010 that while EPC express haematospecific factors like RUNX1, they also express endothelial-specific genes such as Tie-2 and endothelial nitric oxide synthase 3 (eNOS) (Medina et al., 2010). In a direct comparison with eEPCs, BOEC showed increased expression of VE-Cadherin and VWF. In addition BOEC also express: Flk1, CD31 and CD144 (Ingram et al., 2004). These

cells possess the ability to form tubes on Matrigel and can be isolated from peripheral blood using standardised protocols (Martin-Ramirez et al., 2012, Ormiston et al., 2015). Using a microarray analysis comparing HUVEC and BOEC, the expression of the microvascular HOX transcriptome appeared to be shared between these two cells types (Toshner et al., 2014). Extensive characterisation of BOEC have facilitated their use to characterise endothelial defects in diseases such as HHT, PAH and COPD proving that they are a useful tool for the study of endothelial biology (Fernandez-L et al., 2005, Toshner et al., 2009, Paschalaki et al., 2013, Reinhold J. Medina et al., 2012).

1.5.4.2 Cellular basis of VWD: BOEC studies

BOEC provide an alternative approach to understanding a disease as heterogeneous as VWD. Up until BOEC were identified, studies on the cellular function of wild type (WT) and mutant VWF have been carried out by expressing recombinant VWF in non-endothelial cells (generally HEK). These studies have provided significant insights into the cellular basis of VWD and will continue to do so. Isolation of HUVEC from affected patients has also been reported; however this occurs in extremely rare circumstances, thus availability and ease of access of EC from VWD patients remains a challenge.

In 2009 Berber and colleagues used BOEC to demonstrate that a VWF mutation commonly associated with type 1 VWD: R924Q was likely leading to aberrant VWF splicing and contributing to a type 2N phenotype (Berber et al., 2009). In this study, BOEC were isolated from a 16-year-old patient who inherited both a common type 2N mutation (R816W) and the R924Q mutation. Her sister and father were heterozygous for the R924Q mutation, whilst the mother was heterozygous for the R816W mutation. FVIII levels were found to be normal in the father, mother and sister of the proband. Using BOEC this study identified that the VWF mRNA was aberrantly spliced generating a premature stop codon. This suggested that the aberrantly spliced VWF could be degraded and only the R816W mutant VWF would be expressed. As a result the patient had decreased FVIII levels from the R816W mutation in the D domain of VWF and decreased plasma levels due to splicing events of the mutated R924W VWF.

Our laboratory has pioneered the use of BOEC to investigate the role of VWF in endothelial cells (Starke et al., 2011). In this study, BOEC isolated from VWD patients showed enhanced angiogenesis compared to healthy controls. These data will be discussed in detail later (section 1.7.2.4). Two other studies, from our lab and from the Eikenboom lab, provide the first endothelial characterisation of VWF synthesis, processing and storage in patients with type 1 and type 2 VWD (Starke et al., 2013, Wang et al., 2013). Most type 1 BOEC showed defects in synthesis, storage and release, whilst type 2 patients showed defects in release. In particular, Starke and colleagues demonstrated a marked heterogeneity in the cellular phenotypes of four type 1 patients with different mutations and a range of plasma phenotypes. BOEC analysis showed that all 4 type 1 VWD patients had decreased synthesis of VWF, which is in line with the decreased circulating levels of VWF(Starke et al., 2013). It has been recognised that both basal and stimulated release of VWF contributes to the circulating pool. In this study, in two of the type 1 patients, Starke and colleagues identified a "mesh like" appearance of VWF staining in BOEC reminiscent of the ER. Immunostaining of these BOEC revealed that VWF in these patients was retained in the ER, and electron microscopy showed significantly enhanced ER in the cells. In this patient, the mutation was found in VWF exon 4-5 corresponding to the D1 region of the VWF pro peptide; protein analysis showed that the mutation prevented processing of the VWF pro-peptide. Thus these studies demonstrate how access to BOEC from VWD patients allows us to identify the cellular defects underlying the patients' plasma phenotypes.

Studies from the Eikenboom group have utilised a similar approach to study VWD in BOEC isolated from patients, and provided evidence for 3 mutations resulting in ER retention: Ser1285Pro, Leu1307Pro and Cy2693Tyr. The resulting BOEC WPB phenotype was abnormal, showing reduced PMA responses. While the effect of these mutations on synthesis of VWF in this study was not explored the Cys26693Tyr mutation showed reduced VWF production. In both studies carried out by Starke and Wang, a patient with Tyr1583Cys mutation was studied. Strikingly, BOEC from these patients had opposite responses to PMA induced VWF release and basal release. While BOEC from the patient reported in Starke and colleagues showed a decrease in basal VWF production and PMA induced release, the study carried out by Wang et al did not find any significant abnormalities in this patient's BOEC, (Starke et al., 2013, Wang et al., 2013). Since this mutation occurs in the A2 domain, the study from Wang

et al., proposed that increased clearance may be the reason for reduced levels. Interestingly, plasma levels of the two patients were different, despite the same mutation in the VWF gene: the patient in Wang et al had VWF levels almost twice the level of the patient in Starke et al,. This suggests that mechanisms other than gene mutation control plasma VWF levels.

Both these reports have identified a bona fide approach for studying the cellular and molecular basis of VWD in a reliable system. Phenotypes which were otherwise hidden are slowly being uncovered using this approach. While isolation of these cells have yet to be standardised across labs, this approach is facilitating phenotyping of VWF characteristics across various mutations. VWD BOEC are also proving to be a novel and very valuable tool to investigate the vascular, non-haemostatic functions of VWF, such as regulation of angiogenesis; this will be discussed below (section 1.7.2).

1.6 Vascular malformations and gastrointestinal bleeding in Von Willebrand Disease

1.6.1 Congenital VWD and vascular malformations

Angiodysplasia, fragile malformed vascular lesions in the GI tract, have been recognised to appear in elderly patients, potentially due to an increased frequency of colitis, varices or cancers in this patient population. Examination of VWD patients demonstrated an increase in the frequency of GI bleeding in Type 2A and Type 2B patients who lack HMWM and in Type 3 VWD patients (Castaman et al., 2012, Abshire et al., 2013). Due to a lack of efficient and non-evasive diagnostic procedures, it remains a concern whether these numbers truly the reflect the incidence of the condition.

Angiodysplasia in the GI tract is not the only type of vascular malformation identified in VWD. Microscopy of the nail bed in patients with VWD revealed abnormalities of vessels including tortuosity (Blackburn, 1961). Following this, Koscielny and colleagues carried out quantitative analysis of the capillary vascular bed in VWD patients and found not only increased tortuosity but also increase vascular leakage (Koscielny et al., 2000). These data provided the first quantitative analysis of vascular malformations in VWD patients.

1.6.2 Acquired VWD and angiodysplasia

In 1958 Dr. Edward Heyde reported the presence of gastrointestinal (GI) bleeding of unknown origin in patients with aortic stenosis (Heyde, 1958). A systematic review of patients with aortic stenosis later revealed that this diagnosis was associated with an apparent 100-fold increase in chance of GI bleeding compared to the general population at the time (Cody et al., 1974). Later, it was reported that GI bleeding associated with aortic stenosis appeared to be from the cecum and ascending colon. More recently however, this has been revised; bleeding has been found to occur higher in the GI tract in regions such as the stomach. Presentation of this GI bleeding phenotype can be as occult blood and resulting anaemia, but in some cases, severe gross bleeding can occur. Later visualisation of these regions of the colon demonstrated vascular lesions with a “coral reef”-like morphology (Warkentin et al., 2003). Detailed endoscopic analysis showed that these lesions have fragile walls and resembled

telangiectasia (dilated blood vessels usually on skin or mucosal surfaces). These lesions were often thought to be linked to dysregulated endothelial barrier function or disruption of the function of haemostatic VWF multimers.

1.6.3 Anti angiogenic intervention in VWD associated angiodysplasia

The mainstay of treatment in VWD is control of bleeding by VWF replacement or DDAVP-induced VWF release. Unfortunately, for some patients with VWD-related angiodysplasia this treatment seemed ineffective (Sohal and Laffan, 2008, Alikhan and Keeling, 2010). In addition, a retrospective analysis of GI bleeding in VWD patients by the VWD Prophylaxis Network (VWD PN) demonstrated that treatment of these patients with VWF replacement only produced a 50% reduction in bleeding episodes(Abshire et al., 2013). Surprisingly, isolated case reports have shown that anti-angiogenic therapy has been successful in treating patients with VWD who do not respond to VWF replacement therapy (Hirri et al., 2006, Heidt et al., 2006). These reports suggest a potential underlying cellular defect in VWD- angiodysplasia

1.6.3.1 Anti-angiogenic treatment of GI bleeding in VWD: thalidomide

A few studies in patients with GI angiodysplasia have demonstrated a response to thalidomide therapy (Heidt et al., 2006, Hirri et al., 2006). In these reports the reduction in GI bleeding with thalidomide was also associated with decreased VEGF levels. This suggested not only one potential pathway for the regulation of angiodysplasia but also a therapeutic intervention. For elderly patients, where GI bleeding is most common, this treatment regime does not have the added complication of reported birth defects (Tan et al., 2012, Bauditz et al., 2004). In addition, a meta-analysis of therapeutic interventions in angiodysplasia revealed that in studies using thalidomide up to 71% of patients displayed side effects such as severe drowsiness and fatigue. Together these findings demonstrates that while thalidomide has showed some therapeutic promise, issues with patient compliance may arise with long term management of angiodysplasia.

1.6.3.2 Anti-angiogenic treatment of GI bleeding in VWD: Statins

In a case report of VWD-associated angiodysplasia, Sohal and Laffan presented a Type 1 VWD patient with severe GI bleeding who was unresponsive to VWF replacement therapy or thalidomide. The patient did however show improvements with a 40 mg daily dose of atorvastatin (Sohal and Laffan, 2008). In addition, Alikhan and Keeling reported a similar result in a Type 2A patient with VWD (Alikhan and Keeling, 2010). Statins provide a very interesting alternative for the management of angiodysplasia in VWD patients. Statins have been thought to decrease angiodysplasia via regulation of pathways in involved in angiogenesis. VEGF levels have been investigated in past studies, however the role of other growth factors such as Ang-2 remain unknown. A recent study has shown that in angiodysplasia Ang-2 is increased (Holleran et al., 2015, Ghosh et al., 2015). In an interesting study, statins were demonstrated to reduce expression of Ang-2 both *in vitro* and *in vivo* in a model of inflammation (Ghosh et al., 2015). This provided speculative evidence for one potential mechanism through which statins may be regulating angiodysplasia.

Over the last 10 years, a number of studies have shown the potential of statins in regulating angiogenesis. In particular, simvastatin and atorvastatin have been demonstrated to increase angiogenesis at low concentrations but decrease angiogenesis at high concentration (Weis et al., 2002). Interestingly, both statins were shown to have these concentration-dependent functional outcomes despite increasing VEGF expression across all concentrations (Frick et al., 2003). In addition, water soluble statins have been shown to decrease EC proliferation (Asakage et al., 2004). This suggests a global effect of statins to inhibit angiogenesis at high concentrations. Statins target the HMG-CoA pathway leading to a reduction in lipid. New data demonstrates additional pathways for the action of statins. In fact, studies have demonstrated that statins regulate the phosphorylation of Akt (Wang et al., 2008, Bonifacio et al., 2015, Dimmeler et al., 2001). This leads to phosphorylation of the transcription factor FOXO1, inhibiting its activity (Potente et al., 2005, Daly et al., 2004). Together this studies suggest statins can regulate angiogenesis through pathways alternative to traditional HMG-CoA reductase.

1.6.4 Mechanism of VWF-related angiodysplasia

Formation of pathological vessels by the angiogenic process has been attributed to an imbalance between pro and anti-angiogenic factors. One of the best characterised of these growth factors is VEGF, which has been shown to induce vascularisation and has been implicated in several pathological conditions. In addition, Junquera et al showed enhanced expression of VEGF (Junquera et al., 1999), and another angiogenic growth factor bFGF (Montesano et al., 1986) in regions of the colon affected by angiodysplasia. The increased growth factor expression could account for the aberrant vascularisation as well as enhanced permeability seen in these vascular lesions. In line with this evidence, Bauditz et al and Tan et al have demonstrated that levels of VEGF and bFGF were reduced in the presence of thalidomide both *in vitro* and in patients (Bauditz et al., 2004, Tan et al., 2012). More recently, the pro-angiogenic mediator, Ang-2 has been shown to be elevated in both the circulation and tissues of patients affected by angiodysplasia (Holleran et al., 2015). Collectively these studies support the hypothesis that defective blood vessel formation is responsible for angiodysplasia.

1.7 VWF roles in the vasculature besides haemostasis

1.7.1 VWF in inflammation

The role of VWF in controlling inflammation was initially thought to be indirectly through the regulation of P-selectin localisation in WPB (Denis et al., 2001). P-selectin was first identified in platelets and megakaryocytes however later studies revealed synthesis in ECs and storage in WPBs (McEver et al., 1989). P-selectin functions as a cell adhesion molecule binding P selectin glycoprotein ligand 1 (PSGL-1) on leukocytes to mediate slow rolling of inflammatory cells as an initial response during inflammation. In the absence of P-selectin, leukocyte rolling is impaired resulting in decrease extravasation (Mayadas et al., 1993). It was subsequently demonstrated that in the absence of VWF, P-selectin trafficking to the EC surface is decreased (Denis et al., 2001). In this study, the authors revealed that P-selectin is targeted for degradation in the absence of VWF. This subsequently leads to decrease leukocyte recruitment during models of inflammation.

Pendu et al., showed that VWF contained a binding site for PSGL-1 in the A1 domain (Pendu et al., 2006). This suggests that VWF could directly bind to leukocytes and may mediate slow rolling. A study from Koivunen and colleagues suggested VWF could also play a role in firm leukocyte adhesion step during inflammation (Koivunen et al., 2001). In this study, the authors demonstrated that THP-1 monocyte cell line bound to VWF in a β2 integrin dependent manner. This interaction was thought to occur through a leu-leu-gly (LLC) motif in the A2 domain of VWF. Using immobilised fragments of VWF, Pendu and colleagues found that an A1-A2-A3 fragment and D'-D3 fragment sustained binding to polymorphonuclear leukocytes (PMNs) (Pendu et al., 2006). Transfected fibroblasts with αXβ2 and αLβ2 integrins suggested that both the D'-D3 fragment and A1-A2-A3 fragment sustained binding to β2 integrins. These data suggested a role for VWF in both rolling and firm adhesion of leukocytes on the EC surface.

Our understanding of the role of VWF in inflammation comes primarily from animal models. Studies from several groups reported that loss of VWF appears to be protective in models of atherosclerosis and inflammation (Hillgruber et al., 2014, Petri et al., 2010, Methia et al., 2001). Additionally reduced leukocyte recruitment appeared to provide protection and increased survival in a model of endotoxemia using cecal ligation and puncture (CLP) (Lerolle et al., 2009). Surprisingly, in a different study using VWF-deficient mice, Kasuda et al have shown that CLP appeared to cause a decrease in survival (Kasuda et al., 2016). This discrepancy was suggested to be induced by experimental model variability and thrombus formation. This new "immunothrombosis" mechanism is proposed to act as a response to form a barrier around detrimental microbes (Kasuda et al., 2016). In a novel mouse model of Type 2B VWD generated by the Denis lab, the authors demonstrated that leukocyte recruitment was increased. This provided evidence that VWF-leukocyte interactions may be platelet dependent (Adam et al., 2016). Indeed, in this study mice depleted of platelets demonstrated a reduction in leukocyte recruitment, lending support to this hypothesis. While these observations provide more questions than answers, they suggest at least one indirect role for VWF in controlling inflammation.

Studies in patients have also shown that VWF levels in the circulation predict septic patient survival (van der Heijden et al., 2009a, Ware et al., 2004). This has led to VWF being proposed

as a biomarker of EC activation in inflammatory disorders. In is worth noting however that no study in humans has validated that VWF deficiency protects against inflammation. Since VWF also associates with a number of proteins which regulate inflammation there is sufficient evidence to argue that VWF could be involved in control of inflammation.

1.7.2 VWF in blood vessel formation

1.7.2.1 Endothelial VWF control of signalling pathways involved in blood vessel formation

In 2011, work from our lab provided the seminal studies to demonstrate that inhibition of VWF expression in EC caused increased proliferation, migration and Matrigel tube formation *in vitro* (Starke et al., 2011). Crucially, these studies demonstrated that extracellular VWF was able to reduce the pro-angiogenic phenotype of these cells. *In vivo*, this study demonstrated that Matrigel plugs implanted into VWF-deficient mice showed increased cellular infiltrates compared to littermate controls, which was used as a marker of vascularisation (Starke et al., 2011), and was associated with staining for CD31 and VEGFR2. Consistent with enhanced angiogenesis in the VWF-deficient mouse, Starke et al reported enhanced vascularisation in the VWF-deficient mouse ear compared to littermate controls. This provided the first evidence to show that endothelial VWF could regulate angiogenesis and vascular density. Furthermore, these studies proposed three potentially overlapping mechanisms through which VWF may be regulating angiogenesis.

VWF has been shown to bind integrin $\alpha v\beta 3$ on the EC surface (Cheresh and Spiro, 1987). Since this integrin has been shown to have both pro and anti-angiogenic effects (Hodivala-Dilke, 2008), Starke and colleagues investigated whether loss of VWF affects the expression of $\alpha v\beta 3$. This study found that inhibition of VWF caused increased internalisation and decreased protein levels of the $\beta 3$ subunit of the integrin. Interestingly, neither the αv subunit nor the $\beta 1$ subunit were affected after inhibition of VWF. These data suggested that VWF controls the expression of the integrin $\beta 3$.

The integrin β3 has also been shown to regulate signalling through the VEGF receptor, VEGFR2 (Somanath et al., 2009). In the absence of $\beta 3$ *in vivo,* enhanced VEGFR2 signalling has been

observed (Reynolds et al., 2002). To determine whether VEGFR2 plays a role in the VWF-dependent increase in angiogenesis, Starke and colleagues inhibited VEGFR2 signalling in VWF-deficient ECs *in vitro* using the small molecular inhibitor SU4312, and found that this restored proliferation and migration to baseline levels in these cells (Starke et al 2011; Starke et al unpublished). This indicated a role for VEGFR2 signalling in the VWF-dependent increase in angiogenesis.

Finally, VWF directs the formation of WPB, which store a number of key regulators of essential endothelial processes including angiogenesis. Angiopoietin 2 (Ang-2) is stored in WPB (Fiedler et al., 2004) and is also a key regulator of angiogenesis (Augustin et al., 2009). To determine whether loss of VWF affected Ang-2 levels, Starke and colleagues measured the release of Ang-2 from VWF-deficient cells. Here they found an increase in Ang-2 release from VWF-deficient cells. They speculated that this was due to loss of WPB and an inability to store Ang-2. Interestingly, Ang-2 has also been shown to decrease β3 expression (Thomas et al., 2010). This study provided the initial hypothesis that VWF regulates angiogenesis via intracellular (Ang-2 storage) and extracellular (integrin β3) mechanisms (reviewed in Randi et al, 2013).

1.7.2.2 The role for platelet-derived VWF in blood vessel formation

The endothelium and megakaryocytes are the only sources of VWF. While Starke *et al* demonstrated that endothelial VWF regulates blood vessel formation, the question remains whether platelet-derived VWF, which contributes to 20% of circulating VWF, could also regulate angiogenesis. Elegant studies have demonstrated that both *in vitro* and *in vivo*, platelets are required for angiogenesis (Battinelli et al., 2011, Kisucka et al., 2006). These data have mostly been attributed to the contents of platelet alpha granules, which contain both pro and anti-angiogenic mediators, including VEGF (Coppinger et al., 2004, Italiano et al., 2008). One key concept to help unravel this picture is that although VWF is required for WPB formation, it is not essential for alpha granule formation (Blair and Flaumenhaft, 2009). Since alpha granules appear to be largely intact after loss of VWF, it remains to be seen what role platelet VWF contributes to angiogenesis.

1.7.2.3 Plasma studies of growth factors in VWD

Plasma studies in patients with sporadic bowel angiodysplasia have demonstrated enhanced circulating levels of the pro-angiogenic mediators Ang-2 and VEGF (Holleran et al., 2015). Recent studies from a larger cohort of VWD patients revealed that in 395 Type 1, 239 Type 2 and 21 Type 3 patients, median levels of Ang-2 were significantly reduced compared to 100 controls. An increase in VEGF and Ang-1 was also found in the same cohort, but no difference could be observed in OPG and Gal-3 levels (Groeneveld, 2015). The authors attributed the decrease in Ang-2 levels to a lack of *in vitro* clearance mechanisms. However, interestingly, this study also demonstrated that in patients who experienced GI bleeding due to angiodysplasia, Ang-2 levels were significantly higher when compared to patients with bleeding from elsewhere (Groeneveld, 2015). Since platelets are a large source of Ang-1, it is possible that platelet activation could have contributed to the enhanced levels of Ang-1 in plasma samples. Importantly, the role of Ang-2 in VWF-dependent angiogenesis remains an unexplored area.

1.7.2.4 Angiogenic characterisation in endothelial cells (BOEC) from VWD patients

Work from our group in 2011 was the first to demonstrate that BOEC isolated from VWD patients recapitulated the expected endothelial phenotype of patients. In these studies, Starke et al reported an overall decrease in VWF released from these BOEC. In line with the studies carried out using siRNA to VWF in HUVEC, Starke and colleagues found overall enhanced Matrigel tube formation, proliferation and migration in EC isolated from 9 VWD patients compared to 8 healthy controls (Starke et al., 2011). However, more recent studies from our lab have identified distinct defects in *in vitro* angiogenesis in BOEC based on clinical classification. Here, Starke and colleagues found enhanced proliferation across all VWD groups (Type 1, Type 2 and 1 Type 3 patients); however, enhanced migration was only found in the Type 2 patient and Type 3 patients (Starke et al unpublished).

Studies from Groeneveld et al have shown a decrease in directional migration in VWD type 1 patient BOEC, but normal directionality in Type 2 patient-derived BOEC compared to controls (Groeneveld et al., 2015). This study also demonstrated that while at early passages BOEC

from Type 1, 2 and 3 patients showed increased tube formation on Matrigel, this effect was lost compared to controls at later passages. These authors suggested that the angiogenic capacity of these cells is greater at earlier passages at least in VWD patient-derived cells. Larger studies in VWD patients are needed to further determine the range of VWF expression and angiogenesis in patient EC.

1.8 Hypothesis & Aims

Previous work from our group has established that VWF suppresses angiogenesis both *in vitro* and *in vivo* (Starke et al., 2011). This study highlighted three potential candidate pathways through which VWF may regulate blood vessel formation (shown in figure 1.9). Overall, we hypothesise that VWF in the endothelium acts through both intracellular and extracellular mechanisms to modulate blood vessel formation and function. In particular, for this study I will focus on the role of the angiopoietin-2 (Ang-2) pathway.

The aim of this study is to understand the role of Ang-2 in the VWF-dependent control of blood vessel formation and function. An improved understanding of these pathways could allow potential therapeutic interventions for VWD patients with angiodysplasia to be identified.

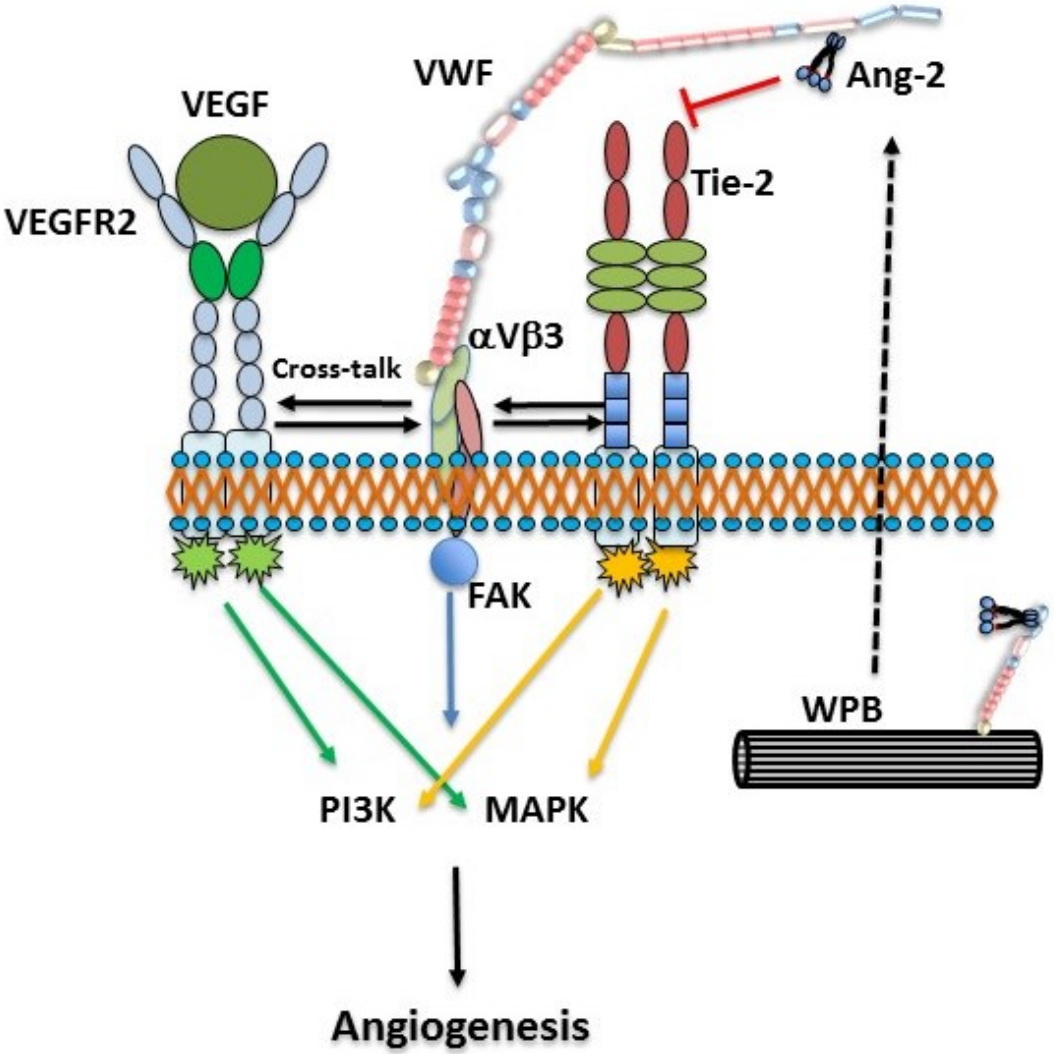

Figure 1.9 Summary of hypothesis. Extracellular VWF binds to and stabilises the integrin β3 which is a regulator of angiogenesis. The integrin αvβ3 interacts with VEGFR2 (Robinson et al., 2009) and also exhibits cross talk with Tie-2 receptor (Thomas et al., 2010; Cascone et al., 2005). VWF inside the endothelium controls the formation of WPB which stores proangiogenic proteins like Ang-2. Loss of VWF causes enhanced release of Ang-2 and decrease expression of integrin β3. We hypothesise that VWF through both intracellular and extracellular pathways can regulate angiogenesis. VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2; Ang-2: angiopoietin 2; FAK: focal adhesion kinase; PI3K: phosphoinositide 3-kinase; MAPK: mitogen activated protein kinase; WPB: Weibel palade bodies;

2 Chapter Two: Materials and Methods

2.1 HUVEC Isolation

Veins from Human Umbilical cords were washed with Hanks Balanced Salt Solution (HBSS; Sigma) containing Ca^{2+} and Mg^{2+} then incubated with 0.5 mg/ml Collagenase-A (Roche) in 20 ml of HBSS for 8 min at 37 °C and 5% CO_2. The cell suspension was centrifuged at 335 g for 8 min and supernatant discarded. Cell pellet was resuspended in "complete" medium: M199 (Sigma) containing 20% fetal calf serum (Labtech), 30 µg/ml endothelial cell growth factor (ECGF; Sigma), 10 U/ml Heparin (CP Pharmaceuticals), 1 U/ml Pencillin, 0.1 mg/ml Streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Cells were transferred to a 25 cm^{-2} flasks (Corning) pre-coated for 30 min with 1% gelatin (Sigma) in PBS. After 24 hrs, media was changed on HUVEC and cells were passaged in a 1:3 ratio into a 75 cm^{-2} flask after reaching 80-90% confluence.

2.2 BOEC Isolation (Dr. Richard Starke, Dr. Koralia Paschalaki, Dr. Anicee Danee)

48 ml of blood was collected in 6 BD Vacutainer® Cell Preparation Tubes containing Ficoll™ Hypaque™ solution (BD Bioscience). Tubes were inverted to mix then centrifuged at room temperature for 30 min at 1600 g. Samples were mixed immediately after centrifugation. Serum was collected along with the monoculear cell layer and added to 8 ml of PBS containing 10% HyClone serum (GE Healthcare Life science). Samples were then centrifuged at 520 g for 10 min at room temperature, with a high brake, twice and each time the supernatant was discarded. The number of viable cells in the cell pellet were counted using trypan blue (Sigma). Cells were resuspended at 3 – 5 x 10^7 mononuclear cells per 4 ml of Endothelial Growth Media (EGM-2) supplemented with 10% FCS. 4 ml of the mixture was added to each well of a 6 well plate pre-coated with 50 µg/ml rat tail collagen (type 1; Corning) and placed in a humid incubator at 37 °C, 5% CO_2.

2.3 Cell Culture

HUVEC were routinely maintained in 75 cm^{-2} flasks and passaged every 2-3 days by trypsin EDTA (Invitrogen) digestion. Cells were washed twice with HBSS without Ca^{2+} and Mg^{2+} (Sigma) and then incubated with 1 ml of 0.05% Trypsin EDTA for 1 – 2 min at 37 °C and 5%

CO_2 then resuspended in "complete" M199 (see section 2.1). HUVEC were used between passages 3 and 4. BOEC colonies were passaged from 6 well plates to 25 cm^{-2} flasks. Media was changed 24 hr post passaging. Cells were passaged 1:3 every 2 days. Cells were washed twice with HBSS without Ca^{2+} and Mg^{2+} then incubated with 1 ml of 0.05% Trypsin EDTA for 1 – 2 min at 37 °C and 5% CO_2 then resuspended in EGM-2 supplemented with 10% hyclone fetal calf serum. BOEC were used at passage P4 to P10 for all experiments.

Human dermal skin fibroblasts (Detroit 551, Public Health England) were purchased from the European Collection of Cell Cultures (ECACC) and maintained in M199 supplemented with 10 % FBS at 37 °C and 5% CO_2. Skin fibroblasts between P2 and P4 were used for all experiments.

2.4 Delivery of specific antisense oligonucleotides in endothelial cells

Expression of human von Willebrand factor (VWF) was inhibited using siRNA against the VWF sequence as shown in table 2-1 and donated as siVWF in text. In parallel All-star negative control siRNA (Qiagen) was used as a control for siVWF1 denoted as siCTL1 in text. Silencer® Select Negative control siRNA (Invitrogen) was used as a control for siVWF2; denoted as siCTL2 in text. These sequences have already been validated in previous studies (Starke et al., 2011). Expression of Forkhead Box O1 (FOXO1) was inhibited using pre validated siRNA (Qiagen) against FOXO1 denoted as siFOXO1 in the text (Dharaneeswaran et al., 2014). In parallel siCTL1 was used as control.

For delivery into HUVEC, cells were seeded at 1 x 10^5 cells per well of a 6 well plate 24 hrs prior to transfection in EGM-2 supplemented with 2% FCS to obtain a 60 – 80% confluent culture 24 hrs post-seeding. After 24 hrs, cells were transfected with siVWF or siCTL (siCTL1: 20 nM; siCTL2: 40 nM) or siFOXO1 (20 nM) and 2 µl per well Actufect01 lipid (final concentration: 1 µg/ml; Silence Therapeutics) prepared as a 5x concentrated solution in OptiMEM® serum free media (Invitrogen) and incubated for 30 min at 37 °C. After mixing, the lipid-siRNA mixture was combined with EGM-2 and added to cells for 24 or 48 hrs as indicated in the experiment details.

Table 2-1 Target sequences used for gene silencing in endothelial cells.

Target	Sequence (5'->3')	Company
VWF	AAGGGCTCGAGTGTACCAAAA	Qiagen
VWF	GGGATCTGTGATGAGAACGGA	Invitrogen
FOXO1	CCGAGTTTAGTAACAGTGCA	Qiagen

2.5 EC treatment *in vitro*

To inhibit mRNA synthesis HUVEC were seeded at 1 x 10^5 cells per well of a 1% gelatin coated - 6 well plate in EGM-2. After 24 hrs HUVEC were transfected with 20 nM siVWF1 and corresponding control siRNA or 40 nM siVWF2 or corresponding control siRNA. Wells were then treated with Actinomycin D from Streptomyces (Sigma) at concentrations indicated in figure legends for 2, 4 or 8 hrs. The cells were then lysed, RNA isolated and subjected to qRT-PCR.

For inhibition of VEGFR2 signalling *in vitro*, cells were seeded at 1 x 10^5 cells per well of 1% gelatin coated plates. After 24 hrs HUVEC were transfected with siRNA to VWF or corresponding control siRNA. After a further 24 hrs cells were treated with VEGFR2 tyrosine kinase inhibitor SU4312 (Sigma S8567) at 4 µM or corresponding DMSO controls. After 24 hrs RNA or protein lysates were collected and subjected to analysis.

For treatment with VEGF, HUVEC were seeded at 1 x10^5 cells per well of 1% gelatin coated plates. After 24 hours HUVEC were treated with siRNA to VWF or control sequences as indicated above. HUVEC were then serum deprived as indicated in the figure legend in M199 supplemented with 1% FCS. Media was then changed at the time point indicated in the figure legend to M199 supplemented with 25 ng/ml VEGF165 (Peprotech) or PBS control and 1% FCS for the time indicated in the text.

For inhibition of Angiopoietin 2 (Ang-2) function in HUVEC, HUVEC were seeded at 1 x10^5 cells per well of a 6 well plate for 24 hours. Cells were then transfected with siRNA to VWF or control sequences for 24 hours in EGM-2. Media was then changed to EGM-2 supplemented

with IgG control antibody (MedImmune LLC) or AZ.3.19.3 (MedImmune LLC) at 5 nM. After 24 hours, RNA and total cell lysates were collected for analysis.

For treatment of HUVEC with VWF, HUVEC were seeded at 1 x10^5 cells per well for 24 hours. In house purified VWF (hVWF) was a gift from Dr. Tom McKinnon purified by affinity chromatography from plasma derived VWF (Canis et al., 2010). VWF concentrate (cVWF) was obtained from a commercially available source. Cells were then transfected as indicated above with siRNA to VWF or control sequences for 24 hours in EGM-2. For reseeding experiments, cells were reseeded at 40,000 cm^{-2} into 6 well plate precoated for 1 hr with 1 µg/ml of VWF or gelatin 1%. For supernatant treatment, media was changed to EGM-2 supplemented with VWF at 4 µg/ml or PBS control for a further 24 hours. Total cell lysates and RNA were then collected for analysis.

For treatment of HUVEC with Lipopolysaccharide (LPS), HUVEC were seeded at 1 x10^5 cells per well for 24 hours. Cells were then transfected as indicated above with siRNA to VWF or control sequences for 24 hours in EGM-2. Media was then changed to EGM-2 supplemented with LPS at 100 ng/ml (Sigma) for 24 hours. RNA was then collected for analysis.

2.6 Adenovirus transduction of HUVEC

The volume of empty Adenoviral vector control (Adeno0) or constitutively active Akt virus (CA-Akt) used to infect cells was calculated using the formula:

$$\frac{Total\ cell\ number}{\#\ of\ wells\ \times\ desired\ MOI} = Total\ infectious\ unit\ needed\ (ifu)$$

$$\frac{ifu}{viral\ stock\ (\frac{ifu}{ml})} = amount\ of\ viral\ stock\ to\ get\ desired\ moi\ (ml)$$

To validate CA-Akt adenovirus, HUVEC were seeded at 2 x 10^5 cells per well for 24 hours. Cells were transduced with adenovirus at MOI indicated in text. To prevent VWF-dependent upregulation of Ang-2 expression, HUVEC were seeded at 1x10^5 cells on 1% gelatin coated 6 well plate. After 24 hrs cells were transduced with Adeno0 or CA-Akt at MOI 40 for 2 hrs in basal M199 with no GF or serum. Media was then changed to EGM-2 for a further 22 hrs after which time the cells were transfected with siVWF or siCTL at 20 nM. 48 hrs later cells were collected for RNA or protein expression analysis.

2.7 RNA isolation from HUVEC and mouse tissue

Mouse tissues were collected from animals after cervical dislocation and immediately snap frozen in liquid nitrogen and stored at -80 °C. Mouse tissues were then placed in 300 µl RLT buffer containing 3 µl of 2-mercaptoethanol with 1 Qiagen bead and homogenized using TissueLyserII (Qiagen) at frequency of 20 Hz for 2 min. Lysates were treated with 590 µl RNAse free water containing 10 µl proteinase K and incubated at 55 °C for 10 min. The mixture was then transferred to a new 1.5 ml Eppendorf tube and centrifuged at 10,000 g for 3 min. Supernatants were collected and used in the RNA extraction procedure.

RNA lysate solution containing mouse tissue or HUVEC in RLT buffer was passed through a 20-gauge needle (0.9 mm diameter) fitted to an RNAse-free 1 ml syringe. 1 volume of 70% ethanol was mixed to HUVEC lysates or 1 volume of 100% ethanol to tissue lysates prior to transferring to an RNeasy spin column placed onto a 2 ml collection tube. The column was centrifuged at 10 000 x g for 15 seconds. The flow-through was discarded. 350 µl of RW1 Buffer (Qiagen) was added to the RNeasy column. The column was centrifuged for 15 sec at 10, 000 g. The flow-through was discarded. 10 µl DNase 1 (Qiagen) diluted in RNAse- free water was added to 70 µl RDD Buffer (Qiagen) and added to the RNeasy column membrane for 15 min. 350 µl of RW1 buffer (Qiagen) was added to the RNeasy spin column. The column was centrifuged for 15 sec at 10,000 g and the flow through discarded. 500 µl of RPE buffer containing 4 volumes ethanol (Qiagen) was added to the RNeasy spin column and centrifuged for 15 secs at 10,000 g and the flow through discarded. 500 µl of RPE buffer (Qiagen) was added to the spin column again and centrifuged for 2 min at 10,000 g and the RNeasy column was placed in a new 2 ml collection tube. The RNeasy column was centrifuged at 18 000 g for

1 min and then placed into a clean RNAse-free 1.5 ml Eppendorf tube. 30 µl RNAse free water (Qiagen) was added to spin column membrane. The column was then centrifuged for 1 min at 18 000 g to elute RNA. RNA concentration and purity were analysed using NanoDrop ND-1000 spectrophotometer.

2.8 First strand cDNA synthesis

1 µg of RNA was added to 1 µl oligoDT (Promega) and 1 µl 10 mM dNTP mix (Invitrogen) and the final volume made to 14 µl with sterile H_2O. The reaction was heated to 65 °C for 5 min and incubated on ice for at least 1 min. 4 µl of 5X First-strand Buffer (Invitrogen), 1 µl 0.1 M Dithiothreitol (DTT) and 1 µl of Superscript III Reverse Transcriptase (Invitrogen) were added to the mixture. The reaction was incubated at 50 °C for 60 min to promote first strand cDNA synthesis. The reaction was terminated by heating to 70 °C for 15 min.

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

For 96 well plate analysis, 5 µl of pre-diluted template cDNA was added to 12.5 µl of PerfeCTa SYBR Green Fastmix (Quanta Biosciences), 10 µl of 10 µM forward, 10 µl of 10 µM reverse primer and 5.5 µl H_20. Cycling consisted of DNA denaturing for 3 min at 95 °C followed by amplification that was repeated for 40 cycles at 95 °C for 15 sec followed by the required annealing temperature as indicated in table 2-2 for 30 seconds. All PCR primer efficiencies were between 90 – 110%. 96 well plate analysis was performed using Bio-Rad CFX Manager Software version 2.0

Table 2-2 Oligonucleotides for qRT-PCR analysis.

Target	Primer sequences (5'-> 3')		Annealing Temp (°C)
	Forward	Reverse	
18S (mouse)	GGACAGGATTGACAGATTGATAG	CTCGTTCGTTATCGGAATTAAC	60
Angiopoietin-2 (human)	CGACGTGAGGATGGCAGCGTTGA	ATCAAACCACCAGCCTCCTGTTAGCA	56.4
Angiopoietin-2 (mouse)	TCTGGTTCTGCACCACATTC	ACAACACACAGTGGCTGATGA	60
BMPR4 (human)	GTCCAAGCTATCTCGAGCCT	GGGATGGCACTACGGAATGG	60
CD63 (human)	AATGTTACTGTGGGCTGTGG	GAGGAGGCTGAGACC	60
CD63 (human)	GTGTGAAGTTCTTGCTCTAC	CCACTGCGATGATGACC	60
CXCR4 (human)	CCAGTAGCCACCGCATCT	ATAGTCCCCTGAGCCCATTT	60
Cyclin D1 (human)	TGAGGCGGTAGTAGGACAGG	GACCTTCGTTGCCCTCTGT	60
Cyclin G2 (human)	ACTTGGCAGGTCATGAAGGG	CCGGGGTAGCCTCAATCAAA	60
Elk-3 (human)	TGTCAGCATGGAAAGTCGGG	GCACTCTCCATACCCAGATGT	60
eNOS (human)	GTGATGGCGAAGCGAGTGAAGG	ACCACCAGCACCAGCGTCTC	60
FOXO1 (human)	AGCTCGGCGGGCTGGAAGAATT	GCTCGGCTTCGGCTCTTAGCA	60
FOXO1 (mouse)	ATGTGTTGCCCAACCAAAGC	ATGTAGCCTGCTCACTAACTCTT	60
Galectin 3 (human)	CTGCTGGGCCACTGATTGT	TGTTATCAGCATGCGAGGCA	60
GAPDH (human)	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG	60
GAPDH (mouse)	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	60
Interleukin 8 (human)	CAGAGACAGCAGAGCACAC	AGTTCTTTAGCACTCCTTGGC	60
p21 (human)	CACTCAGAGGAGGCGCCATGT	CGCTGTCCACTGGGCCGAAG	60
p27 (human)	TGCAACCGACGATTCTTCTACTC	TTTGACGTCTTCTGAGGCCAG	60
Tie-2 (human)	CTCCCAGTGCGCTGGATGCC	GCAGTAGGGTGTGCCTAAGC	63
von Willebrand factor (human)	GCAGTGGAGAACAGTGGTG	GTGGCAGCGGGCAAAC	65
von Willebrand factor (mouse)	CTCACACAGAGCCACAAAGG	AACTGCGAGAGCTCTTCTGG	60

2.10 Ang-2 plasmid construct preparation

The Ang-2 promoter construct was a kind gift from Professor Hellmut Augustin in Germany (Hegen et al 2004). The plasmid was transported as a spot on filter paper. To retrieve the plasmid, the spot was cut from the filter paper and transferred to a 1.5 ml Eppendorf tube. 100 µl of TE buffer was added and incubated at 4 °C overnight. The filter paper was removed and the sample stored at -20°C. DNA was prepared as outlined below and the plasmid vector was cut with HindIII and KpnI restriction enzymes by incubating 5 µl of DNA with 1 µl of buffer 2.1 (New England Bioscience), 2 µl of dH_20, 1 µl of HindIII and 1 µl Kpn1 and incubating at 37

°C for 1hr then 65 °C for 20 min. Samples were electrophoresed on a 1% agarose gel and imaged on using as described below.

2.11 Agarose Gel Electrophoresis

Digestion products were run on agarose (Sigma) gels (percentage specified in figure legend) in Tris Acetate- EDTA (TAE) buffer containing 1:10,000 GelRed (Cambridge Bioscience). Gels were loaded with sample DNA and 4X loading dye alongside a 1 kb DNA molecular weight ladder (New England BioLabs) and electrophoresed at 100 V for 1 hr. Gels were imaged using an Ultra Violet Transilluminator (BioImaging Systems).

2.12 Luciferase reporter assays

2.12.1 Cell transfections:

For analysis of Ang-2 promoter activity, HUVEC were seeded at 1 x 10^5 cells per well of 6 well plate pre coated with 1% gelatin in EGM-2 for 24 hours. Cells were then transfected with siRNA to VWF or control sequences for 24 hours. Cells were then transfected with 6 µl genejuice (Merck chemicals), 1 µg of Renilla plasmid and 1 µg expression plasmid (pGL3-Ang-2) or empty control for 24 hours. SV40 plasmid was used a positive control. Cell lysates were then collected using passive lysis buffer from Dual-Luciferase reporter assay system (Promega).

2.12.2 Reagent preparation:

200 µl of 50X Stop & Glo substrate (Promega) was added to 10 ml of Stop & Glo buffer in the amber stop & Glo bottle provided (Promega). The mixture was then vortexed for 10 sec. Prepared mixture can be stored at -20C for up to 15 days. Lyophilised luciferase assay substrate was resuspended in 10 ml of Luciferase Assay buffer II (Promega).

2.12.3 Measurement:

BioTek Synergy microplate reader dispenser 1 and dispenser 2 were primed with 1X LARII (Promega) and 1X Stop & Glo respectively. 20 µl of sample was placed in triplicate into a clear bottom white walled 96 well plate. Plate was read using Synergy BioTek microplate reader.

2.13 Protein binding assay *in vitro*

2.13.1 Preparation of coated plates

1 µg/ml of recombinant Ang-2 (rhAng-2; R&D systems) diluted in PBS was added to 96 well plate overnight at 4 °C. Wells were emptied and blocked using 3% BSA for 30 mins at 37 °C. 10 nM VWF was added to necessary wells for 45 mins. Wells were blocked again using 3% BSA for 30 min. Wells were kept in PBS until ready.

2.13.2 Preparation of cells and assay

Cells were trypsonised using 0.05% trypsin EDTA and resuspended in complete M199 supplemented with 20% FCS. Cell suspension was centrifuged at 335 g for 8 mins. Media was aspirated and cells resuspended in plain M199 supplemented with 0.5% BSA. Cell count was determined. Cells were then resuspended in M199 supplemented with 0.5% BSA at 10^6 cells per ml. 5 µl of Vybrant cell dye (Thermoscientific) per ml of cells was added and incubated in a waterbath at 37 °C for 20 mins. Cells were then centrifuged and wash twice with fresh media. Cell count was then determined. Cells numbers (indicated in figure legends) were added to matrices in 96 well plate for 40 mins at 37 °C. Plate was washed once to remove no adherent cells and read on Synergy BioTek microplate reader excitation: 530/25 and emission 590/35.

2.14 Enzyme Linked Immunosorbent Assay

2.14.1 Human VWF

Polyclonal VWF antibody (Dako A0082) was diluted 1:500 in carbonate-biocarbonate buffer (Sigma) and 100 µl was added per well of a 96 well maxisorp plate (Thermo scientific) and incubated for 1 hr at room temperature. The plate was washed 3 X with Dulbeccos A

Phosphate Buffered Saline (Sigma) containing 0.1% Tween-20 (Sigma). Wells were then blocked with 100 µl per well of 2% Bovine Serum Albumin (Sigma) for 1hr. Normal human plasma (Technoclone) was diluted 1:5 in PBS supplemented with 0.1% tween 20 (PBS-T) to form the first standard of 2 µg/ml and a dilution series was created by further dilution. PBS-T was used a blank control. Cell lysates were diluted 1:5 and Supernatants 1:1 in PBS-T. All wells were then washed 3 X with PBS-T and 50 µl of standard or samples were added to the plate for 1h. The plate was then washed 3 X in PBS-T. Anti-VWF antibody conjugated with HRP (Dako P0226) was diluted 1:1000 in PBS-T and added to each well for 45 min. The plate was then washed 3 X using PBS-T. 100 µl of Colour Fast OPD (Sigma) solution was added to each well and the reaction stopped with 50 µl of 1M H_2SO_4. The plate was then read at 492 nm using a Synergy BioTek microplate reader.

2.14.2 Human angiopoietin 2

The human Ang-2 ELISA was performed using a human angiopoietin-2 DuoSet ELISA kit (DY623) from R&D systems. 100 µl of capture antibody (R&D system) was added to each well of a 96 well maxisorp plate (Thermoscientific) and incubated over night at room temperature. The antibody was removed and the plate washed with phosphate buffer saline containing 0.05% Tween-20 (PBS-T) 3 times. The plate was blocked using 200 µl of 1% BSA. After 1 hr the plate was washed with PBS-T and 100 µl of samples or standard were incubated on the plate for 2 hrs at room temperature. The plate was then washed with PBS-T and 100 µl of detection antibody (R&D systems) was then incubated per well at room temperature. After 2 hrs the plate was washed and 100 µl of streptavidin-HRP was incubated in each well for 20 min at room temperature away from direct sunlight. The plate was washed with PBS-T then 100 µl of colour fast OPD (Sigma) was added per well and the reaction stopped using 50 µl of 1M H_2SO_4. The plate was then read at 492 nm using a Synergy BioTek microplate reader.

2.14.3 Murine angiopoietin 2

The mouse Ang-2 ELISA was performed using mouse/rat Angiopoietin-2 quantikine ELISA kit (MANG20) from R&D systems. 50 µl of assay diluent RD1-40 was added to each well of pre-coated microplate (R&D systems). Standards were diluted from 2500 pg/ml to 39.1 pg/ml in

calibrator diluent RD5-26 (R&D systems). 50 µl of standard or samples were added to the plate and incubated for 2 hours at room temperature. Wells were then washed with the wash buffer provided 4 times. 100 µl of mouse/rat angiopoietin-2 conjugate was added to each well and incubated for a further 2 hrs. The wash step was repeated and 100 µl of substrate solution was added for 30 min at room temperature. 100 µl of the stop solution provided was added to each well. Optical density was determined using a Synergy BioTek microplate reader at 570 nm and 450 nm. Values at 570 nm were subtracted from readings at 450 nm.

2.14.4 Human Interleukin-8

The human IL-8 ELISA was performed using an anti-human IL-8 ELISA mini kit (Peprotech). Capture antibody at 0.5 µg/ml in PBS was added to each well of a maxisorp 96 well plate (Thermoscientific) for 24 hr at room temperature. The plate was then washed using PBS supplemented with 0.05% Tween-20 (PBS-T). 300 µl of 1% BSA in PBS was added to each well for 1 hr at room temperature. The wash step was repeated as described previously. The standard was diluted from 1 ng/ml to 0.1 ng/ml in 0.1% BSA in PBS containing 0.05% Tween 20. Supernatant samples were diluted 1:1 in diluent and lysates were diluted 1:10 before 100 µl of standard or sample were added to the plate in duplicate. After 2 hr the plate was washed and 100 µl of 0.5 µg/ml detection antibody (Peprotech) was added to each well for an additional 2 hr. The plate was then washed as described above. Avidin-HRP conjugate (Peprotech) was diluted 1:2000 and 100 µl was added per well and incubated for 30 min at room temperature. The plate was then washed and 100 µl of Colour Fast OPD (Sigma) was added per well and the reaction stopped using 50 µl of 1M H_2SO_4. The plate was then read at 492 nm using a Synergy BioTek microplate reader.

2.15 Immunofluorescence analysis of HUVEC and BOEC

2 x 10^4 HUVEC or BOEC were grown on 1% gelatin (HUVEC) or 50 µg/ml Collagen (BOEC) coated 13-mm diameter glass coverslips in 24-well plates for 24 hrs. Cells were then treated as described above with siRNA to VWF or control for 48 hrs. Cells were fixed using 4% Paraformaldehyde (PFA) in HBSS containing Ca^{2+} and Mg^{2+} for 20 min. Cells were permeabilised using 0.4% Triton X100 for 4 min. Cells were blocked using 3% BSA/PBS for 10

min. Cells were stained using the following primary antibodies: rabbit anti-VWF (1:1000; DAKO A0082), goat anti-Ang-2 (1:500; Santa Cruz Biotechnology), mouse anti-FOXO1 (1:200; Cell Signalling), goat anti-VE-cadherin (1:200; Santa Cruz Biotechnology), anti pTie-2 (1:200; R&D system). All primary antibodies were incubated at room temperature for 1 hr. Secondary antibodies used were anti-rabbit Alexa Fluor (AF) 488, anti-mouse AF 555, anti-goat AF 555 (1:500; Invitrogen). Nuclei were visualised using DAPI (1:500; Invitrogen). Secondary antibodies were incubated at room temperature for 1 hr. Coverslips were mounted onto glass slides using Fluoromount G™ (eBioscience). Images were captured using a Carl Zeiss LSM510 or LSM-780 Confocal microscope. Image analysis was performed using Volocity 6.3 (Perkin Elmer).

2.16 Immunofluorescence of mouse tissue and Matrigel plugs

Tissues were collected and fixed in 4% PFA for 2 hours at room temperature then transferred to 70% ethanol (ETOH) for 2 hours. Samples were then embedded in paraffin blocks and sectioned into 4 µm slices by Ms. Lorraine Lawrence, Histology facility South Kensington. Tissue sections were dewaxed using histoclear (National Diagnostics) for 5 min. Sections were then dehydrated in 100% ETOH for 5 min then rehydrated in 70% ETOH for 5 min, then washed in distilled water (dH_2O) for 5 min. Antigen retrieval was performed by heating samples in citrate buffer at pH 6.5 for 6 min in a microwave. Slides were cooled for 30 min then the samples outlined using a hydrophobic liquid blocker pen (Sigma). Sections were blocked in 3% BSA with 0.4% Triton X100 in PBS for 30 min. Sections were incubated with primary antibodies overnight in 3% BSA in PBS. Primary antibodies used were FITC conjugated Isolectin B_4 (1:250; Vector Labs), Rat anti-Endomucin (1:500; Santa Cruz Biotechnology), biotinylated anti-Isolectin B_4, (1:250; Vector Labs), goat anti-Angiopoietin 2 (1:500; Santa Cruz Biotechnology), rabbit anti-TRITC (1:200; Abcam), biotinylated anti-FITC (1:200; Vector Labs), HRP conjugated anti-VWF (1:100; DAKO) and rabbit anti-VWF (1:100; DAKO). Following washes, slides were incubated with the following secondary antibodies: anti-rabbit AF 488, streptavidin AF 555, anti-Rat AF 633, anti-rabbit Texas Red for 1 hour. Nuclei were detected using DAPI or DRAQV. Slides were mounted in Fluoromount G (eBioscience). Images were captured using a Carl Zeiss LSM510 or LSM-780 Confocal microscope. Image analysis was performed using Volocity 6.3 (Perkin Elmer)

2.17 Immunoblotting

2.17.1 Preparation of total cell lysates

Cells were washed twice with cold PBS. 100 µl of Cell lytic MT (Sigma) supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 2 and 3 (Sigma) and Phenylmethylsulfonlyl (PMSF) at 1:100 was used to lyse the cells. Lysates were centrifuged for 15 min at 10 000 g at 4°C. Supernatants were collected and processed for SDS-PAGE.

2.17.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The total protein concentration in samples was determined by incubating 10 µl of sample with 1000 µl of precision red (Universal Biologicals) and detecting the absorption measured at 600 nm in a spectrophotometer. SDS-PAGE immunoblotting was carried out using 20 µg of total cell lysate. Samples contained 20 µg of protein in loading buffer (Thermoscientific) containing 20% 2-mercaptoethanol (Sigma) (for reducing conditions) were heated at 95 °C for 5 min. Proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Licor). Membranes were blocked in PBS containing 3% BSA for 1 hr at room temperature and incubated with primary antibodies diluted in Tris buffered saline supplemented with 0.1% Tween20 (TBST-T) overnight at 4 °C.

Blots were labelled with the following primary antibodies: anti-GAPDH (1:10,000; Millipore), anti-phospho FAK Y397 (1:1000; thermofisher), anti-FAK (1:1000; Santa Cruz), anti-phospho Akt S473 (1:1000; Cell Signalling), anti-pan Akt (1:1000; Cell Signalling), anti-phospho FOXO1 Thr-24 (1:1000; Cell Signalling), anti-FOXO1 (1:1000; Cell Signalling), anti-Tie 2 (1:1000; Cell Signalling), anti- Integrin β3 (1:1000), anti-S6 Ribosomal protein S235/236 (1:1000; Cell Signalling), anti-S6 Ribosomal protein (1:1000; Cell Signalling), anti-p44/42 MAPK (1:1000; Cell Signalling) and anti-p44/42 (T202/Y204) MAPK. Primary antibodies were detected using either fluorescently labelled secondary antibodies: goat anti-rabbit IgG Dylight 680 and goat anti-mouse IgG Dylight 800 (Thermoscientific) or horseradish peroxidase linked anti-rabbit IgG (Cell Signalling) and anti-mouse IgG (GE healthcare)**.** Detection and quantification of fluorescently labelled antibodies were performed using Odyssey CLx imaging system (LI-COR

Biosciences) and Odyssey 2.1 software. Detection of chemiluminescence signals were performed using ECL (Amersham GE Healthcare) on Kodak Bio Max Light Film. Quantification of signal was performed by densitometry and normalised against loading controls using Image J.

2.18 Fibrin Bead Assay

HUVEC were treated with siCTL or siVWF 24 hr prior to coating onto cytodex 3 beads (GE Healthcare). To coat cells onto the beads, cells were incubated with beads at 400 cells per bead in 1.5 ml of complete EGM with 2% FCS. The bead-HUVEC mixture was gently shaken every 20 min for 4 hr at 37°C and 5% CO_2. After coating the beads with cells they were transferred into a 25 cm^{-2} flask in 5 ml of complete EGM containing 2% FCS. The following day cells were collected from the flask by gentle washing. The bead-HUVEC mixture was allowed to settle then media removed and the beads washed three times with complete EGM-2 containing 2% FCS. Cells were then resuspended at 500 beads per ml in 2 mg/ml fibrinogen (Sigma) in PBS containing 0.15 units/ml aprotinin (Sigma). 500 µl of fibrinogen-bead solution was added to 24 well containing 0.625 units thrombin (Sigma) and mixed gently. Mixture was allowed to solidify for 5 min at room temperature then incubated at 37 °C and 5% CO_2 for 20 min. 20,000 fibroblasts were seeded on top fibrin clot in 1 ml complete EGM-2 supplemented with 2% FCS, IgG control or Ang-2 neutralising antibody at 5 nM. For experiments using Tie-2-fc, 2.5 ng/ml was added to complete EGM-2 or PBS control. Media was replaced every 48 hours.

2.18.1 Quantification of sprouts *in vitro*

Images of beads were captured at day 5 on Olympus IX70 microscope using 10X objective. Images were analysed using image J. The number of sprouts per bead was determined. A minimum of 30 beads per condition was analysed.

2.19 BrdU *in vitro* proliferation assay

HUVEC were seeded for 24 hr on 1% gelatin coated 6 well plates. The following day the cells were transfected with siCTL or siVWF for 24 hr. After 24 hr cells were serum starved in M199

with penicillin/strepamycin, L-glutamine and 1% FCS. After 4 hr cells were seeded into a 96 well plate at 2,000 cells per well in either M199 supplemented with 1 U/ml Pencillin, 0.1 mg/ml Streptomycin (Sigma), 2 mM L-glutamine (Sigma) and 10% FCS or cEGM2 containing 2% FCS. Cells were then stimulated with VEGFA-165 (peprotech) at 100 ng/ml. Cells were then treated with the concentration of inhibitors outlined in figures and incubated at 37°C and 5% CO_2. After 72 hr, 1X BrDU reagent (Roche) was added to the cells for 4 hr. The supernatant was removed and 200 µl of fixative reagent (Roche) was added to each well. After 30 min, 100 µl of anti BrDU antibody was incubated with the cells for 90 min. The plate was then washed with 1X wash buffer (Roche) then 100 µ of substrate solution (Roche) was added, The colour development was stopped with 1M H_2SO_4. The plate was then read at 450 and 690 nm using Synergy BioTek microplate reader.

2.20 Matrigel tube formation assay *in vitro*

HUVEC were seeded for 24 hr on a 1% gelatin coated 6 well plate (Corning) before being transfected with siCTL or siVWF. After 24 hr, cells were serum starved in M199 supplemented with 1% Fetal calf serum, penicillin/streptomycin and L-Glutamine for 4 hr. Growth factor reduced (Corning) Matrigel ® was thawed overnight on ice kept at 4 degrees. 130 µl of Matrigel was added to the wells of a pre-chilled 48 well sterile plates and placed at 37°C, 5% CO_2 for 30 min to allow the Matrigel to polymerize and solidify. 50,000 cells per well were seeded in M199 supplemented with 1% Fetal Calf Serum, 1 U/ml Pencillin, 0.1 mg/ml Streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Cells were photographed 24 hr after seeding using Olympus IX70 microscope. Quantification of the capillary network formed was performed using the Image J Neuron J plugin.

2.21 Matrigel tube formation assay *in vivo*

For Matrigel analysis, mice were anaesthetised using Isoflurane gas and injected subcutaneously (s.c) in the abdomen with 250 µl of Matrigel (BD Bioscience) supplemented with 64 U/ml of heparin (CP Pharmaceuticals) and 100 ng/ml murine $VEGF_{165}$ (Peprotech) or PBS (control) and allowed to recover for 7 days. All mice were culled by cervical dislocation.

2.22 Dextran injection *in vivo* (Neil Dufton)

Mixtures containing 1:1 of 10 mg/ml 2 x10^6 MW FITC conjugated Dextran and 4.4 x 10^3 MW TRITC conjugated Dextran in PBS were prepared in 7 ml Bijus wrapped in aluminium foil. Mice were placed in a warming chamber at 30°C for 15 min prior to the procedure. Mice were then anaesthetized using Isoflurane gas and injected IV with 100 µl of the dextran mixture. Mice were allowed to recover for 15 min then culled by cervical dislocation and the tissues harvested.

2.23 LPS treatment *in vivo*

Mice used were aged between 8-12 weeks. A mixture containing LPS at 500 µg/ml PBS. Mice were injected with 100 µl diluted LPS i.p and sacrificed 6 or 12 hours later. Tissues were collected at time points indicated in the figure legends.

2.24 *In vivo* permeability using evans blue

Mice aged between 8-12 weeks were treated with 50 µg i.p LPS (E. coli 0111:B4) as described above. After 11 hrs mice were anaesthetised with Isoflourance gas and injected i.v. with 2% evans blue dye. Mice were then recovered for 15 min then culled by cervical dislocation. Abdomen of mice were then opened and mice were perfused with 10 ml PBS via left ventricle. Hearts and lungs were collected into 1.5 ml eppendorf tube and weight determined. Miles assay was performed as described in (Radu and Chernoff, 2013).Evans blue was extracted by placing tissue sample in 500 µl formamide solution (Sigma) for 48 hours at 50 °C. Samples were centrifuged at 13000 g and supernatant removed. Standard curve of Evans blue was generated to determine evans blue quantity. Absorbance was measured using Synergy BioTek microplate reader at 610 nm. Evans blue values were normalised to tissue weight.

2.25 Data Analysis

Data is presented as mean ± standard error of mean (SEM) of at least three independent experiments (unless otherwise stated). Data were analysed with student t-tests when comparing data between two groups or Analysis of Variance (ANOVA) for multiple

comparisons using GraphPad Prism software 6.0. Results were considered significant when the P value was < 0.05.

3 Chapter Three: VWF regulates Ang-2 storage and levels in endothelial cells

3.1 Introduction

3.1.1 VWF and Ang-2 storage in Weibel Palade Bodies

Colocalisation of VWF and Ang-2 in WPB has not only been observed in EC *in vitro* but also in tissues such as the colon from healthy individuals (Fiedler et al., 2006). As described in Chapter 1, Ang-2 is stored in WPB for rapid release upon endothelial activation (Fiedler et al., 2004). Following endothelial activation, both Ang-2 and VWF are released after WPB exocytosis (Kümpers et al., 2009, Fiedler et al., 2004, Hyseni et al., 2014). Previous work from our lab has shown that inhibition of VWF expression causes enhanced release of Ang-2 into the supernatant (Starke et al., 2011). Since VWF directs the formation of WPB, it is likely that loss of the storage organelle causes enhanced release of Ang-2 (reviewed in Randi et al, 2013). This may not be the case for all WPB components, since P-selectin is targeted for degradation after inhibition of VWF expression(Denis et al., 2001).

3.1.2 VWF and Ang-2 levels in circulation

The contribution of environmental and genetic factors have both been suggested to modulate plasma VWF levels (reviewed in Casari et al, 2013). In particular, both the contribution of the ABO blood group and mutations in the VWF gene itself have been suggested to contribute to VWF hereditability (Antoni et al., 2011, Franchini et al., 2007). In addition, unmodifiable factors such as age have also been shown to positively correlate with VWF levels (Rydz et al., 2015, Coppola et al., 2003). VWF released from WPB has been shown to contribute to most of the circulating levels of VWF (Lopes da Silva and Cutler, 2016). Moreover, large differences in circulating Ang-2 concentrations can be observed in healthy individuals, ranging from 300pg/ml-5000pg/ml (Chong et al., 2004, Groeneveld, 2015, Petersen et al., 2016, Yeo et al., 2008). To date, no explanation for this variability in Ang-2 circulating levels has been proposed. In patients with inflammatory disease, Ang-2 and VWF have been shown to be increased in plasma (van der Heijden et al., 2009b, Nossent et al., 2016, Orfanos et al., 2007).

3.1.3 VWF binds to Ang-2 in EC and after release

VWF and Ang-2 have been described to interact physically (Mckinnon et al., 2011). Using a purified system, McKinnon, in collaboration with our lab, showed that VWF binds to Ang-2 in

conditions of high Ca^{2+} and low pH. These conditions are reminiscent of those in the trans-golgi network (Wagner et al., 1986), which suggests that Ang-2 may associate with VWF before entry into the WPB.

Since VWF and Ang-2 bind at pH 6.5, McKinnon *et al* asked whether this interaction persists in the circulation where pH is 7.4. Immunoprecipitation of VWF from the circulation of healthy individuals showed the presence of Ang-2 in complex with VWF (figure 3.1). This confirmed that VWF binds to Ang-2 in WPB and that this interaction persists after release from EC. As outlined in chapter 1, VWF is released through at least 3 known mechanisms: constitutive, basal and regulated. McKinnon *et al* demonstrated the presence of Ang-2 on VWF strings, after regulated release (Mckinnon et al., 2011). It remains to be seen whether Ang-2 is released under constitutive or regulated mechanisms after inhibition of VWF expression.

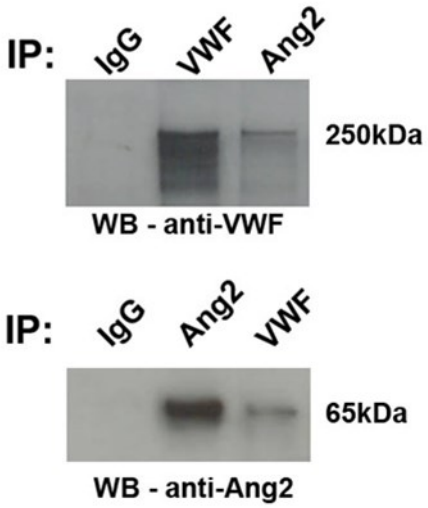

Figure 3.1 VWF and Ang-2 remain bound in the circulation of healthy individuals. Pooled plasma from 4 donors was immunoprecipitated with dyna beads coupled to either anti-VWF or anti-Ang2 antibodies or control IgG. Bound proteins were eluted in 2X SDS gel loading buffer with 2% β-mercaptoethanol. Samples were run on 4-12% Bis-tris gel and immunoblotted for anti-VWF (top) or anti-VWF (bottom). Data reproduced with the permission of Dr. Tom McKinnon, Department of Haematology, Imperial College London.

3.1.4 VWF controls protein stability in the circulation after release from endothelial cells

VWF released from endothelial cells is known to bind to and protect coagulation factor FVIII from degradation (Weiss et al., 1977, Lenting et al., 2007). In patients with decreased VWF, decreased circulating levels of FVIII have been observed (reviewed in Miesbach and Berntorp, 2015). Additionally, in some patients, where VWF levels are normal but function is impaired, FVIII levels are decreased. In these patients, mutations in the D1-D3 domains of VWF (FVIII binding site in VWF) causes the decreased circulating levels of FVIII (Berber, 2012, Nishino et al., 1989). Similar to the intervention in patients with severe VWD, restoration of FVIII levels in VWD mouse by administration of VWF has also been reported (Pergolizzi et al., 2006).

Recently, VWF has also been suggested to act as stabiliser of other endothelial proteins. Galectin 1 and Galectin 3 have been found in WPB associated with VWF (Saint-Lu et al., 2012). Additionally, these carbohydrate-binding proteins are associated with VWF after release from WPB and form complexes with VWF in the circulation. Measurement of both Galectin 1 and Galectin 3 in the circulation of VWF-deficient mice revealed a significant decrease compared to littermate controls (Saint-Lu et al., 2012). These data suggest that VWF can also regulate the stability of these two proteins in the circulation; however further experiments directly investigating proteolysis and protein stability are needed to confirm this hypothesis.

3.1.5 Hypothesis and Aims

Inhibition of VWF expression in EC caused enhanced release of Ang-2. Moreover, VWF directs the formation of WPB which store Ang-2. This would suggest that VWF regulates the storage and release of Ang-2 and potentially other WPB proteins. In addition, VWF and Ang-2 are released together in the circulation. VWF is known to regulate the levels of proteins such as FVIII in the circulation. Whether VWF regulates the stability of Ang-2 remains unknown. Finally, Ang-2 expression in EC is low, however whether this affected by inhibition of VWF remains unknown.

The aims of this chapter are to:

- Investigate whether VWF controls Ang-2 release from endothelial cells via control of Weibel Palade Bodies formation
- Determine the effect of loss of VWF on other WPB constituents
- Determine circulating levels of Ang-2 *in vivo*, in the VWF-deficient mouse and patients with VWD
- Determine whether VWF affects Ang-2 expression in EC

3.2 Results

3.2.1 Inhibition of VWF expression in EC results in loss of regulated Ang-2 release via loss of WPB

Previous work from our lab showed that inhibition of VWF expression by siRNA for 48 hours caused enhanced release of Ang-2 from EC (Starke et al 2011). To study whether inhibition of VWF causes constitutive release of Ang-2, HUVEC were treated with control or VWF siRNA for 24, 48 and 72 hours. Total cell lysates and supernatants were collected at all time points for VWF ELISA and Ang-2 ELISA respectively. After treatment of HUVEC with siVWF in EC for 24, 48 and 72 hours (figure 3.2A) VWF protein expression was inhibited by 90- 95%. Ang-2 release was ≈2-fold higher than in control siRNA treated cells at 24, 48 and 72 hours (figure 3.2B) suggesting enhanced release. To test whether the increased Ang-2 release was due to loss of the storage organelle WPB, we used a well-characterised activator of WPB exocytosis: phorbol 12-myristate 13 acetate (PMA) (Fiedler et al., 2004). HUVEC were treated with control or VWF siRNA for 48 hours. 75 minutes before collection of supernatants and total cell lysates, the media was changed and cells were stimulated with 100 ng/ml PMA or PBS control for 75 minutes. PMA treatment resulted in a 3-fold increase in VWF levels in the supernatant of control cells, confirming WPB exocytosis; no release was detected from VWF-deficient cells, in line with lack of WPB (figure 3.3A). In control cells, VWF release was associated with a significant reduction in VWF intracellular levels by about 50%, as expected, with no change in VWF-deficient cells (figure 3.3B). Measurement of Ang-2 in the same supernatants revealed that PMA induced a significant increase in Ang-2 release in control cells (figure 3.3C; white bars). In line with WPB exocytosis, PMA induced a 50% reduction in intracellular levels of Ang-2 in control cells (figure 3.3D; white bars). However, PMA treatment of VWF-deficient cells did not cause an increase in Ang-2 levels in the supernatant (figure 3.3D; black bars), nor reduced intracellular Ang-2 levels (figure 3.3D; black bars), indicating no stimulated release. These results suggest that constitutive Ang-2 release from VWF-deficient cells does not occur via a regulated, secretagogue-induced pathway and may be due to loss of WPB.

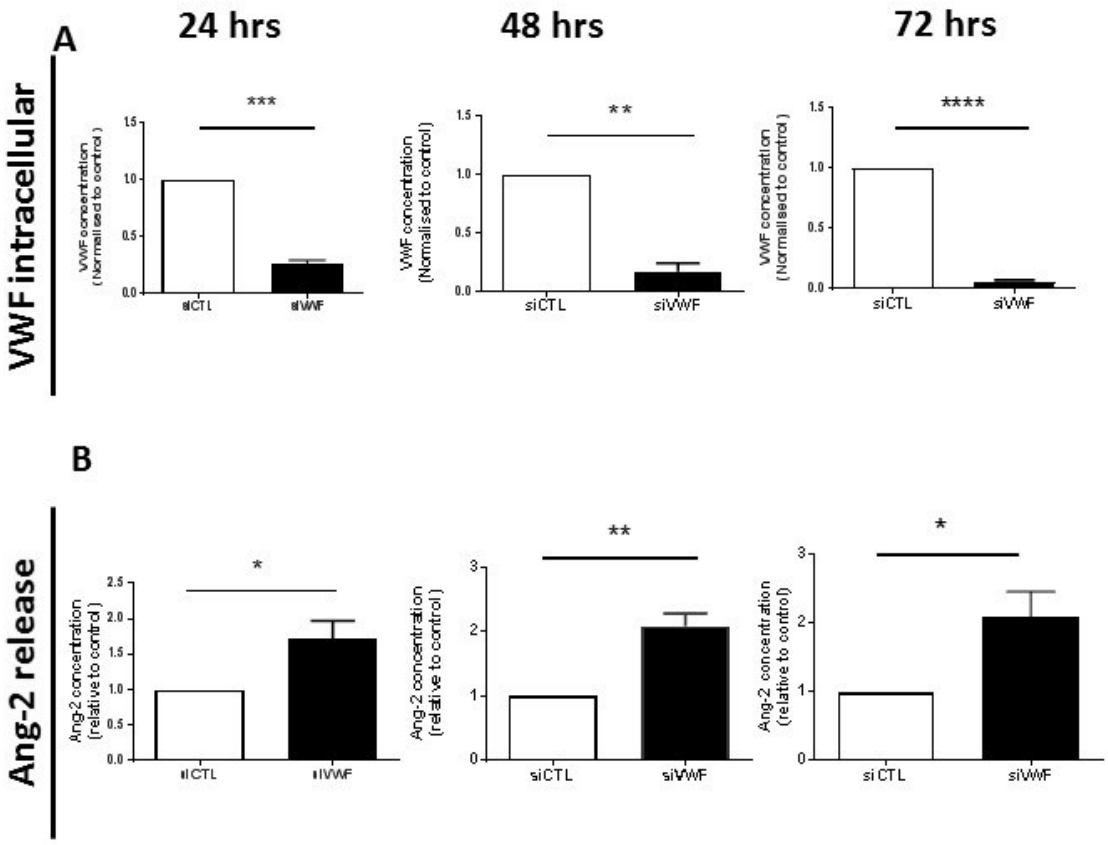

Figure 3.2 Dynamics of Ang-2 release from EC after inhibition of VWF. HUVEC were transfected with either siRNA to VWF (siVWF) or corresponding siRNA control sequence (siCTL). After 24, 48 and 72 hrs intracellular VWF protein levels were measured by ELISA (A). Ang-2 protein levels were measured by ELISA from supernatants collected from the same time points (B). Values were normalized to total protein and expressed relative to siCTL. Data shown as mean ± SEMof n=4. Statistical significance determined by student's t-test. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001

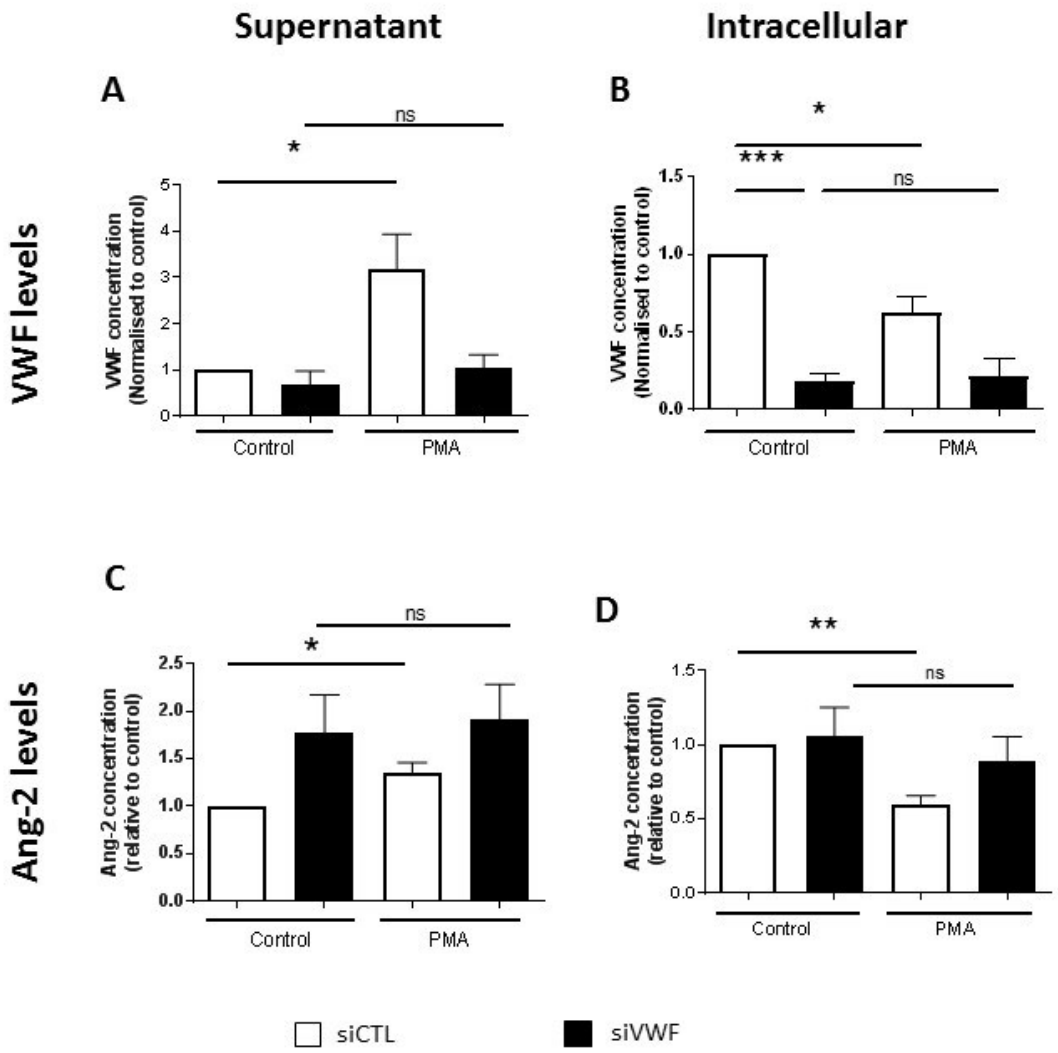

Figure 3.3 Absent PMA response in VWF-deficient cells. HUVEC were transfected with either siRNA to VWF (siVWF) or corresponding siRNA control sequence (siCTL) for a total of 48 hours. 75 mins before collection of total cell lysates and supernatants cells were stimulated with PMA 100 ng/ml or DMSO control. VWF protein levels in supernatant (A) and total cell lysates (B) were measured by ELISA. Ang-2 protein levels in supernatant (C) and total cell lysates (D) were measured by ELISA. Values were normalised to total protein and expressed relative to siCTL (DMSO) treated cells. Data shown as mean ± SEM of n=5. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$.

3.2.2 Inhibition of VWF expression in EC results in redistribution of intracellular Ang-2

Despite enhanced Ang-2 release from VWF-deficient cells (figure 3.3C; baseline), Ang-2 intracellular protein levels were not significantly changed (figure 3.3D; baseline). This was surprising, given that VWF-deficient cells do not possess WPB, the storage organelle for Ang-2. To help understand this finding, immunofluorescence was performed in control and VWF-deficient ECs. HUVEC were treated with siRNA to VWF or control sequence for 48 hours. Cells were fixed then stained for VWF (green), Ang-2 (red) and nuclei (blue). Isotype control staining (figure 3.4, top panel) demonstrated absent background staining. In control siRNA-treated cells, HUVEC showed typical VWF staining in rod shaped WPB structures (figure 3.4, middle panel; zoomed image). In these structures, VWF and Ang-2 were colocalised, as expected (yellow in merged image). In addition, in some areas, VWF expression was observed with absent Ang-2, suggesting that not all VWF colocalises with Ang-2. When VWF expression was inhibited using siRNA for 48 hours, VWF staining was dramatically reduced (figure 3.4, bottom panel). In these cells, Ang-2 staining was less intense than in controls, with a diffuse pattern in the cytoplasm and increased intensity in perinuclear regions (figure 3.4, bottom panel, zoomed image), a pattern reminiscent of endoplasmic reticulum (ER). These data suggested that VWF-deficient cells retain Ang-2 in non-WPB organelles.

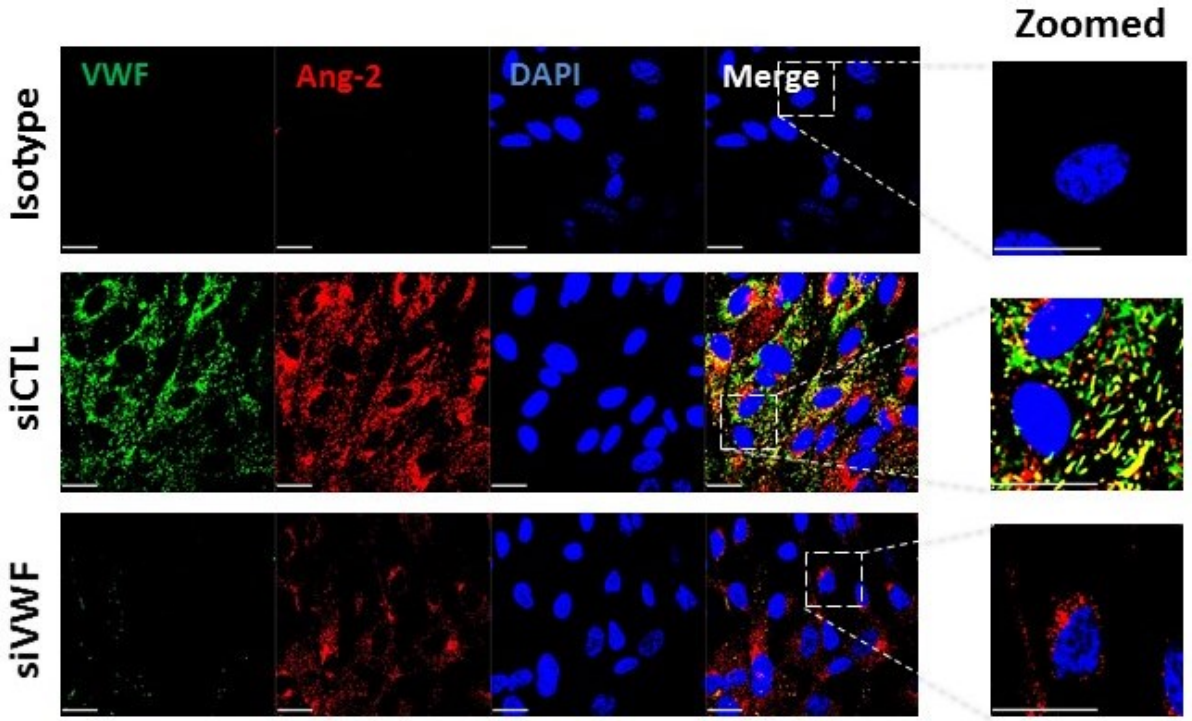

Figure 3.4 Redistribution of Ang-2 protein after inhibition of VWF expression in EC. HUVEC were transfected with either siRNA to VWF (siVWF) or corresponding siRNA control sequence (siCTL) for 48 hours. Representative x63 objective images of cells stained for expression of VWF (green), Ang-2 (red) and DAPI identifying nuclei (blue). Scale bar represents 20 µm.

3.2.3 Inhibition of VWF expression in EC differentially regulates WPB proteins

The results described so far show that inhibition of VWF expression causes loss of WPB and release of Ang-2. We next investigated whether loss of VWF has a generalised effect on WPB proteins. For these experiments we focused on two known constituents of WPB, namely: the transmembrane protein CD63/tetraspanin and the soluble cytokine IL-8. HUVEC were treated with siRNA to VWF (siVWF) or control sequences (siCTL) for 48 hours. Total cell lysates and supernatant were collected. Measurement of CD63 intracellular levels by immunoblotting demonstrated no difference between VWF-deficient cells compared to controls (figure 3.5A). Analysis of IL-8 in total protein lysates revealed that inhibition of VWF expression caused reduced intracellular levels of IL-8 (figure 3.5B). Since CD63 is not released, we focused on IL-8 release. VWF-deficient cells showed a significant reduction of IL-8 levels in the supernatant compared to controls (figure 3.5C). These results show that VWF does not regulate all WPB proteins in the same manner. This may suggest that VWF regulate the synthesis of these proteins differentially or differences in the stability of WPB proteins after inhibition of VWF.

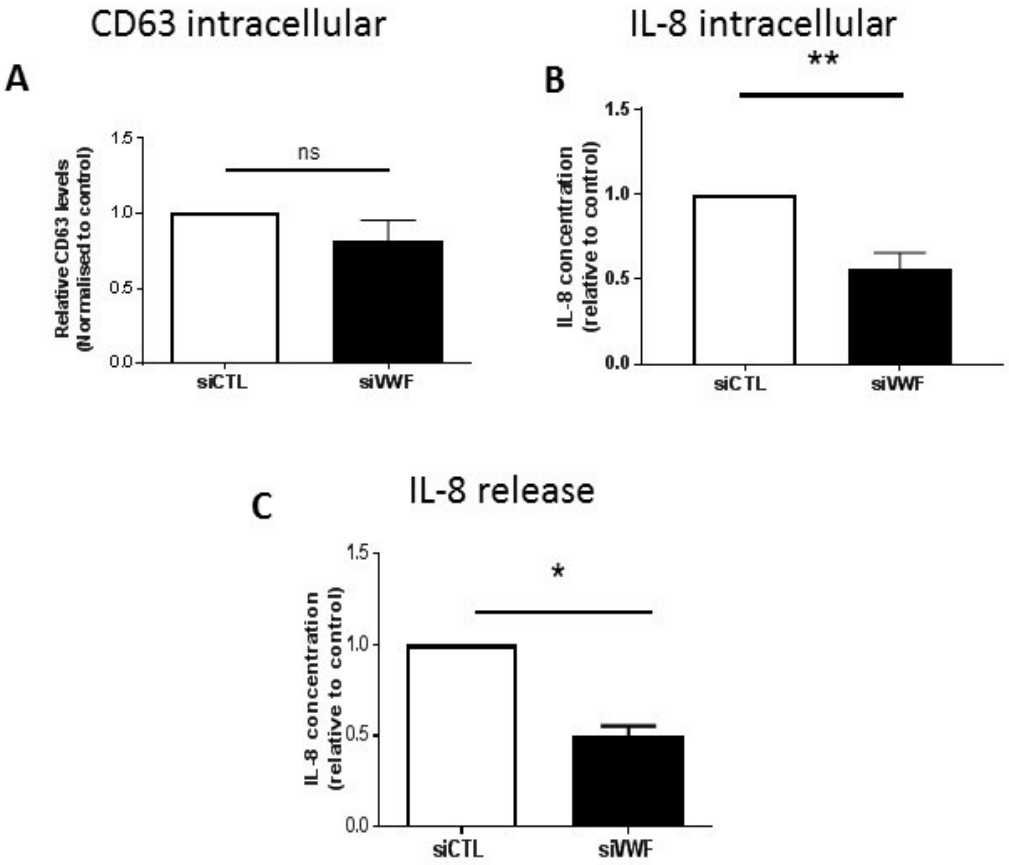

Figure 3.5 Selective regulation of WPB synthesis in EC by VWF. HUVEC were transfected with either siRNA to VWF (siVWF) or corresponding siRNA control sequences (siCTL) for 48 hrs. (A) Quantification of CD63 protein expression by immunoblotting, normalised to tubulin and expressed relative to siCTL (n=3). (B) IL-8 protein levels in total cell lysates (n=5) (C) IL-8 protein released into supernatant were measured by ELISA (n=3). Data was normalised to total protein and expressed relative to siCTL. Data shown as mean ± SEM. Statistical significance was determined by student's t-test. ns= $p>0.05$, * $p\leq0.05$, ** $p \leq 0.01$. (Data shown in A from experiments performed by Dr. Richard Starke)

3.2.4 Enhanced release of Ang-2 in EC from patient with severe quantitative defect in VWF

The studies described so far, showing that VWF regulates Ang-2 storage in EC, were carried out in HUVEC, the commonly used model for endothelial biology. To investigate whether Ang-2 levels are affected in ECs from VWD patients, we used the model of circulating endothelial progenitors, also called blood outgrowth endothelial cells or BOEC. BOEC were isolated from a 37-year-old, female patient with complete absence of VWF in plasma (type 3 VWD, see clinical data in table 3-1). BOEC from healthy volunteers were also used. In culture, BOEC from normal and VWD patient (figure 3.6A; left) showed similar cobble stone morphology as HUVEC (figure 3.6A; right), in line with previous studies. Characterisation of BOEC was carried out as described (Starke et al., 2013). Immunofluorescence staining of BOEC from a healthy control (figure 3.6B; top panel) and HUVEC (figure 3.6B; bottom panel) for VWF (green), VE-CADHERIN (red) and DAPI (blue) further confirmed endothelial phenotype. Using both immunofluorescence (figure 3.7A; bottom panel) and ELISA (figure 3.7B), we showed that BOEC from the type 3 VWD patient had reduced VWF expression compared to healthy controls. Ang-2 protein release into the supernatant of type 3 VWD BOEC patient showed a modest increase compared to controls (62.7 ng/mg vs 49.3 ng/mg) (figure 3.7C), in line with the data from VWF-deficient HUVEC. Statistical analysis was not performed due to only one patient being available. More studies on control BOEC will be required to establish whether the type 3 BOEC data is within normal limits. Immunofluorescence staining for Ang-2 (figure 3.7A; bottom panel) showed a significant reduction in type 3 VWD BOEC compared to healthy controls. Sample was unavailable for intracellular Ang-2 ELISA. These studies lend support the model that VWF controls Ang-2 storage in endothelial cells however more studies are required.

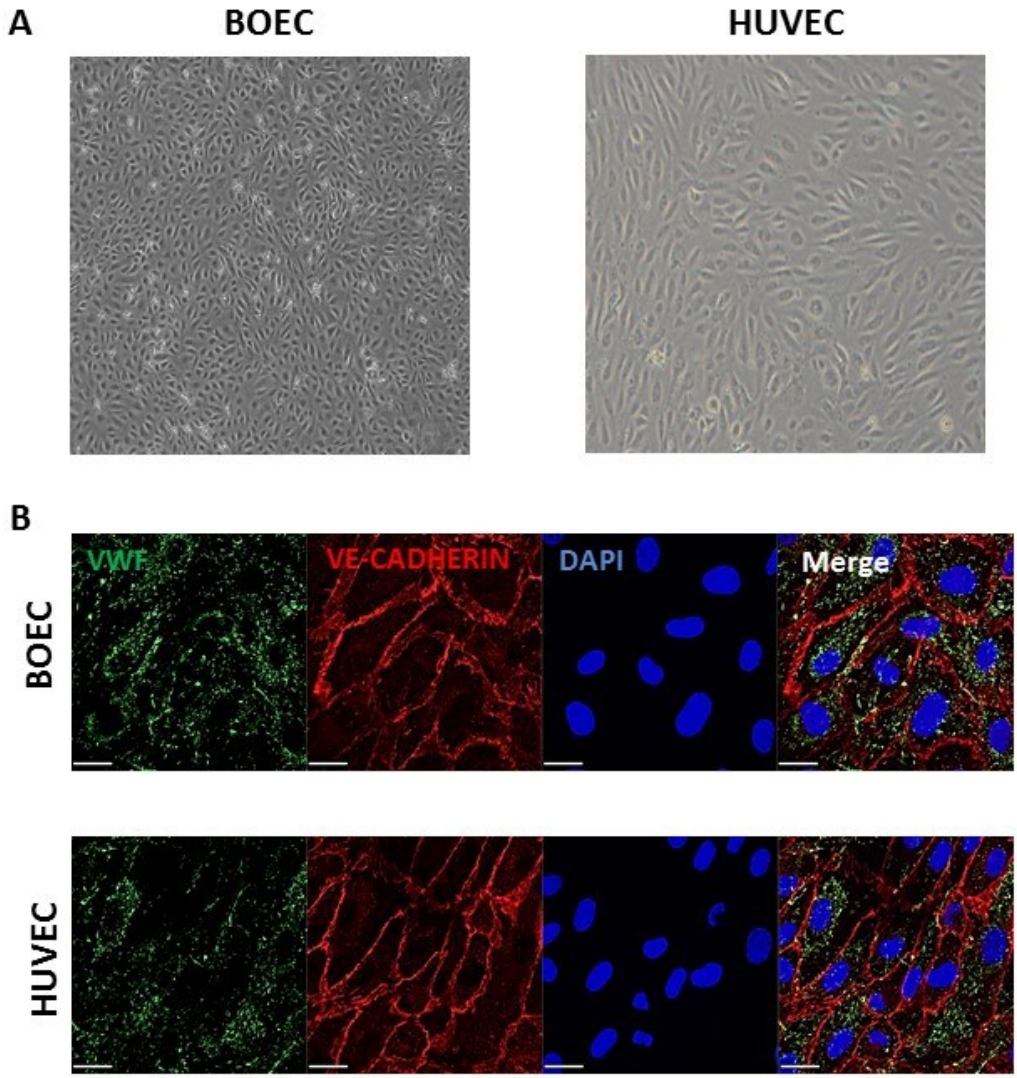

Figure 3.6 Morphological comparison of BOEC and HUVEC. (A) Representative images of established cultures of BOEC (left) and HUVEC (right). (B) Representative images of BOEC (top) and HUVEC (bottom) stained for VWF (green), VE-Cadherin (Red) and DAP (Blue). Scale bar represents 20 µm. (BOEC from healthy volunteer isolated by Dr. Anicee Danaee).

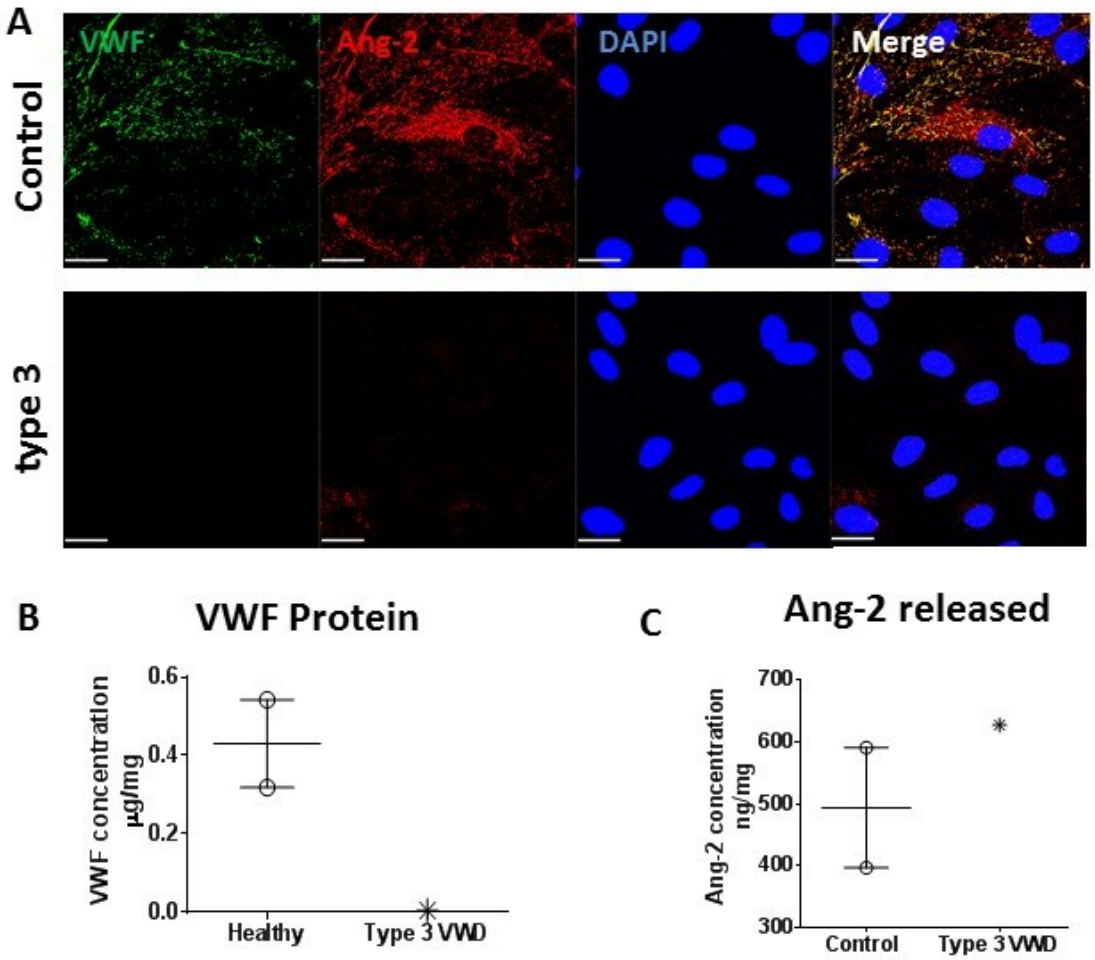

Figure 3.7 Increased Ang-2 release from EC isolated from type 3 VWD patient. (A) Representative image of BOEC isolated from healthy control (top panel) and type 3 VWD patient (bottom panel). Stained for VWF (green), Ang-2 (red) and DAPI (Blue). Scale bar presents 20 µm. (B) VWF protein levels were measured by ELISA in total cell lysates from established cultures of BOEC collected from 2 healthy controls and 1 type 3 VWD patient. Data was normalised to total protein. (C) Ang-2 protein released into the supernatant of established BOEC cultures over 48 hours was measured by ELISA from 2 healthy volunteers and 1 type 3 VWD patient. Data was normalised to total protein. Data presented as mean ± SEM. (BOEC isolated by Dr. Anicee Danaee and Dr. Koralia Paschalaki).

3.2.5 Analysis of levels of VWF and Ang-2 in the circulation

3.2.5.1 Circulating serum Ang-2 levels in patients with VWD

Given that inhibition of VWF caused enhanced Ang-2 release from ECs, we speculated that patients with quantitative defects in VWF may have increased Ang-2 circulating levels. Plasma was collected from 8 patients with quantitative defects in VWF (6 type 1 and 2 type 3). Plasma was also collected from patients with qualitative defects in VWF (5 type 2A and 4 type 2M). Patient clinical details are recorded in Table 3-1. Patient P002 was excluded from analysis due to a diagnosis of AVWS secondary to IgM paraprotein. Additionally given the sensitivity of Ang-2 to inflammation, patient P005 was excluded due to ongoing joint inflammation. Ang-2 levels were measured by ELISA and compared to 15 healthy control volunteers. Considering all VWD patients together, median Ang-2 levels were not different in VWD compared to healthy controls (304.1 pg/ml [IQR; 177-493.7] vs 331.1 pg/ml [254.4-432.2]). In addition, considering all patients with quantitative defects together (type 1 and type 3), again no significant difference in median Ang-2 levels were found compared to controls (429.7 pg/ml [315.8-606.9] vs 331.1 pg/ml [254.4-432.2]). Patient 004 and 006 with type 1 VWD showed higher levels of circulating Ang-2 compared to controls (figure 3.8A), however nothing remarkable was recorded about these patients. Considering patients with qualitative defects in VWF, no significant difference was observed compared to controls, despite a trend towards decreased median levels, (182 pg/ml [113.8-314.4] vs 331.1 pg/ml [254.4-432.2] p=0.0974). Median levels of Ang-2 in type 3 VWD patients (423.6 pg/ml [368.1-479.1]) appeared higher compared to controls and type 2 VWD patients; however, with only two patients in the type 3 VWD group, no reliable statistical analysis could be performed.

Due to the low numbers of patients with severe quantitative defects in VWF, we utilised a murine model of severe VWD. Serum was collected from 5 WT and 7 littermate controls. When Ang-2 levels were measured, no significant difference in the median levels in controls vs VWF-deficient mice was observed (23969.1 pg/ml [20513.2-24927.3] vs 22611.59 pg/ml [20542.1-27208.2]) (figure 3.8B). These data demonstrates that complete absence of VWF does not affect circulating Ang-2 levels. These findings, apparently at odds with the *in vitro* findings, may be linked to the tissue-specific regulation of Ang-2 expression, explored in subsequent sections.

Table 3-1 Baseline characteristics of VWD patients studied

ID	Sex	Age	VWD diagnosis	Mutation	VWF Antigen levels (0.5-2.00)	Multimer analysis	Ang-2 levels (ng/ml)
P001	M	58	Type 1	c.2438dupG and c.2281+4A>G (intronic)	0.07	U/A	214.592
P002	F*	UNK	AVWS	N/A	U/A	N/A	324.954
P003	F	35	Type 1	Not performed	0.43	U/A	208.461
P004	F	41	Type 1	I1343V, V1360A,F1369I,S1378F,R379C	0.13	U/A	858.369
P005	F*	24	Type 1	R1205H	0.11	U/A	931.164
P006	F	36	Type 1	None	0.42	All multimers present	631.044
P007	UNK	UNK	Type 2	NR	NR	U/A	570.202
P008	M	73	Type 2M	R1315C	0.14	All multimers present	171.674
P009	M	28	Type 2A	R1597W	.38	Reduced HMWM	370.9
P010	M	32	Type 2A	R1597W	0.23	Reduced HMWM	379.425
P011	F	40	Type 2A	R1597W	0.48	Absent HMWM	118.487
P012	F	28	Type 2A	D1653V	0.36	All multimers present	62.5717
P013	F	40	Type 2M	No genetics	0.3	All multimers present	193.041
P014	F	52	Type 2M	F1369I	0.5	U/A	295.552
P015	M	36	Type 2M	I1416N	0.2	Reduced HMWM	99.8486
P016	M	37	Type 3	homozygous for c.2435delC (p.P812RfsX31)	<0.01	U/A	312.692
P017	F	35	Type 3	Not performed	0.06	U/A	534.57

*excluded from analysis.
U/A: unavailable
N/A: not applicable
NR: not recorded
UNK: unknown; data not collected

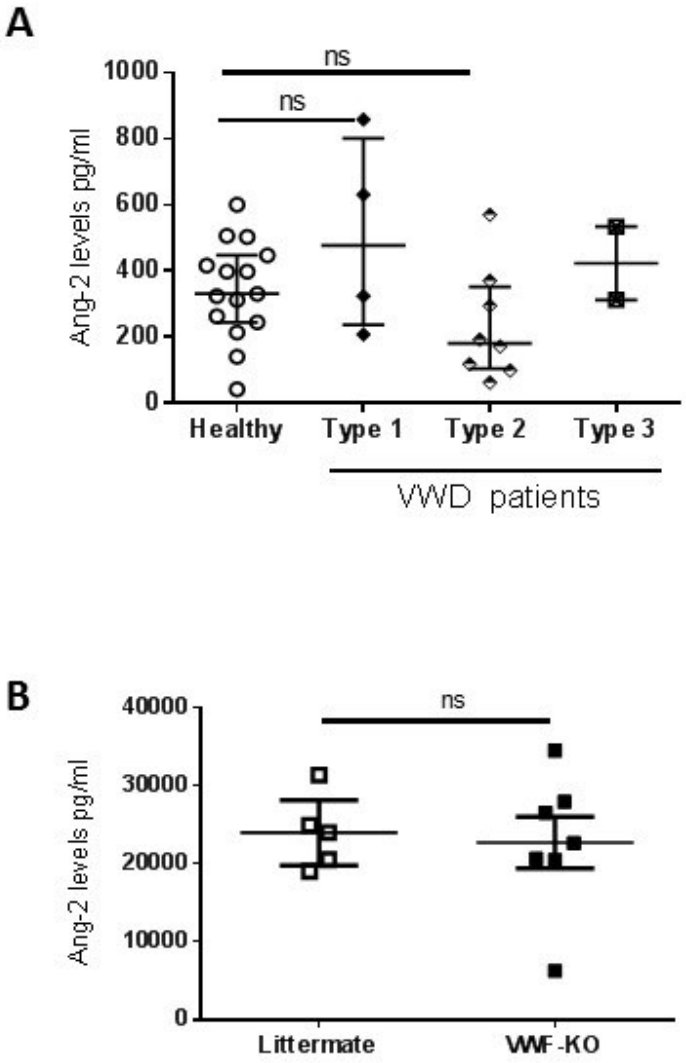

Figure 3.8 The effect of quantitative or qualitative VWD on circulating Ang-2 levels. (A) Plasma samples were collected from 15 healthy controls, 4 type 1 VWD, 8 type 2 VWD and 2 type 3 VWD patients prior to clinical intervention. Ang-2 levels were measured by ELISA. Data presented as median with IQR. (B) Serum samples were collected from 5 WT and 7 VWF-KO mice. Ang-2 levels were measured by ELISA. Data presented as median with IQR. Statistical significance determined by Mann-Whitney test. ns $p \geq 0.05$

3.2.5.2 Regulation of circulating Ang-2 levels in a type 3 VWD patients following treatment with plasma-derived VWF

Having established the basal circulating levels of Ang-2 in patients with VWD, we asked whether an increase in plasma levels of VWF, following treatment with VWF, could affect circulating Ang-2 levels. Five plasma samples were obtained from a 35-year-old, female, type 3 VWD patient. The patient was followed by Dr. Carolyn Millar at the Haemophilia centre, Hammersmith Hospital and was placed on prophylactic weekly treatment with VWF. Two samples were obtained, 20 mins and 4.5 hours after 1000 U of intravenous (i.v) Haemate P (plasma derived VWF). Two follow up samples were collected approximately 45 days following initial assessment, after weekly doses of VWF. All samples were compared to a pre-treatment plasma sample (S1). Treatment with Haemate P rapidly increased plasma levels of VWF back within normal range, as expected; it also reduced Ang-2 levels by approximately 30% (figure 3.9A; S2). Within 4 hours, VWF levels were reduced to the bottom end of the normal range, with not much change in Ang-2 levels (figure 3.9A; S3). This suggested an acute response of circulating Ang-2 to VWF treatment. In the first of the follow-up samples, VWF levels were at the higher end of normal (figure 3.9A; S4). Ang-2 levels in this sample remained around 30% of the pre-VWF sample. In the next sample (figure 3.9A; S5) measured 4 days after S4, VWF levels were back to the bottom end of normal with Ang-2 levels increasing above normal as measured in control patients. To determine whether there was a correlation between VWF and Ang-2 levels, a linear regression analysis was performed. Although not significant ($p=0.2116$), this showed a trend towards a negative correlation between VWF and circulating Ang-2 levels (figure 3.9B). These data are of course not sufficient to draw significant conclusions on the relationship between VWF and Ang-2 circulating levels; nevertheless, they suggest that dramatic changes in VWF levels in the circulation are associated with changes in Ang-2 circulating levels.

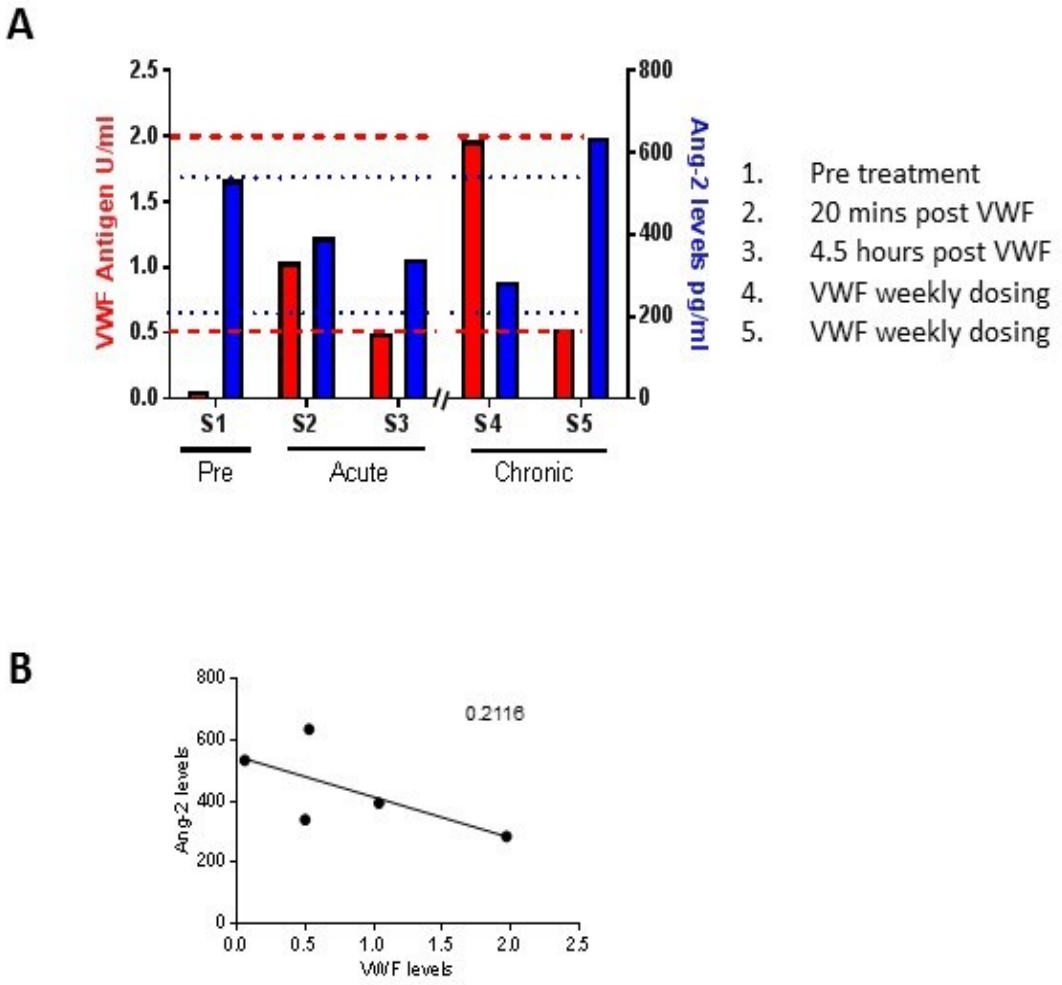

Figure 3.9 The effect of exogenous VWF on circulating levels of Ang-2. Plasma samples were obtained from a severe type 3 VWD patient (A) VWF Antigen levels were measured by Haematology Laboratory Hammersmith Hospital. Ang-2 levels were measured by ELISA. (B) Linear regression plot of VWF and Ang-2 levels in circulation of patient.

3.2.6 Decreased VWF expression in EC results in enhanced Ang-2 mRNA levels

The data described above show that in the absence of VWF, no WPB are formed and Ang-2 is constitutively released. However, intracellular levels of Ang-2 were unchanged. Moreover, Ang-2 staining in VWF-deficient HUVEC appeared to be stronger in regions reminiscent of ER. VWF expression in HUVEC was inhibited using siRNA for 48 hours. SiVWF1 reduced VWF mRNA expression by ≈90% (figure 3.10 A). Analysis of Ang-2 mRNA levels showed a significant 2-fold increase in VWF-deficient cells compared to controls (figure 3.10B). To confirm these findings in HUVEC, a second independent siRNA sequence was used (designated siVWF2). siVWF2 reduced VWF mRNA expression by ≥ 90% (figure 3.10C), resulting in a 2-fold increase in Ang-2 mRNA levels (figure 3.10D). Thus loss of VWF expression in HUVEC causes an increase in Ang-2 expression. In support of this finding, measurement of Ang-2 mRNA in BOEC from the same type 3 VWD patient as measured previously, revealed enhanced levels when compared to 7 healthy controls (figure 3.10E). To determine whether the effect on Ang-2 mRNA levels was a generalised effect on WPB proteins, we measured mRNA levels of CD63 and IL-8, as above. We found CD63 mRNA levels were increased after inhibition of VWF expression (figure 3.11A); however, IL-8 mRNA levels were unchanged (figure 3.11B). These data demonstrate that VWF controls gene expression in endothelial cells, and suggests that this effect is not related to their presence in WPB.

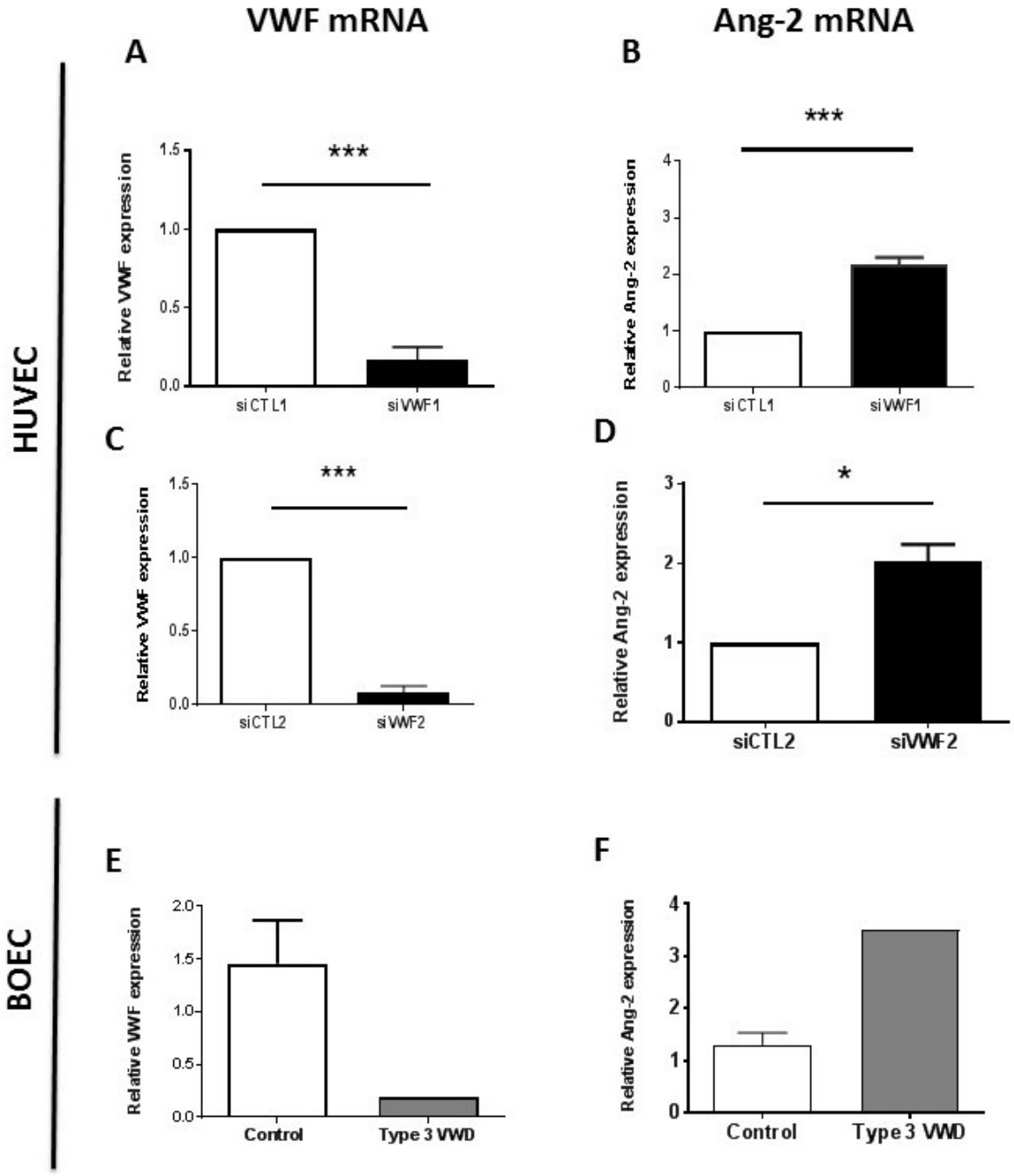

Figure 3.10 VWF suppresses Ang-2 mRNA levels in EC in vitro. HUVEC were transfected with either siRNA to VWF (siVWF1 or siVWF2) or corresponding siRNA control sequences (siCTL1 or siCTL2). After 48 hours, VWF mRNA expression was measured by qRT-PCR (A and C). Ang-2 mRNA expression was measured by qRT-PCR (B and D). Values were normalised to GAPDH and expressed relative to siCTL. Data shown as mean ± SEM of n=3. Statistical significance determined by student's t-test. *p≤0.05, *** p≤0.001. RNA was collected from established BOEC cultures isolated from 7 healthy volunteers and 1 type 3 VWD patient. VWF (E) and Ang-2 (F) mRNA expression was measured by qRT-PCR. Values were normalised to GAPDH and expressed relative to controls. Data presented as mean ± SEM. (Experiment E and F performed by Dr. Richard Starke)

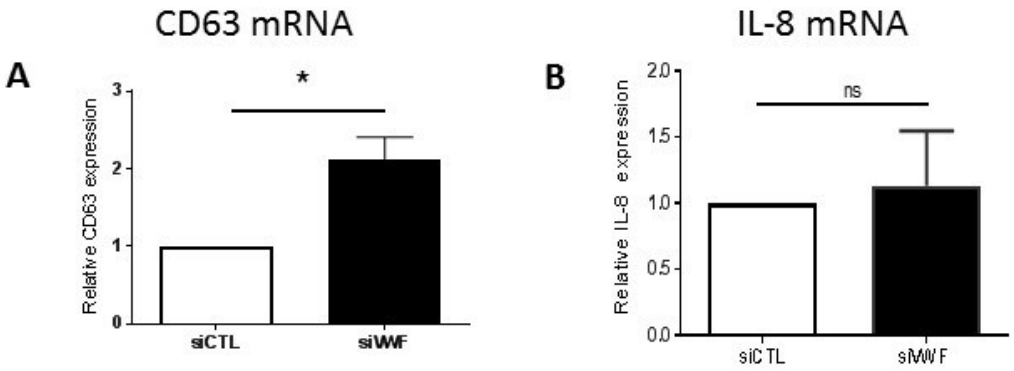

Figure 3.11 Selective regulation of CD63 and IL-8 mRNA levels in VWF-deficient cells. HUVEC were transfected with siRNA to VWF or corresponding control sequence for 48 hours. CD63 expression (A) and IL-8 expression (B) was measured by qRT-PCR. Values were normalised to GAPDH and expressed relative to siCTL. Data shown as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, *$p\leq0.05$.

3.2.7 Increased Ang-2 mRNA levels in heart but not lungs nor liver of VWF-deficient mouse

To validate these findings *in vivo*, we tested whether Ang-2 mRNA levels were increased in the VWF-deficient mouse. VWF expression is known to vary in tissues, with higher expression in lungs over heart and lower in the liver (Yamamoto et al., 1998). This suggests that VWF is regulated in a tissue specific manner. To determine whether Ang-2 is regulated in a similar manner after loss of VWF, lungs, heart, liver were collected from VWF-deficient mice and littermate controls. RNA was collected and Ang-2 mRNA expression was measured by qRT-PCR. Analysis of mouse lungs demonstrated a trend towards increased Ang-2 mRNA levels in VWF-deficient mice compared to controls, although not significant (figure 3.12A). The hearts (figure 3.12B) but not liver samples (figure 3.12C) showed a significant increase in Ang-2 expression compared to littermate controls. These data demonstrated that VWF regulates Ang-2 expression *in vivo* in a tissue specific. While BOEC and HUVEC data show increased Ang-2 release, the data in plasma from patients and the VWF-deficient mouse did not show a consistent difference. We propose that the lack of Ang-2 increase in circulation may be due to tissue specificity of Ang-2 regulation.

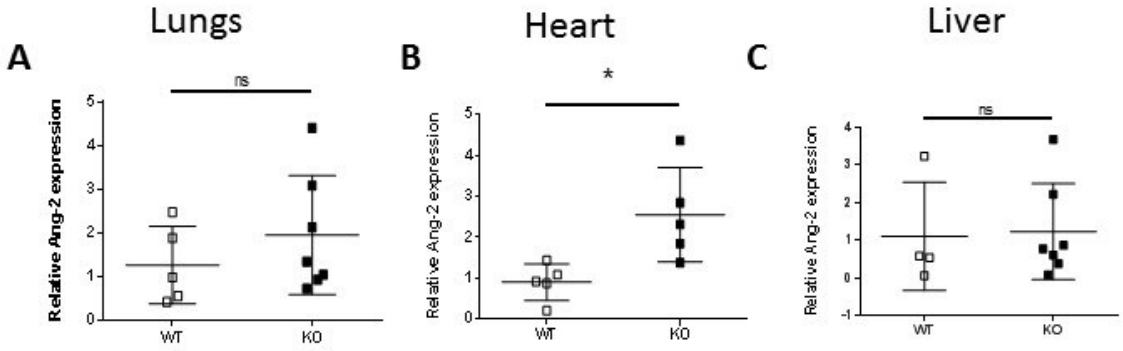

Figure 3.12 Tissue specific regulation of Ang-2 expression in VWF-deficient mouse heart. Ang-2 levels in WT and VWF KO mice was determined by qRT-PCR of whole Lung (A), Heart (B) and liver (C) lysates. Values were normalised to 18s. Data presented as mean ± SEM of 5 WT and 7 KO mice. Statistical significance determined by student's t-test. ns = p> 0.05; *p≤0.05

3.2.8 Decreased VWF and enhanced Ang-2 mRNA levels in lungs of mice after acute LPS treatment

We did not find increased Ang-2 mRNA levels in the VWF-deficient mouse lungs (figure 3.12A). Previous work has shown that treatment of mice with 50 µg lipopolysaccharide (LPS) (2 mg/kg) for 3 hours downregulated VWF expression in the lungs (Yamamoto et al., 1998), whilst a lethal 20 mg/kg LPS dose from 6 to 24 hours was shown to upregulate Ang-2 expression in the lungs of mice (Mofarrahi et al., 2008). To confirm the inverse relationship between VWF and Ang-2 *in vivo,* I utilized a sub-lethal model of LPS-induced endotoxemia. 50 µg LPS was administered to wild-type C57BL/6 mice and whole lung tissue lysates were collected 6 hours later. Analysis of lysates revealed a significant downregulation of VWF mRNA (figure 3.13A) as expected after LPS treatment. In the presence of LPS, Ang-2 mRNA levels were increased 4-fold (figure 3.13B). These data suggest an inverse relationship between VWF and Ang-2 expression levels in mouse lungs after challenge with LPS.

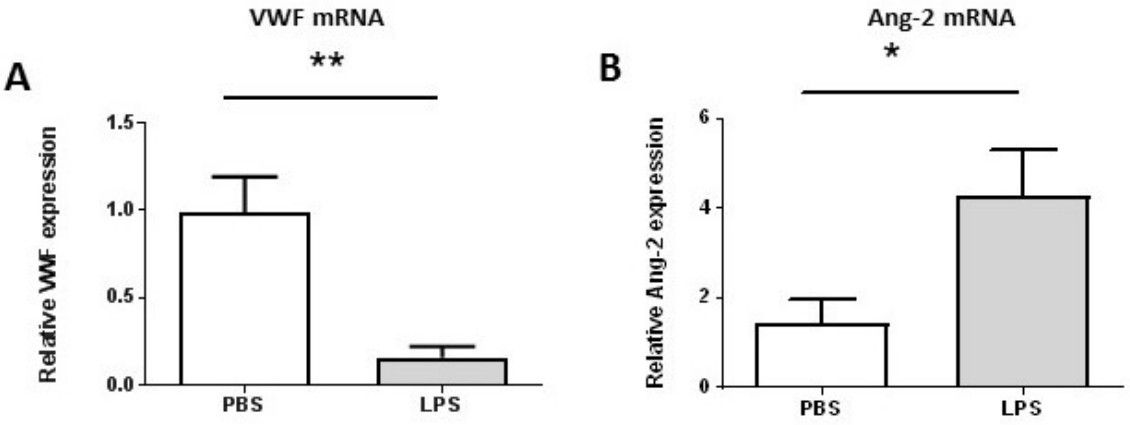

Figure 3.13 Inverse relationship between VWF and Ang-2 in LPS induced inflammation in mouse lungs. WT mice between 8-12 weeks were injected with 50 µg of LPS i.p for 6 hours and lungs collected. VWF (A) (4 mice per group) and Ang-2 (B) (6 mice per group) expression was measured by qRT-PCR and expressed relative to GAPDH. Data presented as mean ± SEM. Statistical significance determined by student's t-test.* p≤0.05, ** p≤0.01

3.2.9 Inverse relationship between VWF and Ang-2 mRNA levels in sparse and confluent EC in vitro

The data so far suggest a complex relationship between VWF and Ang-2 endothelial expression. Ang-2 expression has been reported to be rapidly upregulated during physiological and pathological angiogenesis (Maisonpierre et al., 1997). Microarray analysis from the Dejana lab comparing the gene expression profile of confluent vs sparse endothelial cells (ECs), an *in vitro* model of quiescent versus angiogenic ECs, found that in sparse (angiogenic) ECs VWF expression was decreased compared to confluent ECs (Taddei et al., 2008). To investigate the relationship between Ang-2 and VWF in this model, HUVEC were seeded in sparse (2,000 cell per cm^{-2}) and confluent (40,000 cells per cm^{-2}) conditions in gelatin-coated plates. After 24 hours, mRNA and protein were collected for analysis. As expected, VWF mRNA expression was significantly lower (≈50%) in sparse EC compared to confluent monolayers (figure 3.14A). This was associated with a 14-fold significant increase in Ang-2 mRNA expression (figure 3.14B). In line with the mRNA data, protein levels of VWF, as measured by ELISA, were significantly lower in sparse cells (figure 3.14C). Despite a 1.5 fold increase in Ang-2 protein levels in sparse cells (figure 3.14D), these data did not reach statistical significance ($p=0.0506$). These data suggest an inverse relationship between VWF and Ang-2 mRNA levels in this *in vitro* model quiescence vs proliferating ECs.

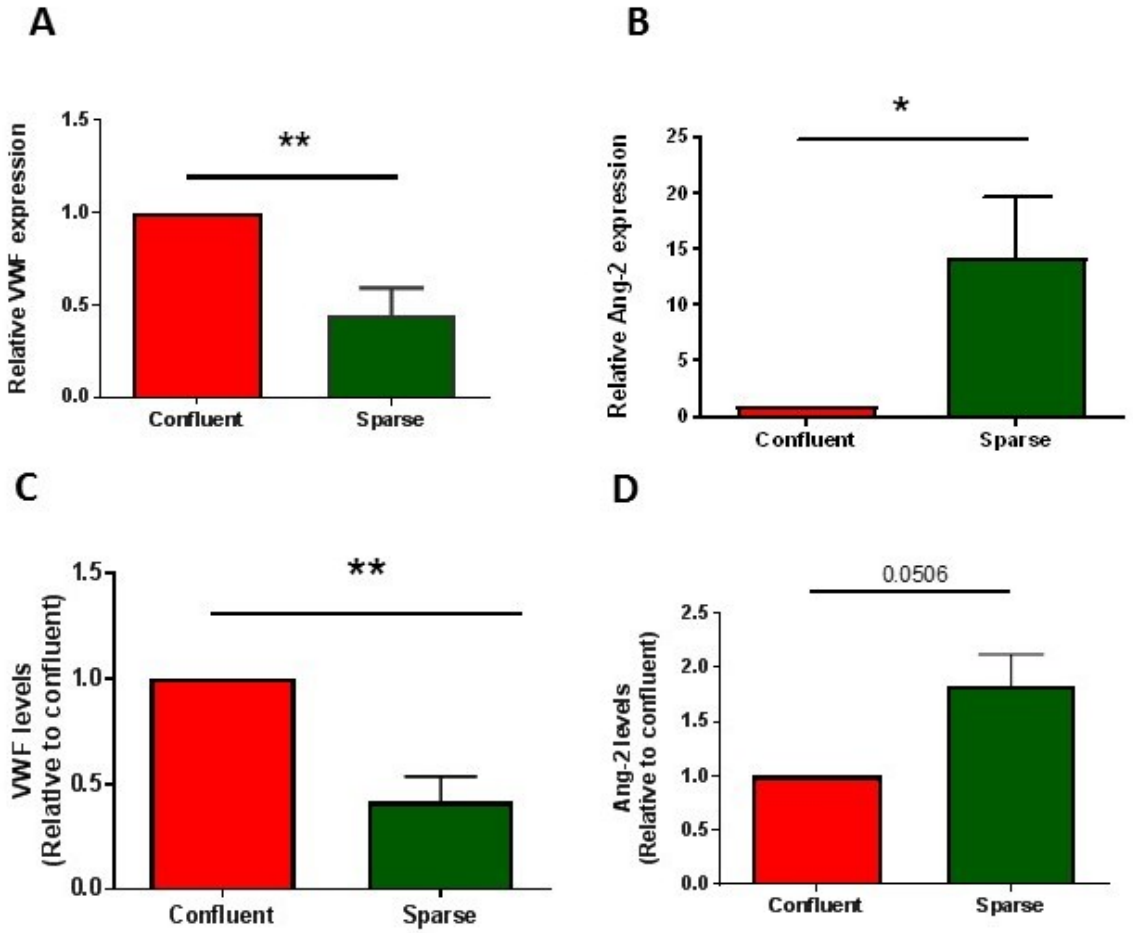

Figure 3.14 Inverse relationship between VWF and Ang-2 in angiogenic and quiescent cells in vitro. HUVEC were seeded at 40,000 cells cm^{-2} (Confluent) or 2,000 cells cm^{-2} (Sparse). After 24 hours VWF expression (A) and Ang-2 expression (B) was measured by qRT-PCR. Values were normalised to GAPDH and expressed relative to confluent cells. VWF protein (C) and Ang-2 protein (D) levels were measured by ELISA. Values normalized to total protein and expressed relative to confluent cells. Data shown as mean ± SEM of n=5. Statistical significance was determined by student's t-test. ns $p>0.05$ * $p\leq0.05$, ** $p\leq0.01$

3.3 Discussion and Future Work

VWF controls release, storage and synthesis of Ang-2 but not of other WPB components in EC

This results described in this chapter shows that inhibition of VWF *in vitro* results in enhanced release of Ang-2 over time. Since VWF controls the formation of WPBs which store Ang-2, we proposed that lack of storage caused the increased release of Ang-2. To investigate this, we used a potent inducer of WPB exocytosis: PMA. In line with our hypothesis, we showed increased Ang-2 release in control but not VWF-deficient HUVEC in the presence of PMA. This suggested that while PMA was able to induce regulated release of Ang-2, the absence of VWF ablated this response. Given that VWF-deficient cells do not contain WPBs, most likely the lack of response to PMA is due to loss of Ang-2 storage. The response of VWF-deficient cells to other WPB secretagogue inducers remains to be explored to confirm these findings. Additionally, reestablishment of WPB in VWF-deficient HUVEC may be another avenue to confirm our observations.

Previous work in Ang-2 overexpressing ECs showed that PMA treatment of ECs depleted WPB stores. These Ang-2 stores start to replenish within 8 hours (Fiedler et al., 2004). We observed that inhibition of VWF expression did not significantly affect Ang-2 intracellular levels whilst immunofluorescence showed decreased Ang-2 protein expression. Ang-2 staining was found increased in perinuclear regions. We also show that increased Ang-2 release is observed in VWF-deficient cells as early as 75 mins. These observations could be in part due to increased Ang-2 synthesis, since inhibition of VWF expression causes enhanced Ang-2 mRNA levels. Therefore, since VWF-deficient cells cannot store the newly synthesised Ang-2 because WPB are not formed in the absence of VWF, this protein is therefore released.

Given that multiple proteins are stored in WPB (Metcalf et al., 2008, Randi et al., 2013, van Breevoort et al., 2012), it is possible to assume that the effect of VWF-deficiency could similarly affect many WPB proteins and cause their release, similar to Ang-2. To test this hypothesis, in this study we focused on CD63/tetraspanin and IL-8. CD63 levels were not affected by inhibition of VWF expression. The lack of effect on CD63 levels could be due to most of intracellular CD63 being localised in lysosomes and some endosomes (Kobayashi et al., 2000). WPB storage of CD63 was shown to account for only about 5% of its intracellular

pool (Vischer and Wagner, 1993). On the other hand, inhibition of VWF significantly reduced IL-8 intracellular levels and release, the opposite to what observed for Ang-2. Previous studies from Denis *et al* demonstrated that P-selectin, another WPB protein, is degraded after loss of WPB (Denis et al., 2001). Together these data suggests that VWF does not regulate all WPB in the same manner. In our study, we identified that inhibition of VWF expression in EC did not affect IL-8 or CD63 mRNA levels while increasing Ang-2 mRNA. Since VWF-deficient cells have decreased IL-8 release and intracellular levels it is possible that IL-8 similar to P-selectin is also degraded. Additional studies carried out in our lab have shown that inhibition of VWF expression also does not change the expression of Galectin-3 (Gal-3) (Smith and Randi, unpublished). Although we have not measured Gal-3 levels in the supernatant of VWF-deficient cells, it is tempting to speculate that the reported decrease in Gal-3 levels in VWF-deficient mouse (Saint-Lu et al., 2012) may be due to lack of WPB storage and degradation. More recently, inhibition of VWF has been shown not to affect intracellular levels of endothelin-1 (ET-1), another WPB protein (Dushpanova et al., 2016). Work from Fieldler *et al* showed that the storage of Ang-2 and P-selectin in WPB is mutually exclusive (Fiedler et al., 2004). Given these findings, studies investigating the localisation of WPB proteins after inhibition of VWF may provide insight into which proteins are degraded and which are selectively released. These studies, together with our work suggest that VWF does not regulate all WPB proteins in the same manner.

To understand whether the regulation of Ang-2 by VWF has implications in patients, we isolated BOEC from a patient with the rare type 3 VWD. Our investigation also found enhanced Ang-2 release and mRNA levels in the type 3 BOEC compared to controls, in line with our *in vitro* studies. Interestingly, while the patient studied in this project had complete absence of VWF from ECs, it has been reported that type 3 patients can be more heterogeneous in the levels of endothelial expression of VWF (Casey et al., 2013). In this study, the authors isolated BOEC from 7 type 3 VWD patients and reported that of the patients studied, the complete absence of WPB structures appeared in only 4 cases. Out of these cases, only 1 presented with a true “null” phenotype, while others showed diffuse VWF staining in ER. Since BOEC have provided useful information for understanding the molecular basis of VWD (Casey et al., 2013, Starke et al., 2013, Wang et al., 2013), they may provide a useful tool for understanding in the role of VWF in controlling WPB proteins.

In this chapter, we show an inverse relationship between VWF mRNA levels and Ang-2 mRNA in an *in vitro* model of proliferating versus quiescent ECs. Decreased VWF expressed in proliferative ECs validated findings from a microarray analysis in carried out in the Dejana lab (Taddei et al., 2008). Increased Ang-2 expression after inhibition of VWF *in vitro* may be a signature of cells undergoing a more "angiogenic" phenotype (Maisonpierre et al., 1997). No signalling pathway has yet been identified for how VWF can control Ang-2 synthesis; this is the main focus of Chapter 4.

Regulation of circulating Ang-2 proteins levels by VWF: studies in VWD patients and in vivo

In this study, we found that reduced VWF expression increased Ang-2 release both *in vitro* and in ECs *ex vivo* from a type 3 VWD patient; thus we speculated that we would observe increase Ang-2 levels in plasma from patients with quantitative defects in VWF. However, we found no difference in circulating Ang-2 levels in patients with either quantitative or qualitative defects in VWF. In our study, median Ang-2 levels in healthy volunteers were around 300 pg/ml, in line with previous reports (Brouwers et al., 2014, Groeneveld, 2015). However, some studies have reported levels ranging from 2000 – 5000 pg/ml, suggesting a huge variability in the healthy population (Chong et al., 2004, Petersen et al., 2016, Yeo et al., 2008). These studies mostly compared Ang-2 circulating levels in the context of inflammatory disease and showed an increased correlation with disease and severity. More recently, a large VWD population study revealed that both type 1 (n=395) and type 2 (n=239) patients had reduced Ang-2 levels compared to controls (n=100) (Groeneveld, 2015). In addition, while the type 3 patients showed decrease circulating Ang-2, this did not reach significance due to low numbers (n=21). In our study we find in the type 2 patient cohort studied a strong trend towards decreased Ang-2 levels in the circulation, albeit not statistically significant. This lends support to the findings from Groeneveld and colleagues. Collectively our VWD patients studied showed similar median levels of VWF:Ag compared to the study of Groeneveld and colleagues (26 IU/dl vs 29 IU/dl). Given the increased power of the Groeneveld study compared to ours it is plausible that VWF controls Ang-2 stability in the circulation

We next explored the possibility that VWF may regulate Ang-2 protein stability. McKinnon and colleagues demonstrated that in a purified system, Ang-2 was able to bind VWF (Mckinnon et al., 2011). Using a similar *in vitro* set up, VWF bound to Ang-2 was subjected to

trypsin which is known to degrade Ang-2. Despite the obvious limitation of the non-specific nature of trypsin, preliminary data from these experiments suggest that VWF does not protect Ang-2 from degradation (Smith and Randi, unpublished). Indirect evidence for a relationship between circulating levels of Ang-2 and VWF comes from our study, where we examined the effect of Ang-2 levels on a type 3 VWD patient after treatment with plasma-derived VWF. Despite analysis of only one patient, increases in Ang-2 levels were not seen after VWF administration which suggests that VWF does not controls Ang-2 protein stability in circulation. Surprisingly administration of VWF appeared to reduce Ang-2 levels initially. However this effect was not observed in S5 sample. One caveat to these studies remain the lack of knowledge surrounding the binding of VWF and Ang-2. It is possible that technical interference with detection of Ang-2 may be a possibility with the interaction between VWF and Ang-2. Further studies are needed to dissect the exact physical interaction between these two proteins.

FVIII is stabilised in the circulation by the presence of VWF. FVIII levels are decreased in patients with quantitative defects in VWF (Type 1 and 3 VWD); its level increase with administration of plasma derived VWF (reviwed in Miesbach and Berntorp, 2015). FVIII circulating levels are also decreased, in a murine model of severe VWD and corrected by delivery of a plasmid containing murine VWF cDNA (Pergolizzi et al., 2006). We measured circulating Ang-2 levels in the VWF-deficient mouse and we found no difference between control and VWF-deficient mice. In line with our findings, two other groups have reported no difference in circulating Ang-2 levels in VWF-deficient mice compared to littermate controls (O'Regan et al., 2016, Yuan et al., 2016). These findings *in vivo* argue strongly against VWF regulating Ang-2 stability. These experiments will need to be followed up using labelled Ang-2 measurements on a VWF-deficient background to conclusively corroborate our findings.

Tissue specific regulation of Ang-2 mRNA levels

Heterogeneity between ECs in different vascular beds has long been recognised (reviewed in Aird, 2012). In particular, VWF has been the focus of intense research into the mechanisms underlying EC heterogeneity (Minami et al., 2002, Yuan et al., 2016, Yuan et al., 2013). In our study, we found increased Ang-2 mRNA levels in the heart but not the lungs nor liver of the VWF-deficient mouse compared to controls. This was in line with the previous findings of

Yuan et al who also reported enhanced Ang-2 mRNA levels in the heart of the VWF-deficient mouse compared to controls (Yuan et al., 2016). However, in ECs in culture, inhibition of VWF expression caused increase Ang-2 mRNA levels.

One potential explanation for this is the concept of stochastic switching which controls VWF mosaic expression (Yuan et al., 2016). In this study, the authors demonstrated *in vivo* that VWF expression by confocal appeared in a heterogeneous manner in capillaries. Using fate mapping, this study showed that all ECs of the heart and lungs but not liver or kidneys expressed VWF at some point during their lifetime. They proposed a mechanism through random biological noise that VWF switches on or off within a cell. They proposed a "nature" (intracellular) rather than "nurture" (extracellular) process in regulating VWF and other gene expression in EC, including ESM-1 and Ephrinb2. The latter in particular was found to be expressed in a heterogeneous manner even in culture, where the external environment is more controlled. This is also observed with Ang-2, whose expression can be heterogeneous in the *in vitro* culture environment. This group then demonstrated enhanced Ang-2 mRNA and protein in ECs from VWF-deficient mouse heart but not kidneys or liver, in line with our findings.

In the lungs of VWF-deficient mice we only find a small trend towards increased Ang-2 levels compared to controls. However, upon LPS challenge in wild type mice we find decreased VWF mRNA levels and increased Ang-2 mRNA levels in the lungs as expected. This may indicate that although under basal conditions VWF did not appear to affect Ang-2 levels in the lungs, in response to inflammatory stimuli, this effect may be different. In addition, a study from Yamamoto and colleagues demonstrated that different tissues had distinct responses to VWF expression in the presence of LPS (Yamamoto et al., 1998). In particular, this study demonstrated that VWF mRNA levels were decreased in the lungs, increased in the heart, and unchanged in the liver following LPS challenge. Additionally, LPS differentially regulated VWF mRNA levels in tissues but overall caused increased VWF in plasma. It is likely that different tissues have different sensitivities to VWF regulation. Possibly this could suggest that the circulating levels of Ang-2 could be a reflection of tissue specific regulation. Further characterisation of Ang-2 expression in other tissues in VWF-deficiency are needed to explore the regulation of Ang-2 *in vivo*.

How do we reconcile our observations in both ECs explored *in vitro* (BOEC and HUVEC) and the tissue specificity *in vivo*? One possibility is the behaviour of ECs in culture, which has been proposed to take on a more "plastic" nature compared to mature ECs (Toshner et al., 2014). In this study, the authors used the HOX transcription factor family to shed light on the differences between mature ECs, pulmonary artery endothelial cells (PAEC) vs HUVEC and BOEC. Particularly, this study showed that HOXB5, a transcription factor expressed at higher levels in developing ECs (Wu et al., 2003) was increased in HUVEC and BOEC compare to PAEC. In addition, this transcription factor has been shown to increase Ang-2 expression after overexpression in ECs (Winnik et al., 2009). Thus, the enhanced Ang-2 expression in BOEC and HUVEC after decreased VWF may be linked to their plastic nature.

In summary, in this chapter we have shown that inhibition of VWF expression in ECs results in increased Ang-2 expression and Ang-2 release into the supernatant. Increased Ang-2 release in VWF-deficient cells may be due to lack of the storage organelle WPB. We also found that in the circulation, quantitative or qualitative defects of VWF are associated with variable Ang-2 levels, without a direct correlation This may be partly due to the fact that *in vivo*, the regulation of Ang-2 levels is tissue specific. Understanding of how VWF controls Ang-2 levels will improve our understanding of this significant pathway.

4 Chapter Four: VWF regulates Ang-2 expression in endothelial cells through an Akt-FOXO1 dependent pathway

4.1 Introduction

4.1.1 VWF regulation of gene expression in EC

The finding that VWF regulates levels of Ang-2 mRNA in EC opens the question as to how VWF could control gene expression in EC. Very limited evidence is available on this. In one report which investigated the effect of VWF on gene transcription, a microarray analysis was performed on HUVEC stimulated with 4 μg/ml VWF for 4 hours (Potapova et al., 2010). Since enhanced VWF expression is associated with endothelial activation, this study first examined the expression of E-selectin, P-selectin, VCAM-1 and ICAM-1. VWF stimulation of HUVEC did not affect adhesion molecule expression; however VWF stimulation of EC did cause a 2-fold increase in levels of the transcriptional regulators Kruppel-like factor 4 (KLF4) and a decrease in levels of the E26-transformation specific (ETS) family member ETV1. This suggests that VWF binding to EC can regulate gene transcription. To identify which pathways were affected by stimulation of HUVEC with VWF, the same study also carried out a phospho-kinase array was performed following HUVEC treatment with VWF (Potapova et al., 2010). This revealed enhanced phosphorylation of p38 MAPK as well as ERK1/2 in the presence of VWF. This study provided the first evidence for extracellular VWF inducing signalling in EC which could regulate downstream transcription.

4.1.2 **The** β3 integrin-FAK-Akt signalling pathway in EC

As described in Chapter 1, VWF binds to and controls the internalisation and stability of β3 integrin subunit. These transmembrane receptors integrate signals from the extracellular matrix to the inside of the cell (Mitra et al., 2005). Integrin activation results in downstream signalling, predominately via recruitment of Src-family kinases to their alpha subunit and focal adhesion kinase (FAK) to their beta subunit. The best characterised FAK signalling event is phosphorylation at tyrosine 397 (Y397) (Lechertier and Hodivala-Dilke, 2012, Mitra et al., 2005, Parsons, 2003). Activation of this residue is thought to provide signals which lead to activation of the Akt-PI3K signalling (shown in figure 4.1) and promote cellular migration (Mitra et al., 2005). In addition, FAK activation can promote cell proliferation and tube formation (Braren et al., 2006). It is clear that the MAPK pathway plays an important role in cell proliferation (reviewed in Zhang and Liu, 2002). The best characterised of these pathways is the ERK pathway, which is activated by FAK recruitment of Src homology 2 (SH2). Integrins

that do not activate ERK appear to not activate shc. This highlights at least two pathways through which integrins interact to regulate angiogenesis.

As described in chapter 1, integrins interact with growth factor receptors. In addition, integrins can interact directly with growth factors. In one report, Ang-2 stimulation of EC has been shown to cause Tie-2-$\alpha v\beta 3$ complex formation and activation of FAK at S910 (Thomas et al., 2010). This signalling event also causes disassociation of the adaptor protein talin and p130Cas; these responses are thought to be associated with focal adhesion turn over important for cell migration (Lamalice et al., 2007). According to a model recently put forward by the Augustin lab, release of Ang-2 from EC during angiogenesis causes activation of $\alpha v\beta 3$ at Y397 which promotes angiogenesis (Felcht et al., 2012).

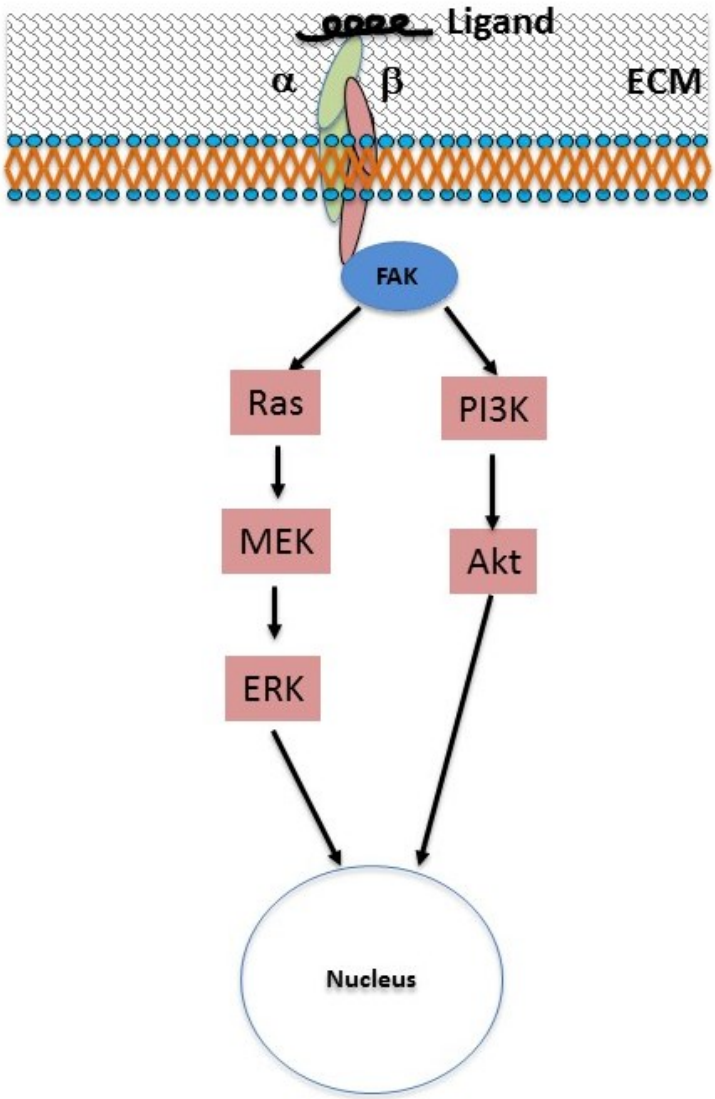

Figure 4.1 Integrin β subunit outside in signalling. Classically integrins recruit FAK to their β subunits which activates downstream signalling via PI3K-Akt dependent manner. On the other hand, FAK recruitment can also activate Ras, MEK and ERK pathway (Guo and Giancotti, 2004).

4.1.3 Endothelial signalling pathways in the induction of Ang-2 expression

Although detectable in resting EC *in vitro* (Hegen et al., 2004, Mandriota and Pepper, 1998, Hasegawa et al., 2004), Ang-2 expression is low in EC *in vivo*, except for tissues such as ovaries and uterus where there is active angiogenesis and vascular remodelling (Maisonpierre et al., 1997). Several agents have been shown to regulate Ang2 expression in EC. Pro-angiogenic growth factors including VEGF and bFGF (Mustonen and Alitalo, 1995) promote expression of Ang-2 (Hegen et al., 2004, Oh et al., 1999). Furthermore, inflammatory stimuli such as LPS, TNF-α, thrombin and Angiotensin II (AngII) can increase Ang-2 expression (Otani et al., 2001, Fiedler et al., 2006, Fiedler et al., 2004). In particular, Otani and colleagues provided key evidence to demonstrate that inhibition of MAPK pathway ablated AngII-induced Ang-2 mRNA levels. In addition, thrombin activation of Ang-2 transcription in EC has been shown to be MAPK dependent (Huang et al., 2002). While the MAPK pathway has been shown to drive Ang-2 expression, activation of the PI3K-Akt pathway by Ang-1 appears to suppress Ang-2 mRNA levels (Tsigkos et al., 2006, Daly et al., 2004) Hypoxia drives the expression of Cyclooygenase enzymes (COX) which catalyse formation of prostanoids (Pichiule et al., 2004). Moreover the production of PGE_2 has been shown to increase VEGF expression in certain cancers (Greenhough et al., 2009). Surprisingly PGE_2 induction of Ang-2 in EC was shown to only be partly VEGF dependent (Pichiule et al., 2004). These studies indicate that a number of growth factors upregulate Ang-2 levels and implicates the MAPK signalling pathway in induction of Ang-2 expression and PI3K-Akt in its suppression.

4.1.4 Regulation of Ang-2 mRNA stability

Hypoxia has been shown to be a potent inducer of Ang-2 expression (Pichiule et al., 2004, Simon et al., 2008). Hypoxia inducible factor (HIF) binding sites have been described in the promoter of Ang-2, and Simon and colleagues showed that hypoxia increases Ang-2 transcription. On the other hand, Pichule *et al.,* showed that hypoxia can also increase the stability of Ang-2 mRNA. This suggests that Ang-2 mRNA are regulated by both transcriptional and post transcriptional modifications.

4.1.5 Transcriptional regulation of Ang-2 mRNA

Multiple transcription factors have been shown to regulate the basal, growth factor and inflammatory expression of Ang-2.The transcription factor GATA-2 regulates Ang-2 by binding to a number of transcription factors. In particular, the transcription factor LMO2, which is important for haematopoiesis, has been shown to assemble TAL1 or LYL1 with GATA-2 to drive Ang-2 expression in EC (Deleuze et al., 2012). In addition, during tube formation *in vitro*, an increase in TAL1 and LMO2 coincided with Ang-2 expression. GATA-2 expression decreased during these assays, suggesting that GATA-2 was only required for basal expression. GATA-2 is thought cooperate with ETS-1 to regulate basal expression of Ang-2 (Simon et al., 2008). On the other hand, ETS-1 has been shown to drive the expression of Ang-2 induced by both VEGF and bFGF (Hasegawa et al., 2004, Hegen et al., 2004). The transcriptional activity of ETS-1 is reliant on phosphorylation at Thr-38 found in the MAPK responsive region. Conversely, phosphorylation of residues S251, S257, S282 and S285 is thought to reduce ETS-1 DNA binding activity (Dittmer, 2003). This suggests that VEGF induced MAPK activation drives the expression of Ang-2 via ETS-1.

4.1.6 FOXO1 regulation of Ang-2 mRNA

The transcription factor (FOXO1) has been shown to drive Ang-2 expression in endothelial cells (Daly et al., 2004, Dharaneeswaran et al., 2014, Potente et al., 2005). siRNA studies demonstrated that inhibition of FOXO1 reduces Ang-2 levels in HUVEC (Potente et al., 2005). FOXOs are regulated by a number of processes including phosphorylation, ubiquitination and acetylation. For the purposes of this chapter I will focus on phosphorylation.

Akt is the major regulator of FOXO1 phosphorylation. FOXO1 contains three conserved phosphorylation sites Thr-24, S256 and S319 (Calnan and Brunet, 2008). Phosphorylation of Akt induces phosphorylation of all three sites on FOXO1. Nuclear translocation is important for the activity of FOXO1. Inhibition of the PI3K/Akt pathway leads to reduced phosphorylation at these sites and cause rapid increase in Ang-2 expression. The growth factor Ang-1 has been shown to suppress Ang-2 expression via the PI3K/Akt pathway (Daly et al., 2004). Mutation of FOXO1 phosphorylation sites to an unphosphorylated form leads to enhanced nuclear localisation which drives Ang-2 expression (Daly et al., 2004). Moreover,

the FOXO1 promoter has been shown to contain a FOXO binding site, suggesting a positive regulation by itself (Essaghir et al., 2009). In study from Dharaneeswaran et al, transgenic mice were generated expressing a constitutively active form of human FOXO1 in ECs. Analysis of the FOXO1 mRNA in lungs and heart demonstrated in like with Essaghir et al that enhanced FOXO1 activity caused increased FOXO1 expression in ECs. Surprisingly however, despite enhanced FOXO1 activity, ECs isolated from the heart but not the lungs showed increased Ang-2 mRNA levels compared to controls (Dharaneeswaran et al., 2014). This suggests that at least *in vivo* enhanced FOXO1 activity appears to selectively increase Ang-2 mRNA levels in the heart but not the lungs *in vivo*.

4.1.7 Hypothesis and Aims

In chapter 3 we demonstrate that inhibition of VWF expression causes increased Ang-2 mRNA levels. It is possible that VWF can control Ang-2 mRNA through regulation of its stability or transcription. Given that VWF has been shown to regulate transcription, we speculated that VWF regulates the transcription of Ang-2. Previous work from our lab has shown that VWF regulates the levels and internalisation of the integrin β3 (Starke et al., 2011). Moreover, VWF and integrin β3 have been shown to regulate pathways which can regulate the expression of Ang-2. I therefore hypothesised that VWF binding to the integrin β3 can regulate its signalling. I therefore propose that VWF suppresses Ang-2 expression via the transcription factor FOXO1.

The aims of this chapter are to:

- Investigate the mechanisms though which VWF regulates Ang-2 mRNA levels
- Determine whether VWF regulates β3 integrin signalling in EC
- Determine the role of Akt in the VWF-dependent increase in Ang-2 mRNA levels
- Investigate whether FOXO1 is involved in the VWF-dependent control of Ang-2

4.1 Results

4.1.1 LPS does not potentiate the VWF dependent increase in Ang-2 expression

In chapter 3 inhibition of VWF expression in EC using siRNA resulted in a significant increase in Ang-2 mRNA levels. Furthermore, in the VWF-deficient mice increased Ang-2 mRNA levels were found in the heart, in line with the model that VWF may repress Ang-2 expression in EC during quiescence. Given that an inverse relationship between VWF and Ang-2 mRNA levels were also found in the lungs of WT mice after LPS (section 3.2.8), we hypothesised that during EC activation, Ang-2 expression would be potentiated in the absence of VWF. To investigate this hypothesise, control and VWF-deficient mice were injected with LPS at 50 µg i.p for 6 hours or PBS control. Mice were then sacrificed by cervical dislocation and tissues collected for RNA. In line with data in previous experiments, genetic deletion of VWF expression did not cause an increase in Ang-2 mRNA levels in the lungs (figure 4.2A). In the presence of LPS, both control and VWF-deficient mice significantly increased Ang-2 expression. The absence of VWF expression was found to increase Ang-2 mRNA levels in the heart, in line with previous evidence (Yuan et al., 2016). However, in the presence of LPS, both control and VWF-deficient mice did not significantly increase Ang-2 mRNA levels (figure 4.2B). This suggested that in an acute model of EC activation, Ang-2 mRNA levels were not potentiated in the absence of VWF.

Given the severity of LPS in long term *in vivo* models, an *in vitro* system using HUVEC was used to test whether, chronic EC activation potentiated Ang-2 expression in the absence of VWF. HUVEC were treated with control or VWF siRNA for 24 hours. Cells were then treated with LPS 100 ng/ml or PBS control for a further 24 hours as previously described (Menden et al., 2015). RNA was collected and analysed for VWF and Ang-2 mRNA levels. As reported, Ang-2 mRNA levels were significantly increased after LPS treatment in control cells (figure 4.3A). As expected, inhibition of VWF expression in control cells significantly increased Ang-2 mRNA levels. However, LPS treatment was unable to further upregulate Ang-2 mRNA levels in VWF-deficient cells. LPS treatment did not significantly affect VWF mRNA levels (figure 4.3B). These data suggests that inhibition of VWF expression does not potentiate Ang-2 mRNA levels in ECs during EC activation.

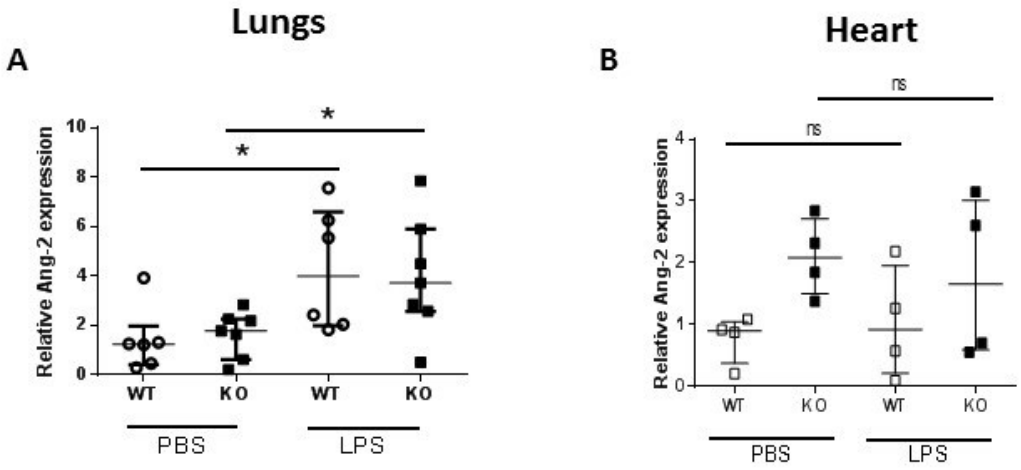

Figure 4.2 Regulation of Ang-2 mRNA expression in VWF-deficient mouse heart and lungs by LPS. Mice aged between 8-12 weeks were treated with 50 µg LPS or PBS control for 6 hours. Mice were then sacrificed and tissues snap frozen for RNA processing. Ang-2 mRNA levels were determined in Lungs (A) and Heart (B) of littermate controls (open shapes) and VWF-deficient mice (black shapes) by qRT-PCR. Values were normalised to 18S and expressed relative to WT control. Data expressed as median ± IQR of n= 4-7 mice per group. Statistical significance determined by ANOVA with Sidak's post hoc test. ns $p>0.05$, *$p\leq0.05$.

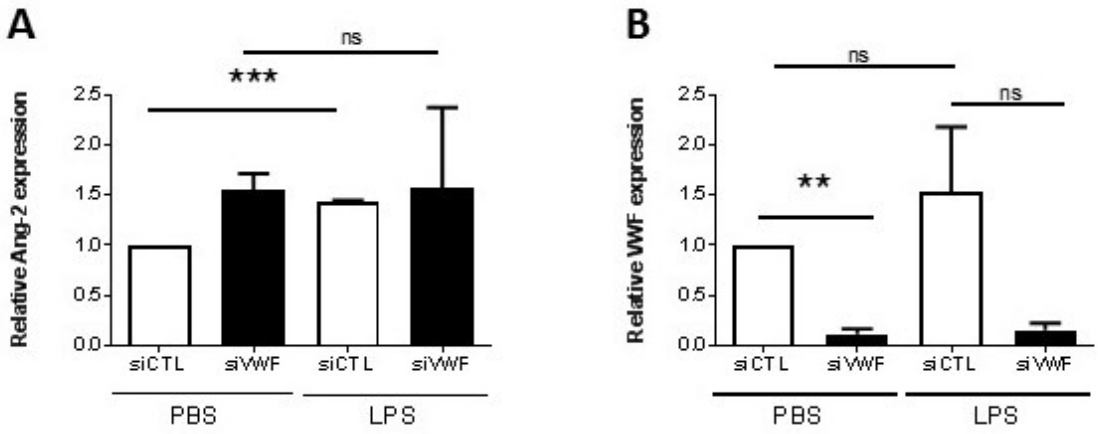

Figure 4.3 Regulation of Ang-2 mRNA expression by LPS. HUVEC were transfected with siRNA to VWF (siVWF) or siRNA control (siCTL) for 24 hours. HUVEC were then stimulated with 100 ng/ml of Lipopolysaccharide (LPS) or PBS control. After 24 hours Ang-2 expression (A) and VWF expression (B) was measured by qRT-PCR. Values were normalised to GAPDH and expressed relative to siCTL (PBS). Data shown as mean ± SEM of n=4. Statistical significance determined by student's t-test. ns $p>0.05$, ** $p\leq0.01$, *** $p\leq0.001$

4.1.2 Inhibition of VWF expression in ECs does not affect the ability of VEGF to induce Ang-2 expression

Given the previous findings, we next investigated whether VWF affected the VEGF induction of Ang-2 mRNA. HUVEC were transfected with control or VWF siRNA sequences for 24 hours. Cells were then serum deprived in media containing 1% serum for 22 hours, to induce EC quiescence and limit growth factors-dependent activation. HUVEC were then treated with VEGF 25 ng/ml or PBS as control for 2 hours to induce Ang-2 transcriptional activity. RNA was isolated from cell lysates for qRT-PCR analysis. VEGF stimulation of HUVEC in these conditions had no effect on VWF mRNA levels (figure 4.4). Interestingly, serum deprivation for 22 hrs, normalised Ang-2 mRNA levels in VWF-deficient cells (figure 4.4B), compared to experiments carried out in full media (see chapter 3, figure 3.10). VEGF treatment significantly increased Ang-2 mRNA levels in both control and VWF-deficient cells by ≈2.5 fold. These experiments show that inhibition of VWF expression does not affect the ability of VEGF to increase Ang-2 mRNA levels. Additionally, since Ang-2 was normalised in VWF-deficient cells after serum deprivation for 22 hrs, this suggests that VWF-dependent increase in Ang-2 mRNA is growth factor dependent.

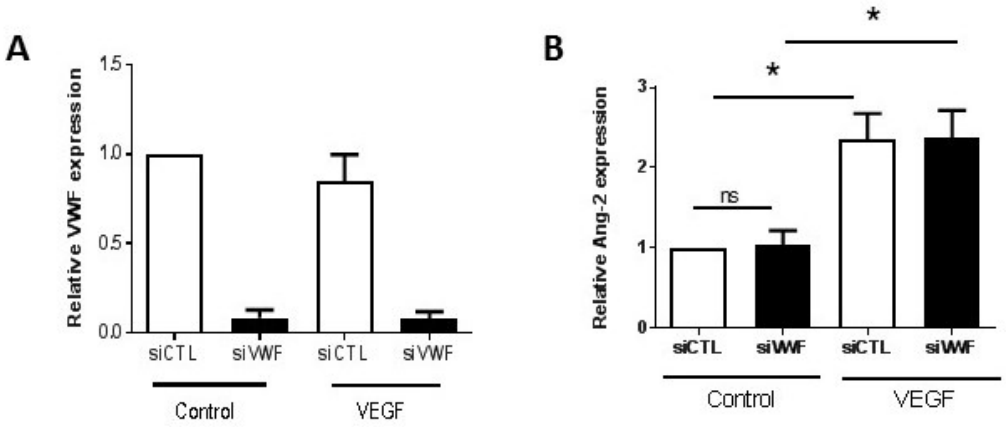

Figure 4.4 Regulation of Ang-2 expression by VEGF. HUVEC were transfected with siRNA to VWF (siVWF) or siRNA control (siCTL) for 24 hours. Cells were then serum deprived overnight then stimulated with VEGF 25 ng/ml. After 2 hours VWF expression (A) and Ang-2 expression (B) was measured by qRT-PCR. Values were normalised to GAPDH and expressed relative to siCTL (Control). Data shown as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$

4.1.3 Inhibition of VWF expression in ECs does not affect Ang-2 mRNA stability

Using two siRNA sequences to VWF, enhanced Ang-2 mRNA levels have been observed (chapter 3, figure 3.10C & D). It is possible that VWF affects Ang-2 mRNA stability. To test this hypothesis, HUVEC were treated with siRNA to VWF or control sequences. HUVEC were then stimulated with Actinomycin D (Act D) at 2.5 ng/ml (figure 4.5A) or 5 ng/ml (figure 4.5B) to block transcription. Treatments were carried out at 1, 4 and 8 hours prior to collection of samples, to ensure the same length of siRNA transfection. RNA was isolated for RT-PCR analysis. To determine the rate of decay of Ang-2 mRNA, values were normalised to 0 hr post-ActD for control and VWF siRNA samples separately. Ang-2 mRNA levels were found to decay at the same rate in siCTL and siVWF-treated HUVEC over 8 hours. These findings indicate that VWF does not modulate Ang-2 mRNA stability in EC.

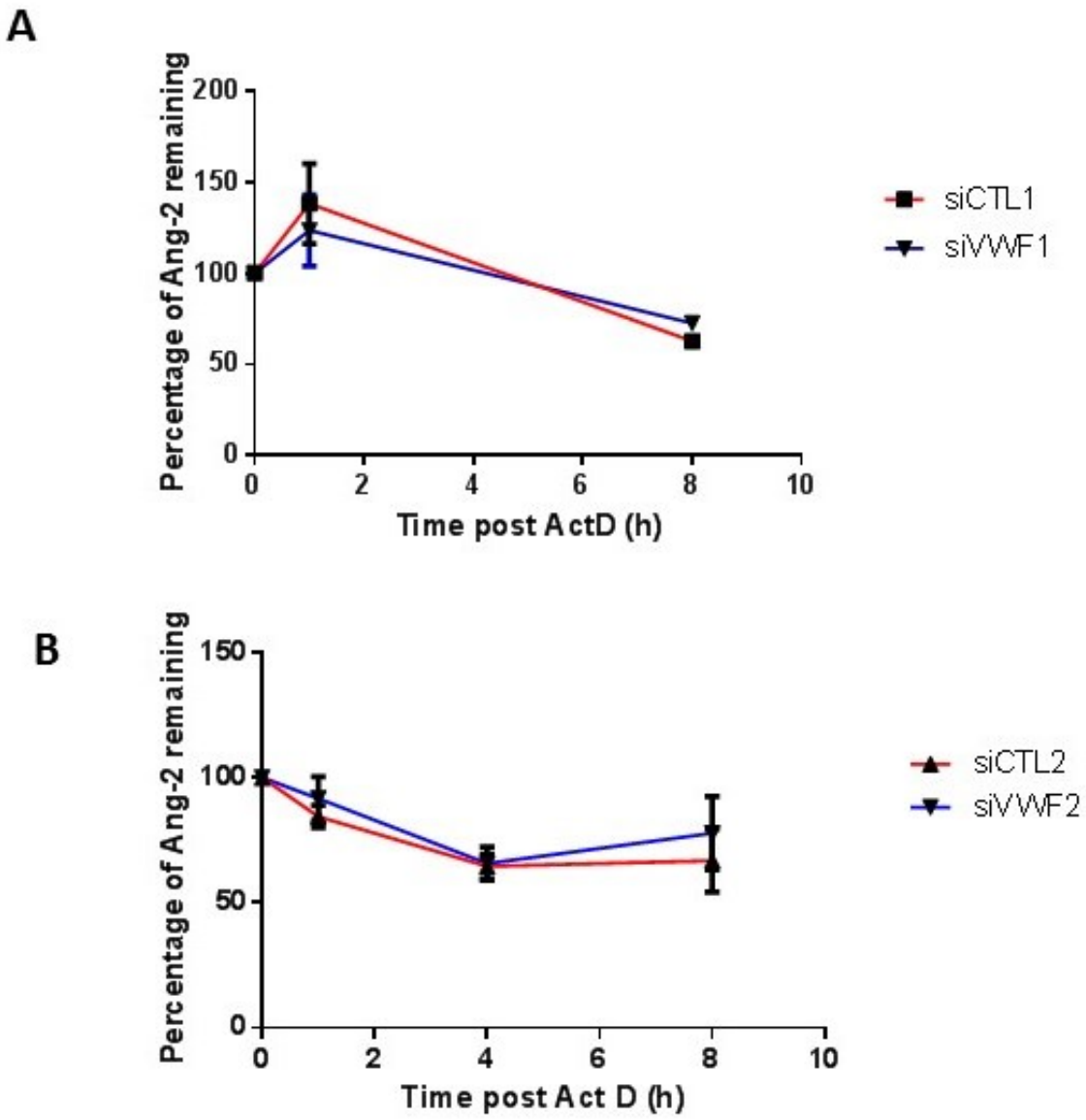

Figure 4.5 No difference in Ang-2 mRNA stability in VWF-deficient cells. (A) HUVEC were transfected with siVWF sequence 1 (see methods: siVWF1) or corresponding siRNA control (siCTL1) HUVEC were then treated with 2.5 ng/ml Actinomycin D (ActD) for 1 or 8 hours. Ang-2 expression was measured by qRT-PCR 48 hrs siRNA treatment. (Experiment performed by Dr. Richard Starke). (B) HUVEC were transfected with siVWF sequence 2 (see methods: siVWF2) or corresponding siRNA control (siCTL2) HUVEC were then treated with 5 ng/ml Actinomycin D (ActD) for 0, 1, 4 or 8 hours. Ang-2 expression was measured by qRT-PCR after 48 hrs siRNA treatment. Data expressed as percentage of Ang-2 of siCTL or siVWF 0 hr treatments. Data shown as mean ± SEM of n=3.

4.1.4 Inhibition of VWF expression in EC induces Ang-2 promoter activity

Since inhibition of VWF expression in EC did not affect Ang-2 mRNA stability (section 4.1.3), we speculated that VWF represses the transcription of Ang-2. Therefore, we set out to test whether inhibition of VWF expression in HUVEC induced Ang-2 transcription. A validated Ang-2 promoter construct was acquired from Professor Hellmut Augustin (Germany) (Hegen et al., 2004) (cloning method shown in appendix 1). This promoter construct contains the region -4903 bp upstream of the coding region of Ang-2, containing the transcription start site (TSS) for Ang-2 (see figure 4.6A & B). Sequencing analysis was performed by the Augustin lab to confirm Ang-2 plasmid (pAng-2) construct identity. To insert the 5' region of Ang-2, the pGL3 vector was cut using a Kpn1 restriction enzyme and the Ang-2 insert added with an Mlul restriction site at the 5' end and blunt ended towards the 3' end into the multiple cloning region. The basic pGL3 vector map revealed upstream restriction enzymes KpnI and downstream HindIII of the proposed cloned product (figure 4.6C). To generate fragments of 4904 bp, containing the Ang-2 promoter, and of 4818 bp, corresponding to the vector, the pGL3-Ang-2 vector was digested with Kpn1 and HindIII. Products were analysed by agarose gel electrophoresis. This revealed the 4818 bp vector, a product at approximately 3kb and other approximately 1.8 kb (lane 2; figure 4.7A). The expected product size was 4903 bp. The uncut super coiled vector was run as a control in comparison (lane 3; figure 4.7A). Given that during the cloning process an internal restriction site was identified in the Ang-2 construct (Hegen et al., 2004), it is not surprisingly therefore to observe two products. Product sizes were approximately 4.8 kb, suggesting the correct insert.

The same Ang-2 construct has been demonstrated to be active in Bovine Aorta Endothelial Cells (BAEC) compared to non-endothelial cell lines such as HEK293 and NHI3T3 (Hegen et al., 2004). To test the hypothesis that inhibition of VWF expression induced transactivation of the Ang-2 promoter, HUVEC were treated with control or VWF siRNA for 24 hours then transduced with empty vector, Ang-2 promoter construct or SV40 construct as positive control for a further 24 hours. Luciferase reporter activity was measured and expressed relative to the empty vector. Compared to siRNA control treated cells, VWF-deficient cells did not show enhanced luciferase activity of the Ang-2 promoter construct (figure 4.7B). This suggested that inhibition of VWF expression in EC did not induce Ang-2 promoter. However,

expression of the SV40 positive control vector were significantly decreased by about 50% in VWF-deficient cells, suggesting an interference of VWF-siRNA in these assays. The SV40 promoter is often used due to its strong activity, however one cited limitation of this is the sequestering of TFs to the SV40 promoter which may interfere with results. In some instances, normalisation to positive controls may serve to correct for interference of the SV40 promoter itself. Considering this effect, normalisation of pAng-2 expression to SV40 positive control reveals enhanced transactivation of the Ang-2 promoter in the absence of VWF (figure 4.7C). These data suggests that inhibition of VWF expression in EC causes enhanced transactivation of the Ang-2 promoter.

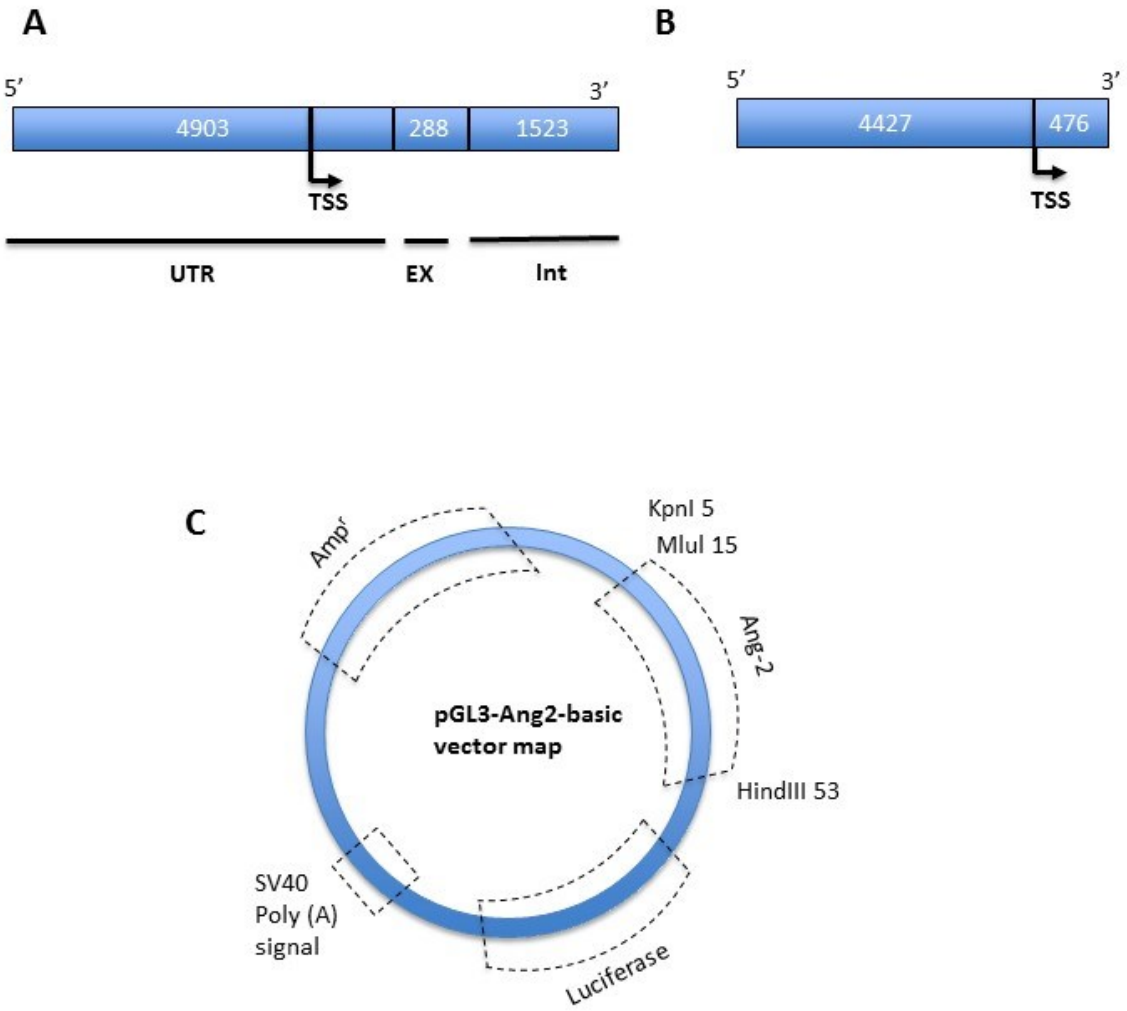

Figure 4.6 Map of Ang-2 promoter and pGL3 Ang2 vector. (A) Cloned region of Ang-2 gene from Hegen et al., 2004 showing 4903bp 5' Untranslated region (UTR), 288bp of first exon (Ex) and the 1523 intronic sequence (Int). (B) The 4903 bp region 5' of the first exon of Ang-2 was used for this cloning strategy (GeneBank accession number: AY563557). (C) Pgl3-basic vector map identifying Ampicillin resistance region (Amp^r). SV40 poly (A) minimal promoter, firefly luciferase and multiple cloning regions. The regions for restriction enzymes Kpn1, MluI and HinIII are also shown.

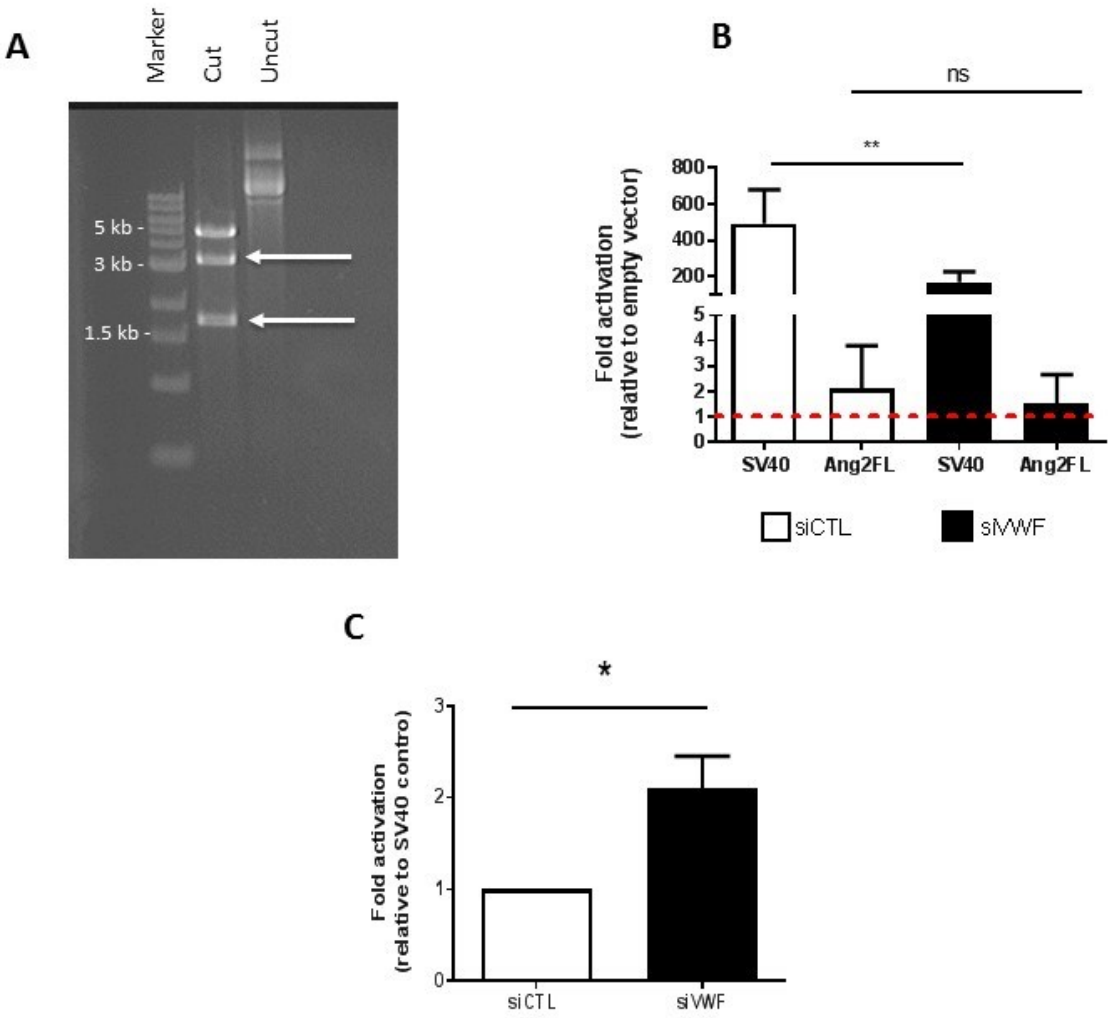

Figure 4.7 Transcriptional regulation of Ang-2 promoter construct in VWF-deficient cells. (A) pGL3 vector containing Ang-2 promoter was digested using Kpn1 and HindIII (Lane 1) and uncut vector (Lane 2). (B) HUVEC were treated for 24 hours with siCTL (open bars) or siVWF (black bars). Cells were then transfected with SV40 positive control, empty vector and Pgl3 Ang-2 containing construct. Luciferase reporter assay was performed after 24 hours. Data presented as mean ± SEM of DLR ratios normalised to empty. (C) Fold activation of Ang-2 promoter in control and siVWF treated HUVEC normalised to SV40 control. Values were expressed relative to siCTL. N=3. Statistical significance determined by student's t-test. ns $p>0.05$, *$p\leq0.05$, ** $p\leq0.01$

4.1.5 Inhibition of VEGFR2 signalling does not reduce the VWF-dependent increase in Ang-2 mRNA levels

Enhanced angiogenesis in VWF-deficient cells was significantly reduced in the presence of a VEGFR2 inhibitor (Starke et al., 2011). This suggested a role for VEGFR2 signalling in VWF-dependent angiogenesis. To test whether VEGFR2 signalling contributes to the VWF-dependent increase in Ang-2 mRNA levels, HUVEC were transfected with control or VWF siRNA sequence for 24 hours then treated with 4 µM small molecule VEGFR2 inhibitor SU4312 (previously described in Lee et al, 2007). RNA and total cell lysates were collected for analysis. Immunoblotting revealed that treatment of HUVEC with SU4312 did not significantly decrease pERK1/2 levels, a known readout of VEGFR2 signalling (Olsson et al., 2006). In addition, inhibition of VWF expression significantly increased pERK1/2 levels in total cell lysates. As shown previously (chapter 3), inhibition of VWF expression in EC caused a significant increase in Ang-2 mRNA levels. In line with no effect on pERK1/2 levels (figure 4.8A & B), SU4312 treatment did not significantly decrease Ang-2 mRNA levels in control and VWF-deficient cells (figure 4.8C). Since inhibition of VEGFR2 signalling did not reduce the pERK1/2 levels in control cells and a number of growth factors are present in normal culture media, this suggested the possibility of compensation by another signalling pathway regulating ERK-Ang-2 axis.

To address the role of VEGFR2-ERK signalling axis in the VWF-dependent increase in Ang-2 mRNA levels, HUVEC were treated with siRNA control or siVWF for 40 hours then serum deprived for 4 hours. Cells were then pre-treated for 30 mins with 4 µM SU4312, followed by VEGF 25 ng/ml for 3 hours. Immunoblotting revealed that serum deprivation normalised pERK1/2 levels in control and VWF-deficient cells (figure 4.9A & B). In the presence of VEGF, pERK1/2 levels increased in both control and VWF-deficient cells, albeit not significant due to variability. In the presence of SU4312, pERK1/2 levels were reduced in both control and VWF-deficient cells, however, the data was very variable and not significant. Analysis of RNA revealed that Ang-2 mRNA levels were upregulated in VWF-deficient cells compared to controls (figure 4.9C). VEGF, as expected, upregulated Ang-2 mRNA levels in both control and VWF-deficient cells; however the effect was quite variable. Surprisingly, while inhibition of VEGFR2 signalling was able to significantly reduce Ang-2 mRNA levels in control cells, SU4312

did not affect Ang-2 mRNA levels in VWF-deficient cells. Taken together, these data suggest that VEGFR2-ERK signalling does not mediate the VWF-dependent upregulation of Ang-2.

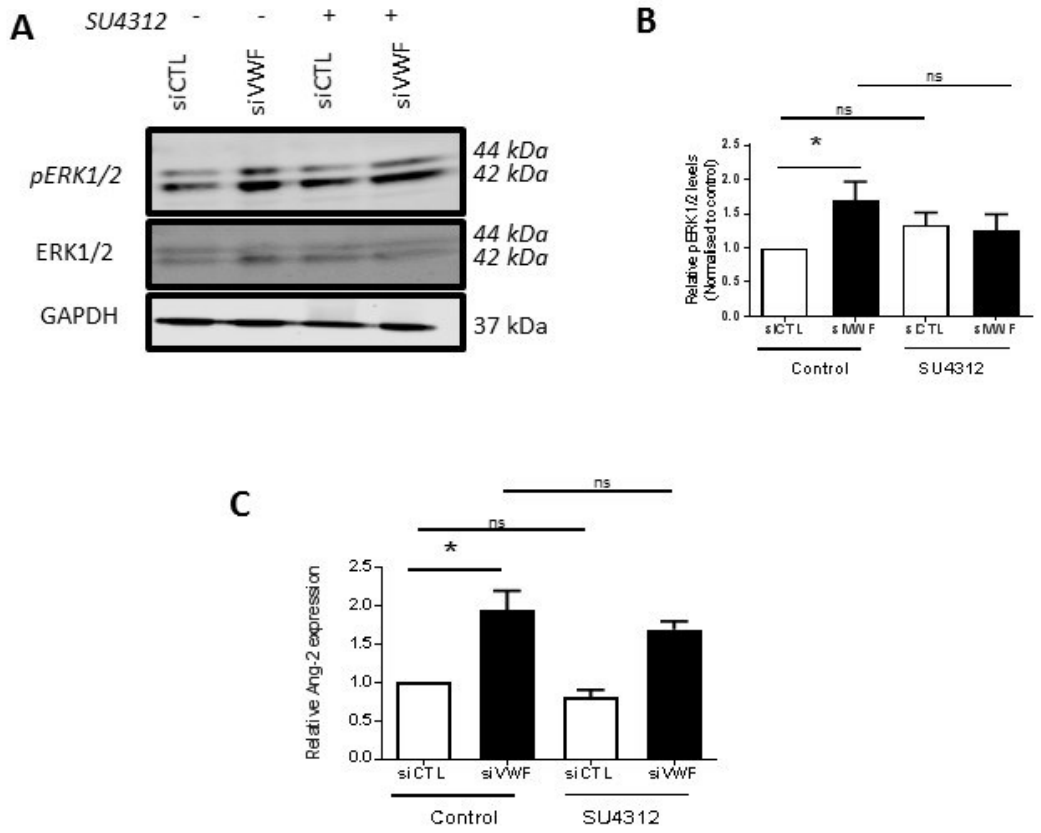

Figure 4.8 Inhibition of VEGFR2 signaling does not affect Ang-2 mRNA levels in basal conditions. HUVEC were treated with siRNA to VWF or siRNA control for 24 hours. Cells were treated with 4 µM VEGFR2 inhibitor SU4312 for 24 hours. (A) Representative immunoblots (B) Expression of pERK1/2 was measured by Odyssey and data normalized to total ERK1/2 expression and expressed relative to siCTL DMSO (C) Ang-2 expression was measured by qRT-PCR and normalized to GAPDH and expressed relative to siCTL DMSO. Data presented as mean ± SEM of n=4. Statistical significance determined by ANOVA with Dunn's post hoc test. ns $p>0.05$, * $p\leq0.05$,

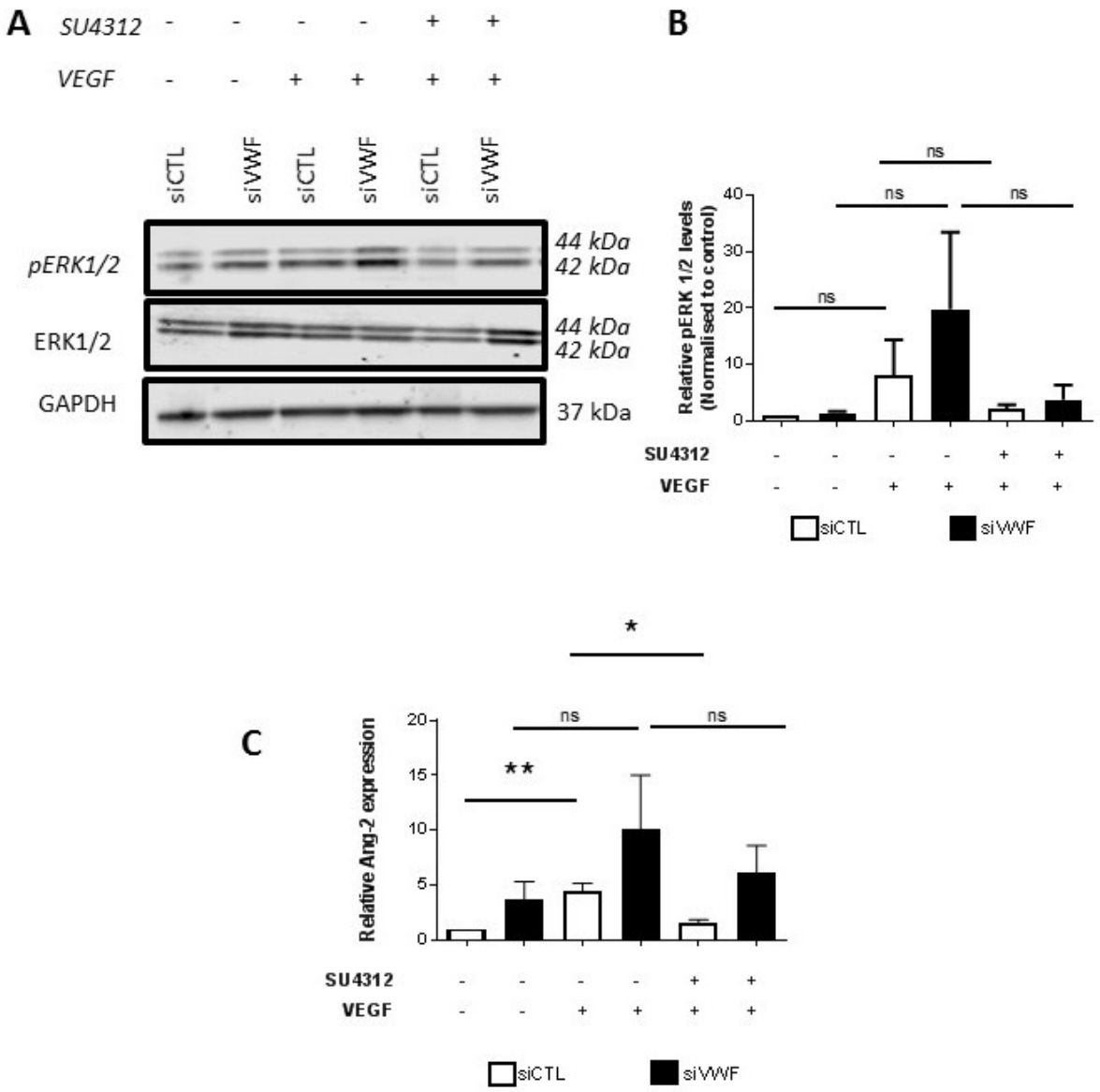

Figure 4.9 Inhibition of VEGFR2 signalling reduces Ang-2 expression in control but not VWF-deficient cells after VEGF treatment. HUVEC were transfected with siCTL or siVWF for 46 hours then pretreated with SU4312 or DMSO control for 30 mins prior to 25 ng/ml VEGF for 3 hours. (A) Representative immunoblots. (B) Expression of pERK1/2 was measured by Odyssey and data normalized to total ERK1/2 and expressed to siCTL DMSO. (C) Ang-2 expression was measured by RT-PCR and normalized to GAPDH and expression relative to siCTL DMSO. Data presented as mean ± SEM of n =4. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$

4.1.6 Inhibition of VWF expression in ECs reduces β3 levels and signalling via FAK Y397 and Akt S473

Since neither VEGFR2 nor ERK signalling appear to mediate VWF-dependent upregulation of Ang-2 mRNA levels, we next investigated whether the PI3K/Akt pathway was involved through activation of integrin αvβ3. Since VWF binds to the integrin αvβ3 and controls its levels and internalisation (Cheresh and Spiro, 1987, Starke et al., 2011), we speculated that inhibition of VWF could affect αvβ3 signalling (illustrated in figure 4.10A). As discussed in chapter 1, "outside-in" signalling via integrins such as αvβ3 occurs via recruitment of adaptor molecules such as FAK inducing tyrosine phosphorylation activating downstream pathways involving Akt (Schlaepfer et al., 1999). To test this hypothesis, HUVEC were treated with control or VWF siRNA for 48 hours and total cell lysates were collected. Immunoblotting was performed for integrin β3, phosphoFAK (pFAK) tyrosine residue 397 and total FAK, phospho Akt (pAkt) S473 and total Akt and loading control GAPDH (figure 4.10B). Inhibition of VWF expression in EC (figure 4.10C) demonstrated a significant reduction in integrin β3 levels (figure 4.10D), in line with our previous study (Starke et al., 2011). In addition, inhibition of VWF expression in ECs caused a decreased FAK phosphorylation at Y397 (figure 4.10E) and Akt phosphorylation S473 (figure 4.10F). These data suggests that inhibition of VWF expression causes decrease β3 integrin signalling via FAK and Akt.

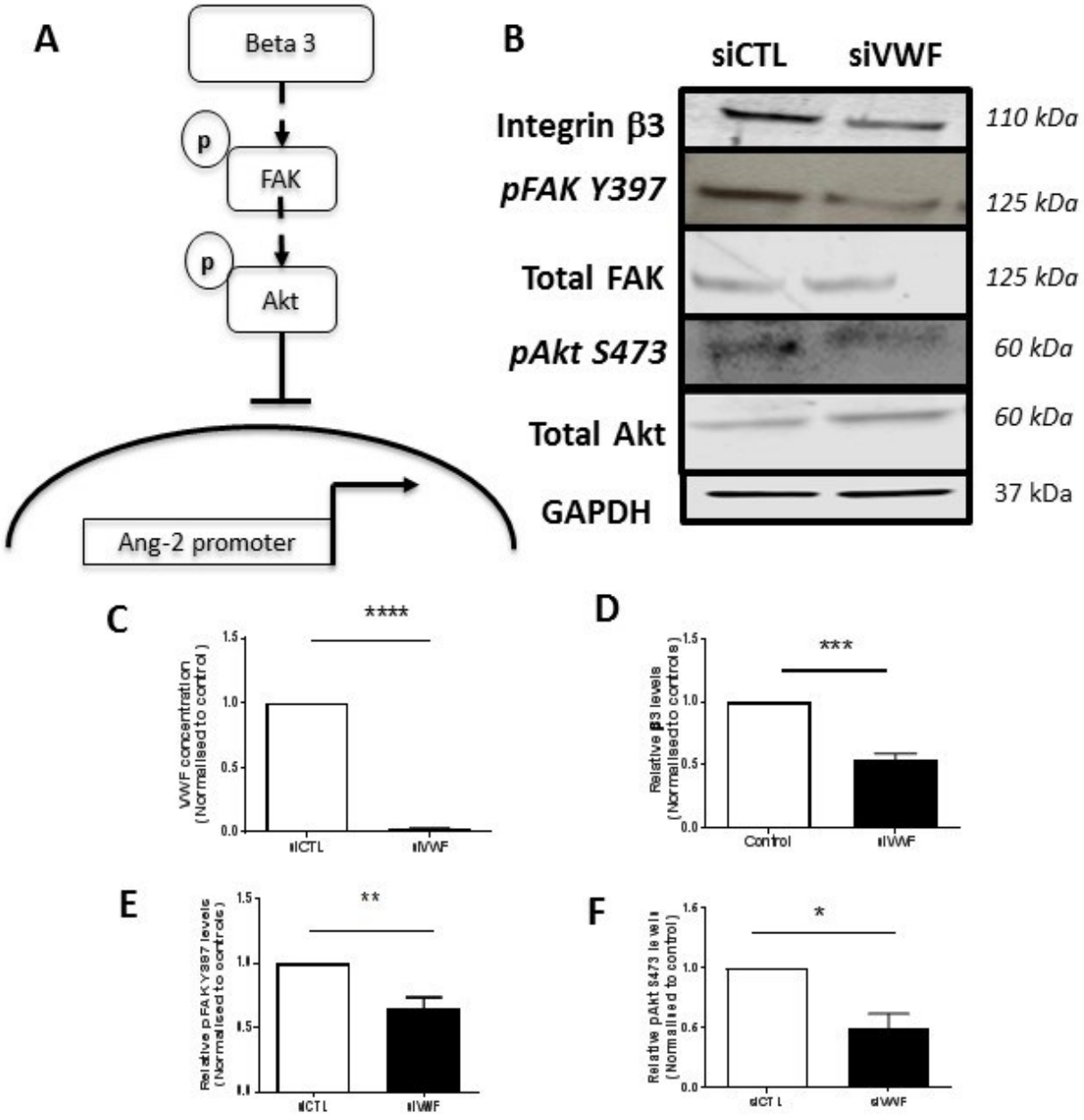

Figure 4.10 VWF regulates integrin β3 levels and signaling via FAK and Akt axis. (A) Representative schematic of β3 signaling cascade via phosphorylation of Focal Adhesion Kinase (FAK) and Akt,. HUVEC were transfected with siVWF or siCTL control siRNA for 48 hours. Total cell lysates were collected and Immunoblotting performed. (B) Representative Immunoblots from siCTL and siVWF treated cells. (C) Quantification of VWF expression in total cell lysates by ELISA normalized to total protein and expressed relative to siCTL. (D) Quantification of Beta 3 expression normalized to GAPDH expressed relative to siCTL (E) pFAK Y397 normalised to total FAK and expressed relative to siCTL (F) Quantification of Akt S473 normalized to total Akt and expressed relative to siCTL. Data presented as mean ± SEM of n=4-6. Statistical significance determined by student's t-test. * p≤0.05, ** p≤0.01

4.1.7 The regulation of integrin β3 levels in VWF-deficient cells is not Ang-2 dependent

The Augustin group has shown that Ang-2 stimulates integrin β3 internalisation and decrease signalling via FAK Y397 (Thomas et al., 2010). We have shown that VWF-deficient cells have increased constitutive release of Ang-2 into the cell supernatant (chapter 3). To determine whether decreased β3 expression in VWF-deficient cells was due to enhanced Ang-2 release, first an Ang-2 siRNA was used. However treatment of HUVEC with Ang-2 siRNA resulted in a significant decrease in VWF expression (appendix 2). To avoid interference with VWF levels, an Ang-2 functional blocking antibody (AZ 3.19.3) was used in control and VWF-deficient cells. HUVEC were treated with control or VWF siRNA sequences for 24 hours. Cells were then treated with AZ 3.19.3 or IgG control at 5 nM, a concentration previously shown to block Ang-2 function (Brown et al., 2010). Immunoblotting was performed on total cell lysates (figure 4.11A). Surprisingly, inhibition of Ang-2 function did not affect integrin β3 expression in control cells (figure 4.11B). In line with our previous findings, inhibition of VWF expression in ECs reduced β3 expression; this was not affected by pre-treatment with the Ang-2 functional blocking antibody. The Anti-Ang-2 blocking Ab had no effect on VWF expression (figure 4.11C). This suggests that Ang-2 is not involved in the decreased β3 integrin expression in VWF-deficient cells.

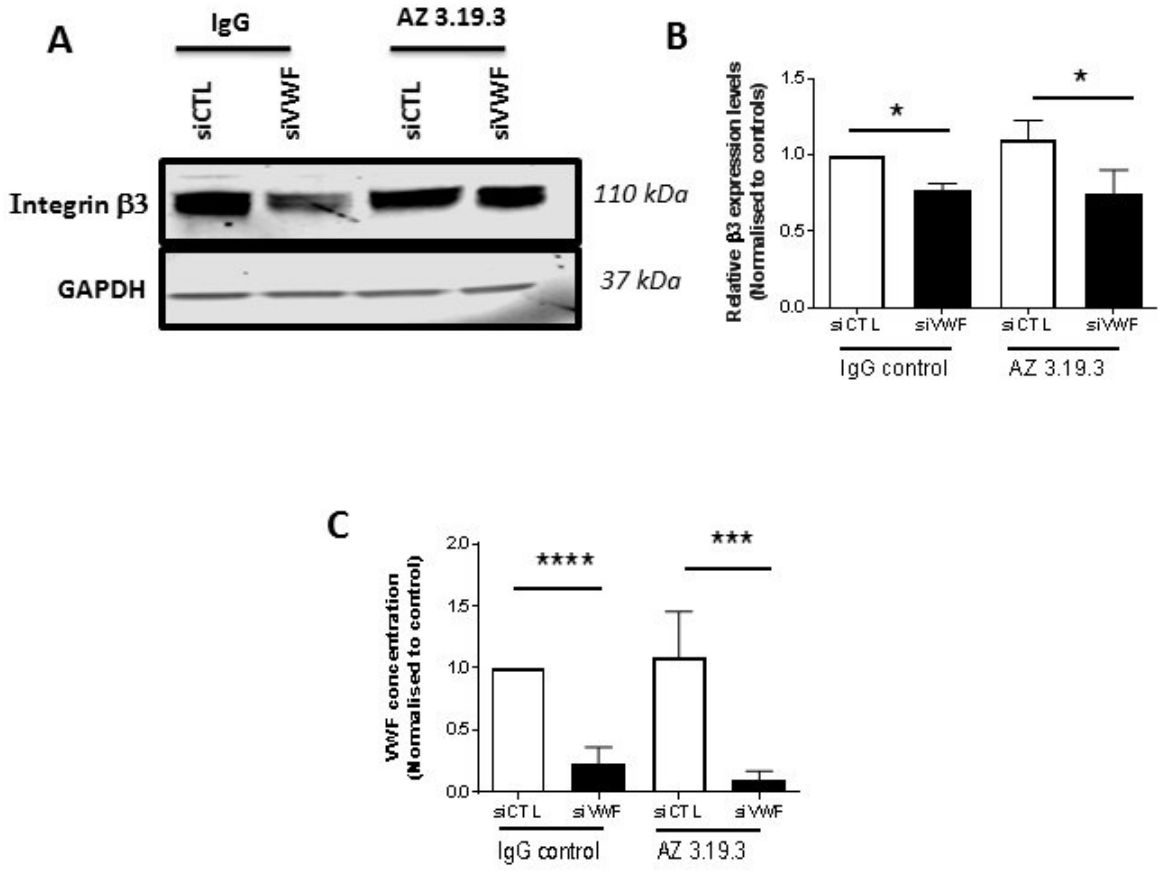

Figure 4.11 Ang-2 does not mediate the VWF-dependent decrease in integrin β3 expression. HUVEC were transfected with siVWF to inhibit VWF expression and necessary control. After 24 hours cells were treated with Ang-2 neutralizing antibody AZ 3.19.3 at 5 nM or IgG control. After 24 hours total cell lysates were collected. (A) Representative Immunoblots of Beta 3 integrin and GAPDH. (B) Expression was quantified and normalized to GAPDH and expressed relative to siCTL IgG. (C) VWF expression was measured by ELISA and normalized to total protein. Data presented as mean ± SEM of n=4. Statistical significance determined by student's t-test. * p≤0.05, ***p ≤0.001, **** p ≤0.0001.

4.1.8 Immobilised extracellular plasma-purified VWF regulates β3 integrin levels and signalling

We hypothesised that VWF regulates Ang-2 levels by interacting with integrin αvβ3 on the cell surface. To test this hypothesis, two different purified, plasma-derived forms of VWF were used, VWF concentrate (cVWF) from a commercial source and VWF purified in-house from Haemate P (hVWF) (Supplied by Dr. Thomas McKinnon). HUVEC were transfected with control or VWF siRNA for 24 hours. After 24 hours, HUVEC were reseeded onto 1 μg/ml of cVWF or hVWF. Total cell lysates and RNA were collected for analysis. Immunoblotting demonstrated that inhibition of VWF expressed caused a decrease in β3 expression, as expected, which was normalised by adhesion to cVWF (figure 4.12A). Inhibition of VWF expression caused an increase in Ang-2 expression, as expected compared to controls. After adhesion to cVWF, the VWF-dependent increase in Ang-2 mRNA levels was normalised compared to siCTL treated cells in the same condition (figure 4.12B). In contrast to cVWF, adhesion of VWF-deficient HUVEC to immobilised hVWF did not have a profound effect on β3 levels (figure 4.12C); however, in these conditions the difference between control and siVWF-treated cells was no longer significant (p=0.0847). As shown with cVWF, adhesion HUVEC to hVWF resulted in normalisation of Ang-2 mRNA levels in VWF-deficient cells compared to control (figure 4.12D). In both experimental scenarios, adhesion of HUVEC to immobilised VWF normalised Ang-2 mRNA levels in VWF-deficient cells. To understand whether this was due to the ability of immobilised VWF to regulate Ang-2 levels in control cells, data was expressed relative to siRNA control on gelatin (figure 4.13). This normalisation did not affect the interpretation of integrin β3 results. In the presence of cVWF, integrin β3 levels were normalised (figure 4.13A). In contrast, on immobilised hVWF, integrin β3 levels remained decreased in VWF-deficient cells (figure 4.13C). On the other hand, normalising Ang-2 expression to control cells on gelatin revealed that in the presence of immobilised cVWF (figure 4.13B) and hVWF (figure 4.13D), Ang-2 mRNA levels were increased in control cells, although no this did not reach significance in hVWF (p=0.0576). After inhibition of VWF expression in EC, Ang-2 mRNA levels were unaffected after adhesion to both cVWF and hVWF. These data suggest extracellular VWF regulates Ang-2 mRNA levels.

Since cVWF was able to normalise integrin β3 expression, we next investigated whether signalling was also normalised. To test this, FAK Y397 was measured in HUVEC seeded on immobilised cVWF compared to gelatin, in control and VWF-deficient cells. Here, despite variability in control cells, adhesion to immobilised cVWF resulted in normalization of FAK Y397 levels (figure 4.14). Together these data suggests that extracellular VWF is able to stabilise integrin β3 levels and normalise signalling via FAK.

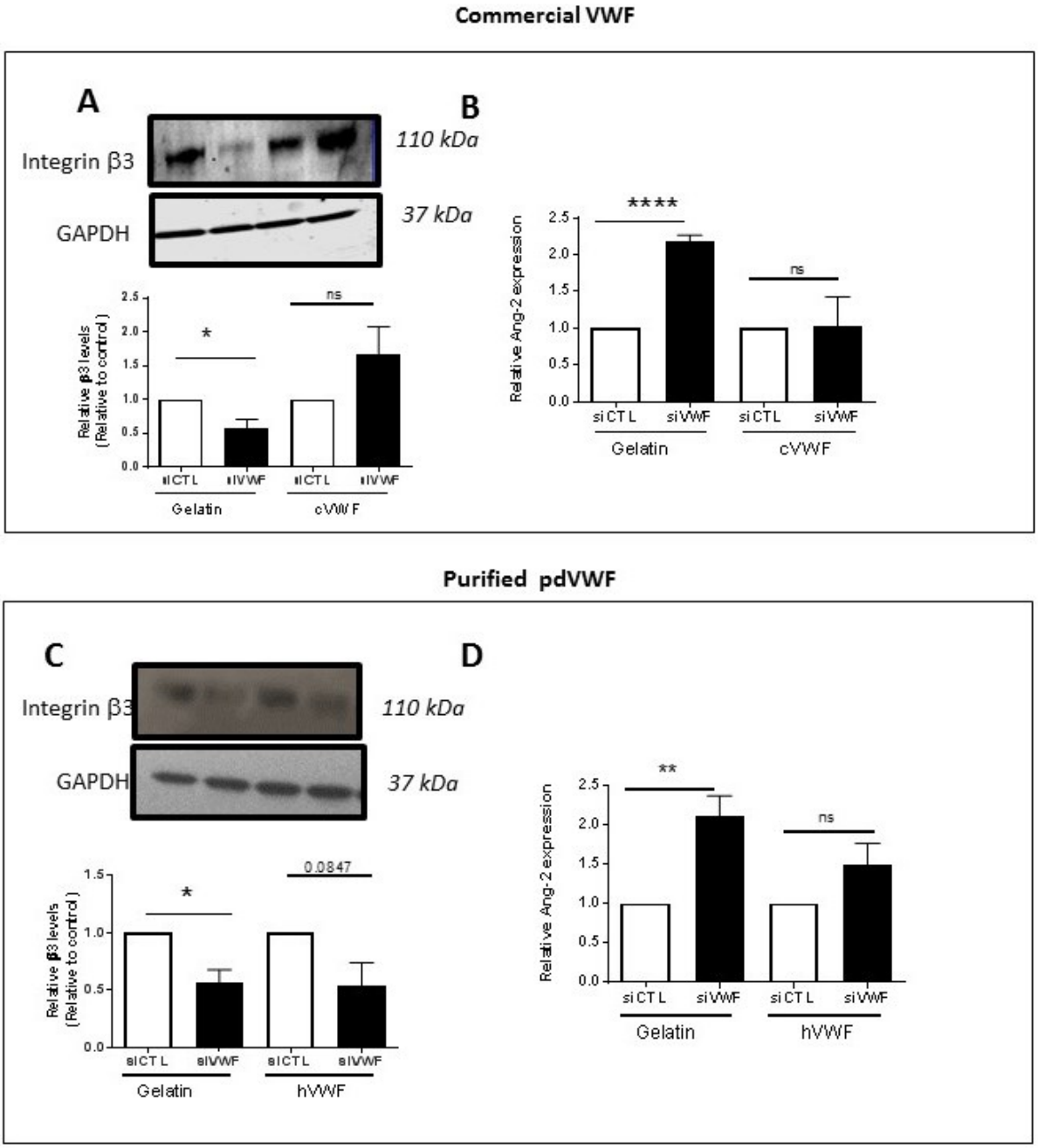

Figure 4.12 Extracellular VWF regulation of integrin β3 and Ang-2 mRNA levels VWF-deficient cells. HUVEC were transfected with siVWF or siCTL. After 24 hours cells were reseeded onto plates coated with 1 µg/ml of VWF concentrate (Top panels) or Purified VWF (bottom panel). Total cell lysates were collected. (A and C) Representative Immunoblot of β3 and GAPDH expression. Quantification of β3 expression normalized to GAPDH and expressed relative to siCTL treated cells. Ang-2 expression was measured by qRT-PCR of cells on Gelatin and VWF (B and D). Data normalized to GAPDH and expressed relative to siCTL. Data presented as mean ± SEM of n=4. Statistical significance was determined by student's t-test. ns $p>0.05$,* $p\leq0.05$,** $p\leq0.01$, **** $p \leq0.0001$

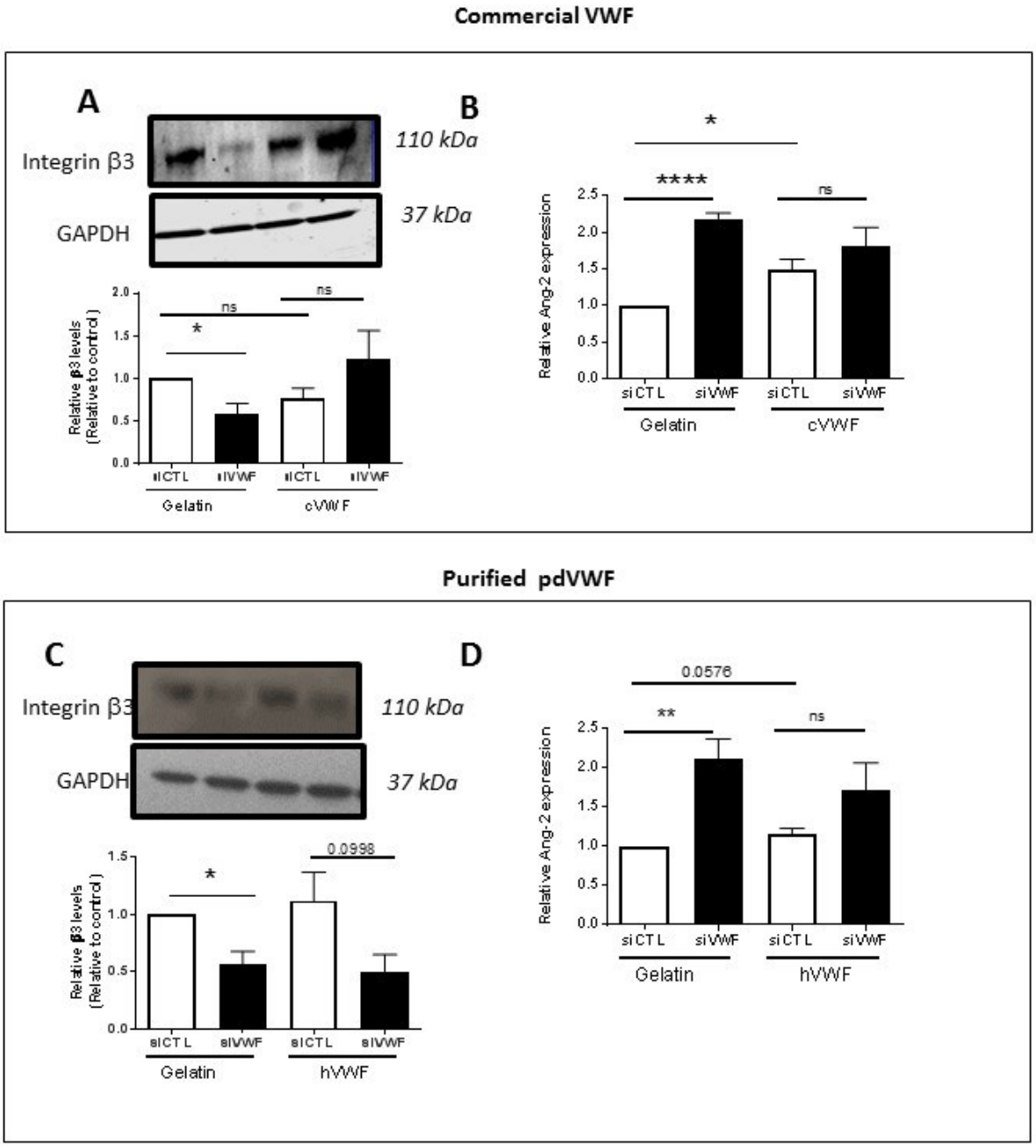

Figure 4.13 Extracellular VWF regulates integrin β3 levels without affecting Ang-2 mRNA levels in VWF-deficient cells. HUVEC were transfected with siVWF or siCTL. After 24 hours cells were reseeded onto plates coated with 1 μg/ml of VWF concentrate (Top panels) or Purified VWF (bottom panel). Total cell lysates were collected. (A and C) Representative Immunoblot of β3 and GAPDH expression. Quantification of β3 expression normalized to GAPDH and expressed relative to siCTL treated cells on Gelatin. Ang-2 expression was measured by qRT-PCR of cells on Gelatin (B and D). Data normalized to GAPDH and expressed relative to siCTL treated cells on gelatin. Data presented as mean ± SEM of n =4. Statistical significance was determined by student's t-test. ns $p>0.05$, * $p\leq0.05$,** $p\leq0.01$, **** $p \leq0.0001$

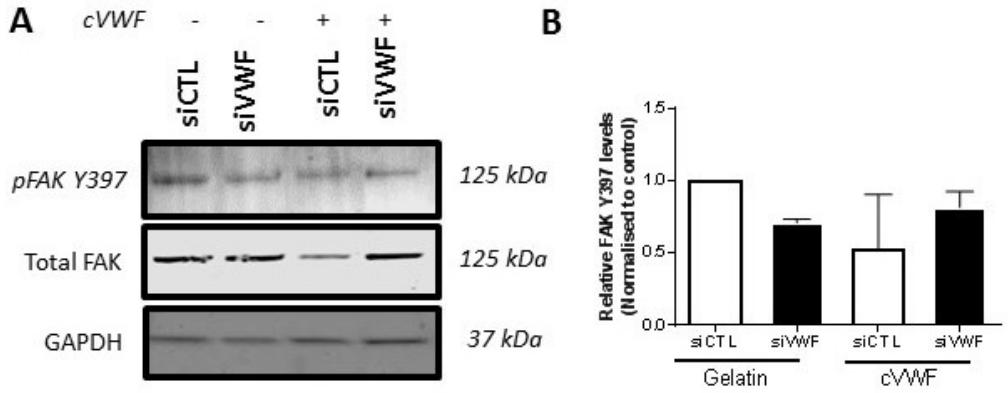

Figure 4.14 Extracellular VWF rescues β3 integrin signaling in VWF-deficient cells. HUVEC were transfected with siVWF or siCTL. After 24 hours cells were reseeded onto plates coated with 1 μg/ml of commercial VWF or Purified VWF. Total cell lysates were collected. (A) Representative Immunoblot of FAK Y397, Total FAK and GAPDH expression. (B) Quantification of pFAK/FAK expressed relative to siCTL treated cells on Gelatin. Data presented as mean ± SEM of n=2

4.1.9 VWF in solution does not regulate Ang-2 expression in endothelial cells

VWF interaction with ECM induces conformational change and exposes its A1 domain allowing binding of platelets (Baruch et al., 1991, Schneider et al., 2007, Siedlecki et al., 1996). Moreover, VWF under flow has been shown to tether to EC surface via to integrin $\alpha v\beta 3$ after release (Huang et al., 2009). To test whether VWF in solution could regulate integrin $\beta 3$ expression as well as regulate Ang-2 mRNA levels, HUVEC were transfected with control and VWF siRNA for 24 hrs then stimulated with 4 μg/ml for 24 hrs. RNA and total cell lysates were collected for analysis. As expected, inhibition of VWF expression in EC caused decreased integrin $\beta 3$ levels (figure 4.15A). In the presence of cVWF in solution, the significant difference in $\beta 3$ levels between VWF-deficient cells and control cells was lost, (figure 4.15B). This is likely to be due to variability in the control siRNA samples, since cVWF addition was unable to increase $\beta 3$ levels in VWF-deficient cells. In line with previous findings, inhibition of VWF expression significantly increased Ang-2 mRNA levels. Addition of cVWF in solution did not modify Ang-2 mRNA levels in control or VWF-deficient cells (figure 4.15C). These data suggests that conformation of VWF may be important for the regulation of Ang-2 levels.

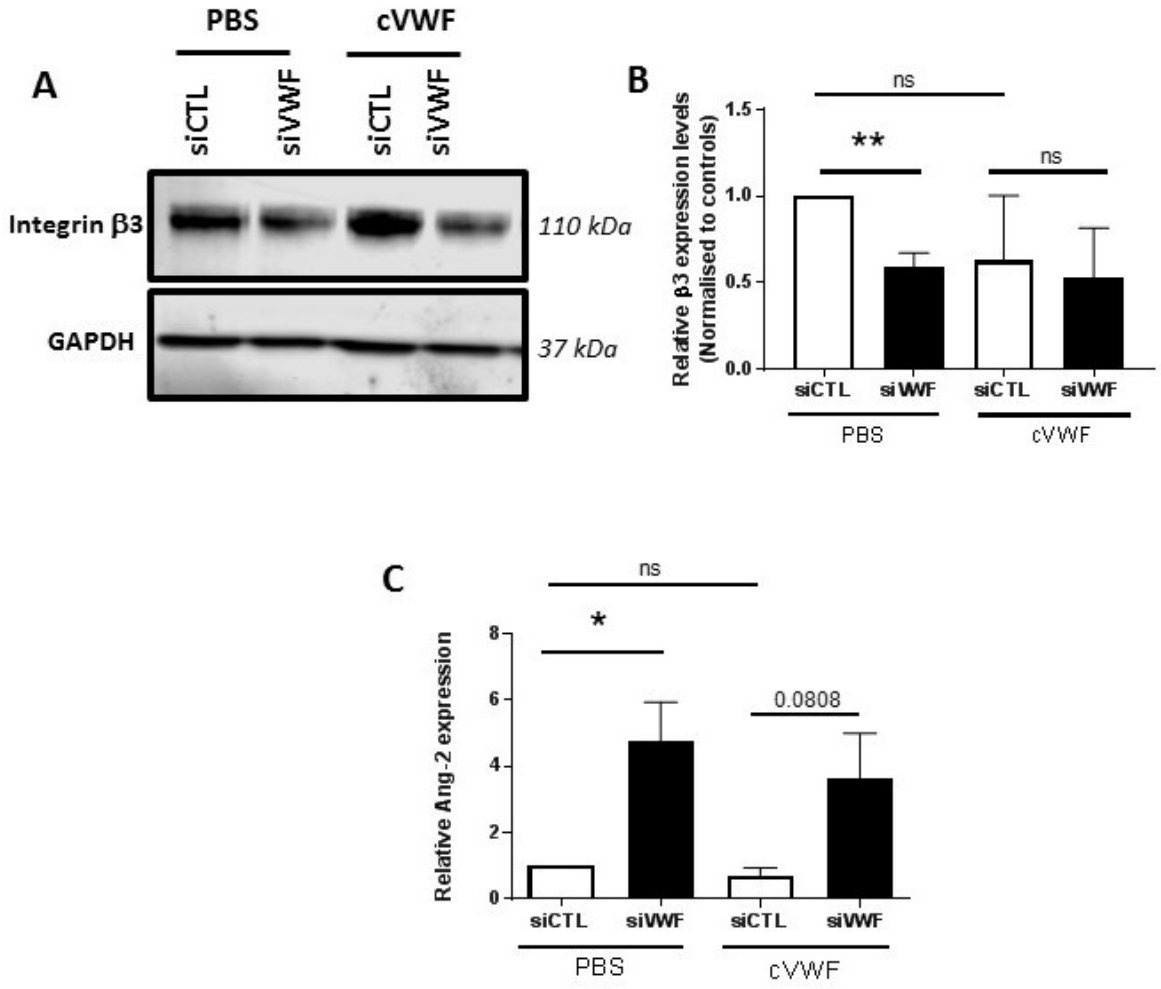

Figure 4.15 No effect on Ang-2 mRNA levels after addition of VWF in solution. HUVEC transfected with control or VWF siRNA for 24 hours then stimulated with 4 μg/ml of commercial VWF (cVWF) or PBS control for 24 hours. (A) Representative immunoblots and quantification (B) of integrin β3 expression in total cell lysates. Values were normalised to GAPDH and expressed relative to siCTL (PBS). (C) Ang-2 expression was measured by qRT-PCR and normalized to GAPDH. Data expressed relative to siCTL (PBS). Data presented as mean ± SEM of n=4. Statistical significance determined by student's t-test. ns $p>0.05$, * $p<0.05$, ** $p<0.01$

4.1.10 Activation of Akt phosphorylation in VWF-deficient cells reduces Ang-2 mRNA levels

4.1.10.1 Validation of constitutively active Akt adenovirus for transduction in endothelial cells

The data so far suggests that inhibition of VWF expression in ECs causes decrease integrin β3 expression and signalling via FAK and Akt. To validate the role of Akt signalling in regulating Ang-2 levels in VWF-deficient cells, an Adenovirus expressing a constitutively phosphorylated (Active) form of Akt (CA-Akt) was used. To optimise the Adenovirus use in HUVEC, CA-Akt was tested at different Multiplicity of Infection (MOI). Increased phosphorylation of S473 and T308 Akt was found with increasing MOI (figure 4.16). To confirm that activation of Akt could reduce Ang-2 mRNA levels (described in section 4.1), HUVEC were transduced with CA-Akt at MOI 80 and an Adeno control (Adeno0). As expected, enhanced activity of Akt in the CA-Akt-treated HUVEC (figure 4.17A) was associated with a decrease in Ang-2 expression (figure 4.17B). Interestingly, VWF mRNA showed a trend towards increase in CA-Akt-treated HUVEC compared to Adeno0 by ≈1.5-fold, although this was not significant (figure 4.17C). No significant difference between VWF protein in CA-Akt and AdenoO control was observed (figure 4.17D). These data demonstrate that activation of Akt using a constitutively active adenoviral construct was able to reduce Ang-2 expression in HUVEC and thus validated this tool for further experiments.

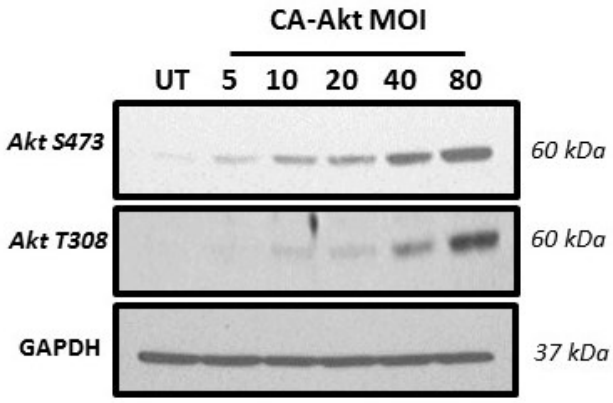

Figure 4.16 Validation of CA-Akt Adeno virus activity in EC. HUVEC were transduced with Adeno viral vector with phosphorylated Akt (CA.Akt) for 24 hours at Multiplicity of infection (MOI) indicated. Cell lysates were collected and analyzed for Akt S473 and T308. n=1

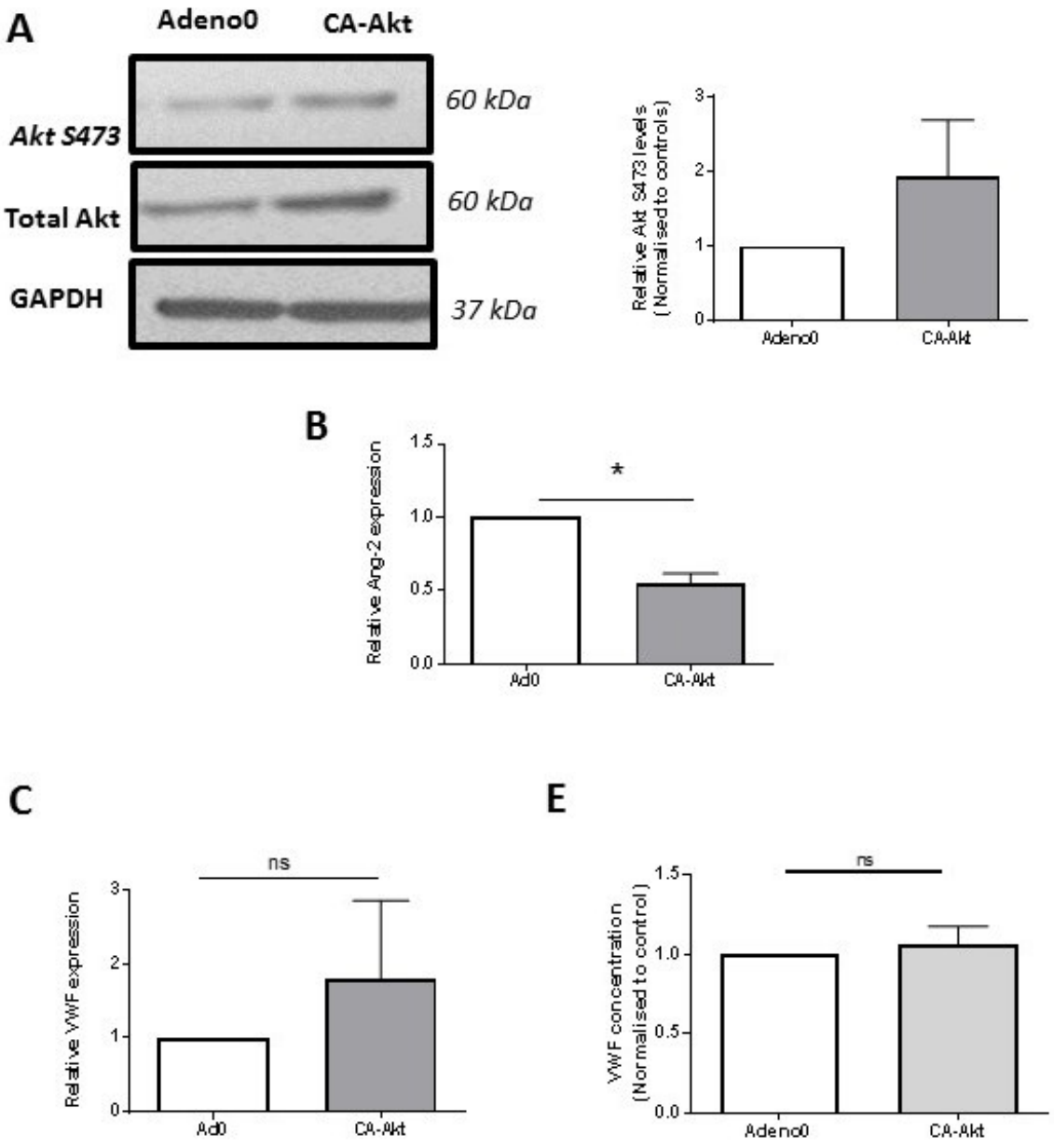

Figure 4.17 Akt activation regulates Ang-2 synthesis in EC. HUVEC were transduced with Adeno viral vector with phosphorylated Akt (CA.Akt) for 24 hours at Multiplicity of infection (MOI) 40. (A) Cell lysates were collected and analysed for Akt S473, Total Akt and GAPDH. Quantification of phosphorylation of Akt normalized to Adeno. (B) Ang-2 mRNA expression was measured by qRT-PCR and normalized to GAPDH. Data expressed relative to Adeno0 control. (C) VWF expression as measured by qRT PCR and normalized to GAPDH. Data presented as expression relative to Adeno0. (D) VWF levels were measured in protein lysates by ELISA and normalized to total protein. Data presented as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$

4.1.10.2 Activation of Akt in VWF-deficient cells reduces enhanced Ang-2 mRNA levels

To investigate whether Akt mediates the effect of VWF on Ang-2 expression, VWF expression was inhibited for 24 hours then HUVEC were transduced with Adeno0 and CA-Akt for 24 hours. Inhibition of VWF expression with this protocol only produced a 30% reduction in VWF in AdenoO treated cells and variable response in CA-Akt treated cells (data shown in appendix 4). Therefore, we decided to modify the protocol. Cells were first transduced with Adeno and CA-Akt for 24 hours, followed by transfection with siRNA for 48 hours. Measurement of VWF levels by ELISA demonstrated that the Adenovirus did not interfere with the ability of siRNA to inhibit VWF expression (figure 4.18A). As expected, the CA-Akt virus was able to increase phosphorylation of Akt S473 compared Adeno0 control (figure 4.18B). AdenoO virus transduction did not interfere with the ability of VWF siRNA to increase Ang-2 mRNA levels (figure 4.18C). CA-Akt adeno virus was able to significantly reduce Ang-2 mRNA levels in VWF-deficient cells (figure 4.18D). These data indicate that in VWF-deficient cells, decreased Akt signalling is involved in the enhanced Ang-2 expression, and suggest that VWF controls Akt signalling in EC

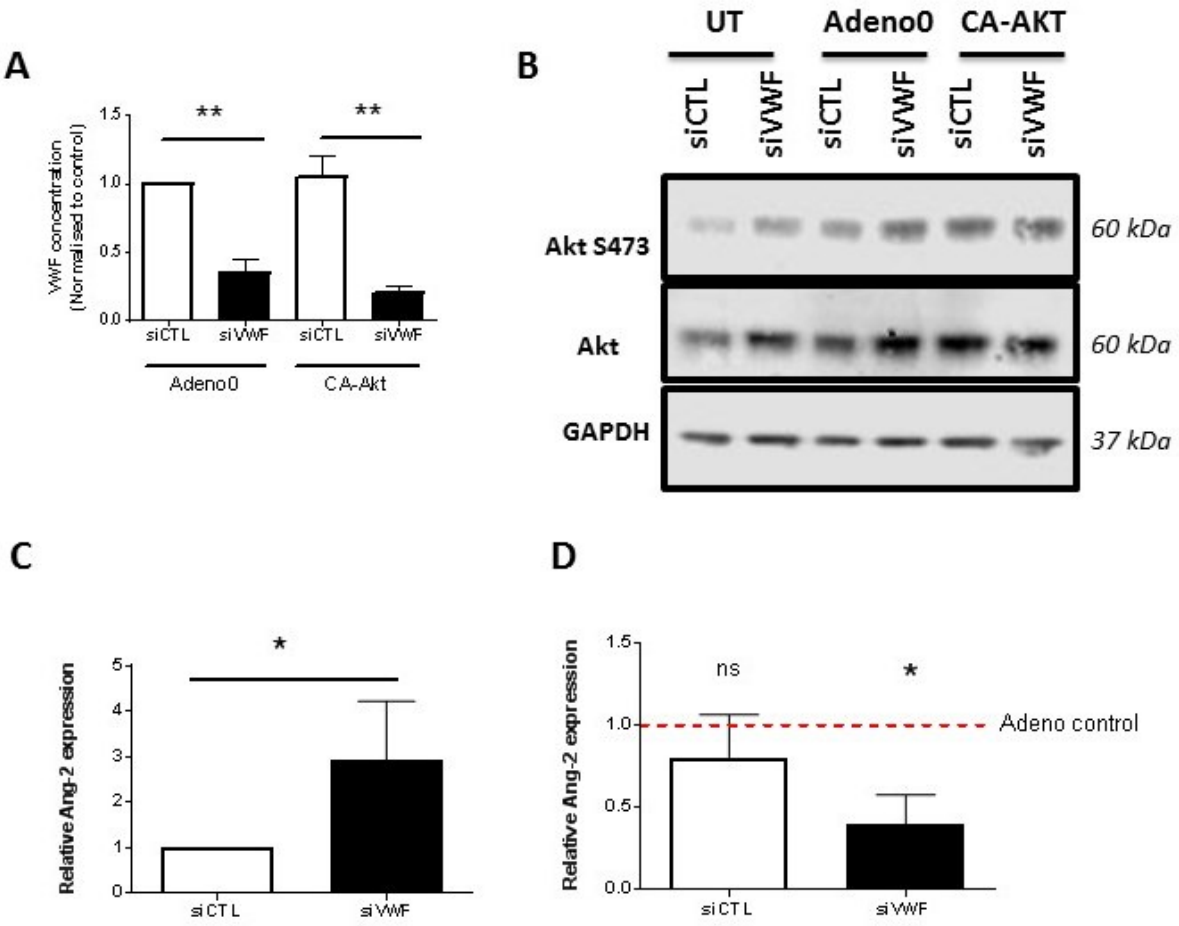

Figure 4.18 CA-Akt reduces enhanced Ang-2 expression in VWF-deficient cells. HUVEC were transduced with CA-Akt at MOI 80 for 24 hours then transfected with siRNA to VWF (siVWF) or control (siCTL) for 48 hours. Lysates were collected and VWF expression (A) was measured by ELISA and normalized to total protein. (B) Expression of pAkt and total Akt was measured by Immunoblotting. (C) Ang-2 expression in Aden0 treated samples was measured by qRT-PCR and normalized to GAPDH. Data expressed relative to siCTL treated cells. (D) Ang-2 expression was measured by qRT-PCR and normalized to GAPDH. Data expressed as a percent of Adeno0 transduced cells in siCTL and siVWF treated cells. Data presented as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$

4.1.11 Inhibition of VWF expression in EC controls FOXO1 localisation and phosphorylation

As described previously (section 4.1.6), Akt regulates Ang-2 expression via the transcription factor FOXO1. In order for FOXO1 to drive Ang-2 expression, FOXO1 must be dephosphorylated and translocate to the nucleus (illustrated in figure 4.19A) (Ghosh et al., 2015). We hypothesised that VWF may control Ang-2 expression via repression of FOXO1 activity in EC. We first tested whether inhibition of VWF expression in ECs affected phosphorylation of FOXO1. HUVEC were treated for 48 hours with control or VWF siRNA sequences. Immunoblotting showed a significant reduction in FOXO1 T24 phosphorylation in VWF-deficient cells, with no significant difference in FOXO1 total levels (figure 4.19B). Since FOXO1 phosphorylation was decreased, we next investigated whether FOXO1 localisation was affected. Control (top panel, figure 4.19C) and VWF-deficient HUVEC (bottom panel, figure 4.19C) were stained for VWF (green), FOXO1 (red) and DAPI (blue) 48 hours after siRNA transfection. Interesting, in a confluent monolayer of control cells, heterogeneity in FOXO1 intensity was observed between cells. Quantification of nuclear intensity of FOXO1 revealed a significant increase in FOXO1 nuclear intensity in VWF-deficient cells compared to controls (figure 4.19D), in line with the decreased phosphorylation. These data suggests enhanced FOXO1 activity in VWF-deficient cells.

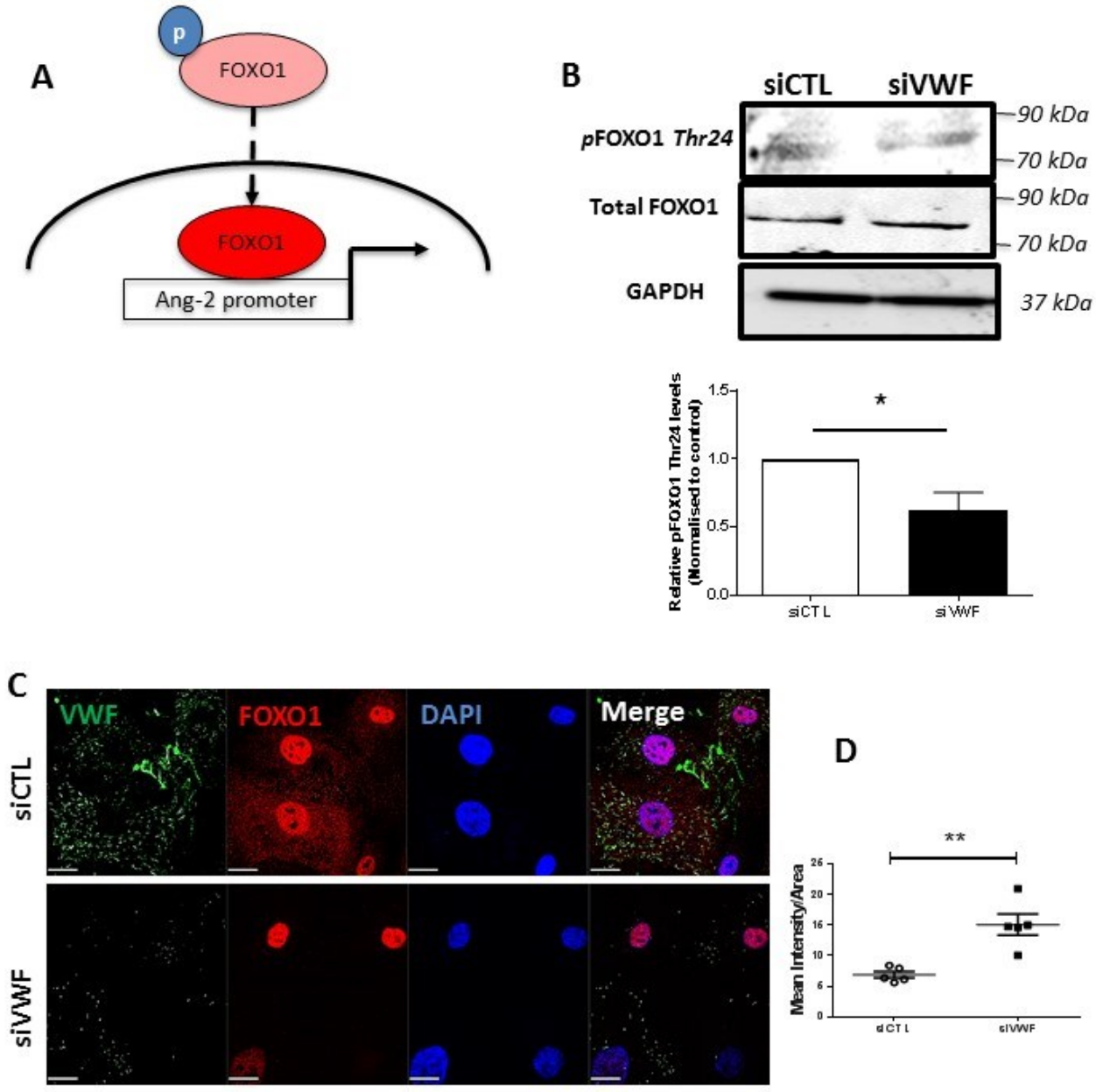

Figure 4.19 Enhanced nuclear localization of FOXO1 in VWF-deficient cells. (A) Cartoon representation of FOXO1 nuclear localization after loss of phosphorylation. HUVEC were transfected with siCTL or siVWF for 48 hours. (B) Representative blot of pFOXO1 Thr24 and Total FOXO1 expression. Quantification of pFOXO1 Thr24 expression by densitometry was normalized to total FOXO1 and expressed relative to siCTL. (C) Representative image of siCTL and siVWF treated HUVEC stained for VWF (Green), FOXO1 (Red) and DAPI for nuclei (Blue). (D) Quantification of 5 x20 fields normalized to nuclear area (n=1). Data expressed as mean ± SEM. Statistical significance determined by student's t-test. * p≤0.05, ** p≤0.01

4.1.12 FOXO1 mediates the VWF dependent increase in Ang-2 levels

Adenoviral transduction of FOXO1 with unphosphorylated sites has been shown to drive Ang-2 expression in ECs (Daly et al., 2004). In addition siRNA to FOXO1 in ECs have been shown to reduce Ang-2 expression (Potente et al., 2005). To determine if FOXO1 mediates the VWF dependent increase in Ang-2 levels, we took an siRNA approach. Pre-validated FOXO1 siRNA was obtained from Qiagen and used at 20 nM. Co-transfection of validated FOXO1 siRNA and VWF siRNA sequences was first optimised. Both siRNA sequences were transfected simultaneously into HUVEC alongside control sequences for 48 hours. RNA and protein was collected. Inhibition of VWF expression in HUVEC for 48 hours caused increase FOXO1 mRNA levels (figure 4.20A). Co-treatment with siRNA to FOXO1 produced a significant reduction of FOXO1 mRNA expression in control cells (≈50%) and VWF-deficient cells (≈60%). At the protein level, no significant difference in FOXO1 was observed after inhibition of VWF expression (figure 4.20B). As expected, inhibition of FOXO1 expression produced a significant reduction in FOXO1 protein in both control and VWF-deficient cells.

To determine whether FOXO1 mediated the VWF-dependent increase in Ang-2 mRNA levels, RNA was extracted from HUVEC treated with VWF siRNA sequences (siVWF1 or siVWF2), co-transfected with siRNA to FOXO1 for 48 hours. As previously shown, inhibition of VWF significantly increased Ang-2 mRNA levels, using both VWF siRNA sequences (Figures 4.20C & D). Inhibition of FOXO1 expression significantly reduced Ang-2 mRNA levels in VWF-deficient cells. Measurement of Ang-2 release from control and VWF-deficient cells revealed that inhibition of FOXO1 was able to also normalise the VWF-dependent increase in Ang-2 release into the supernatant (figure 4.21A & B). These data demonstrate that transcription factor FOXO1 mediates the VWF-dependent increase in Ang-2 levels.

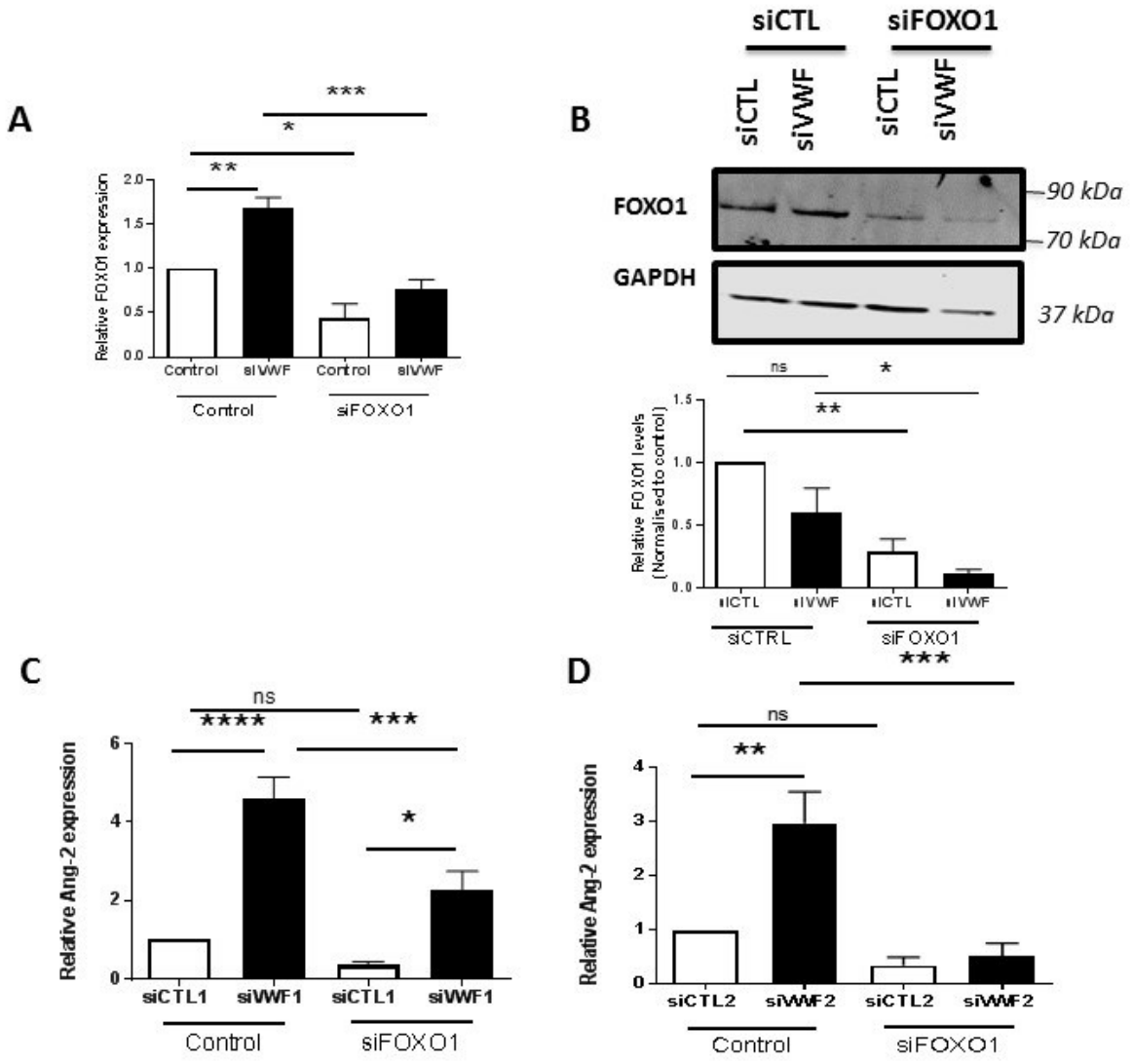

Figure 4.20 FOXO1 mediates VWF dependent upregulation of Ang-2 mRNA levels. HUVEC were transfected with siCTL and siVWF with siCTL or siFOXO1 for 48 hours. (A) FOXO1 expression was measured by qRT-PCR, normalised to GAPDH and expressed relative to siCTL. (B) Representative Immunoblot of FOXO1 and GAPDH. FOXO1 expression was measured by densitometry normalized to GAPDH and expressed relative to siCTL. (C) Ang-2 expression was measured by qRT-PCR after 48 hours transfection with siCTL 1 or siVWF1 in the presence of siFOXO1 or control. (D) Ang-2 expression measured by qRT-PCR after 48 hours transfection with siCTL2 or siVWF2 in the presence or siFOXO1 or control. Data normalized to GAPDH and expressed relative to siCTL. Data presented as mean ± SEM of n ≥ 3. Statistical significance determined by ANOVA with Tukey or Sidak's post hoc test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p \leq 0.001$ **** $p \leq0.0001$

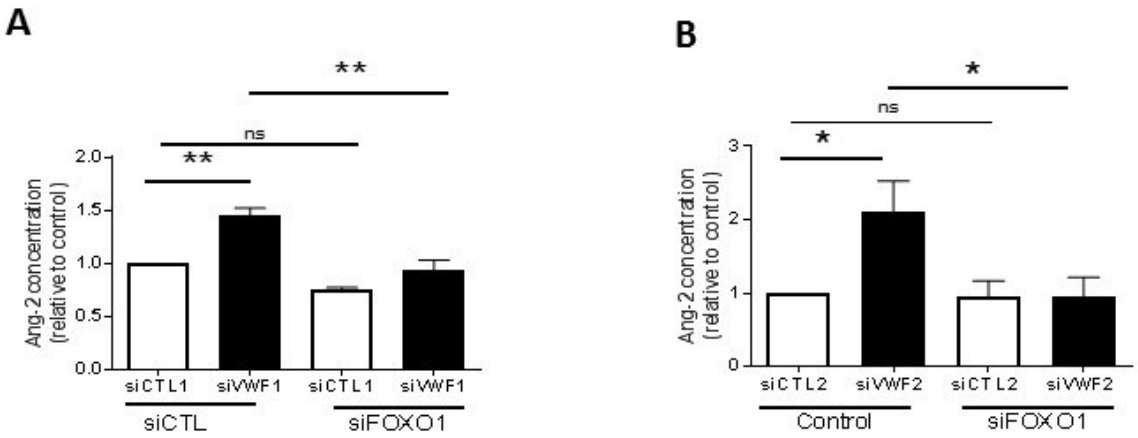

Figure 4.21 Inhibition of FOXO1 reduces Ang-2 released in the supernatant of VWF-deficient cells. HUVEC were transfected with siVWF1 (A) or siVWF2 (B) and corresponding controls with siCTL or siFOXO1 for 48 hours. Ang-2 released into supernatant was measured by ELISA and normalized to total protein. Data expressed relative to siRNA control. Data presented as mean ± SEM of n=3. Statistical significance determined by one way ANOVA with Sidak's post hoc test. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$

4.1.13 Enhanced FOXO1 activity in VWF-deficient cells may regulate distinct gene and cellular functions

4.1.13.1 Inhibition of VWF expression in ECs does not regulate all FOXO1 targets in the same manner

FOXO1 is a key regulator of a number of genes important for vascular maintenance and function (Daly et al., 2004, Potente et al., 2005, Dharaneeswaran et al., 2014). FOXO1 has been shown to drive the expression of CXCR4, BMPR4 and Cyclin G2 (Potente et al., 2005). In addition, FOXO1 has been shown to repress expression of genes such as eNOS and Elk-3 (Dharaneeswaran et al., 2014, Potente et al., 2005). To determine whether enhanced FOXO1 activity in VWF-deficient cells causes enhanced CXCR4, BMPR4 and Cyclin G2, we measured expression of these proteins in VWF-deficient cells. HUVEC were treated with control or VWF siRNA for 48 hours. Inhibition of VWF expression for 48 hours induced a significant increase in FOXO1 driven targets CXCR4 (figure 4.22A), BMPR4 (figure 4.22B) and Cyclin G2 (figure 4.22C) compared to controls. Surprisingly, inhibition of VWF expression in HUVEC also caused an increase in Elk3 (figure 4.22D) and eNOS (figure 4.22E), targets known to be repressed by FOXO1.

To determine whether enhanced FOXO1 activity in VWF-deficient cells was responsible for increased CXCR4, BMPR4 and Cyclin G2 expression, mRNA was measured using qRT-PCR in control and VWF-deficient cells after treatment with control or FOXO1 siRNA sequences for 48 hours. Inhibition of FOXO1 in control and VWF-deficient cells significantly reduced CXCR4 expression (figure 4.23A). Conversely, BMPR4 (figure 4.23B) and Cyclin G2 (figure 4.23C) expression were increased in control cells after inhibition of FOXO1, but unchanged in VWF-deficient cells. Together, these data demonstrate that enhanced FOXO1 activity is also responsible for increased CXCR4 expression in VWF-deficient cells. The results show that inhibition of VWF expression may have a broad effect on FOXO-1 dependent targets. The regulation of all of these genes of course depends on multiple TF; thus it is possible that other pathways controlling ELK3 and eNOS may be affected by VWF deficiency.

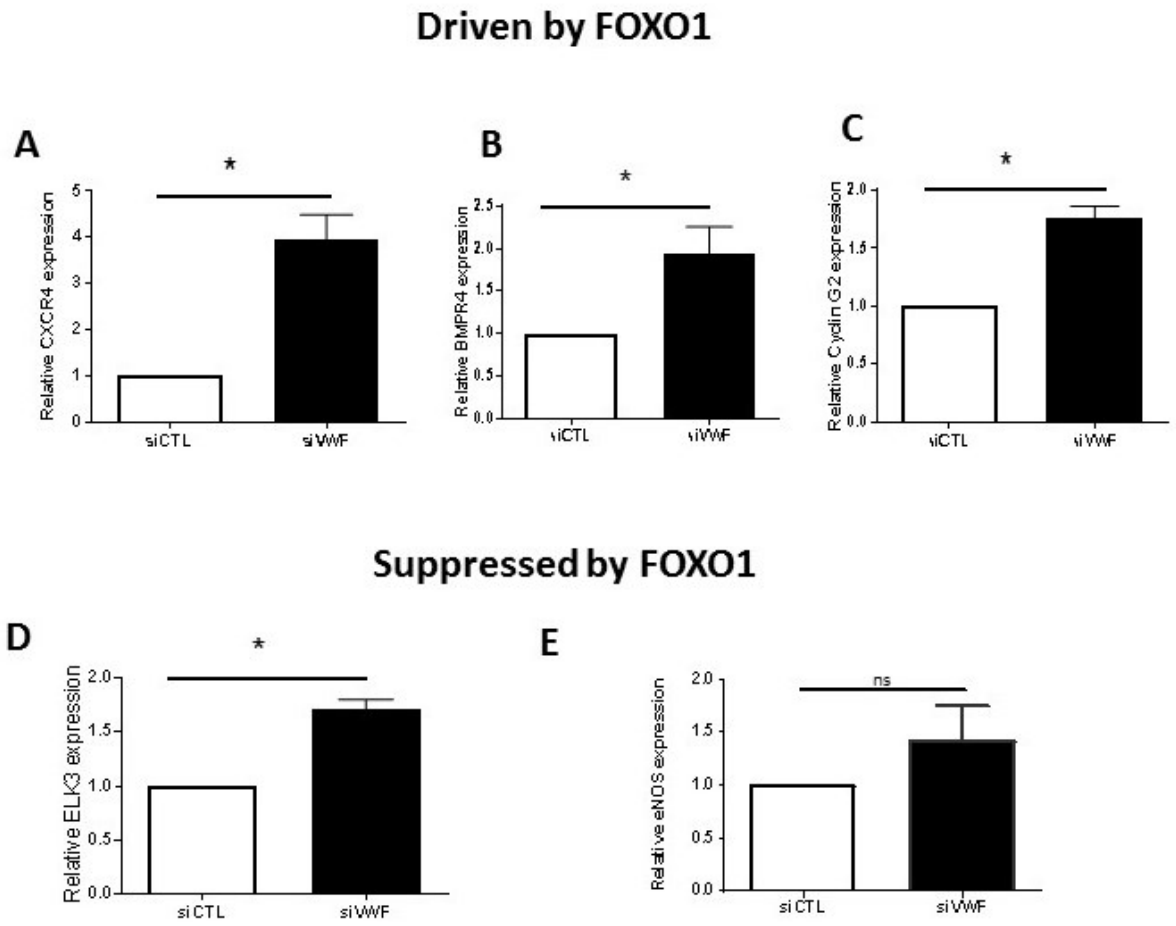

Figure 4.22 Screen of FOXO1 targets involved in Angiogenesis and EC function in VWF-deficient cells. HUVEC were transfected with siCTL or siVWF for 48 hours. RNA was collected and expression of CXCR4 (A), BMP4 (B), Cyclin G2 (C), ELK3 (D) and eNOS (E) was measured by qRT-PCR normalized to GAPDH and expressed relative to siCTL. Data presented as mean ± SEM of n=3-4. Statistical significance determined by student's t-test. * p≤0.05

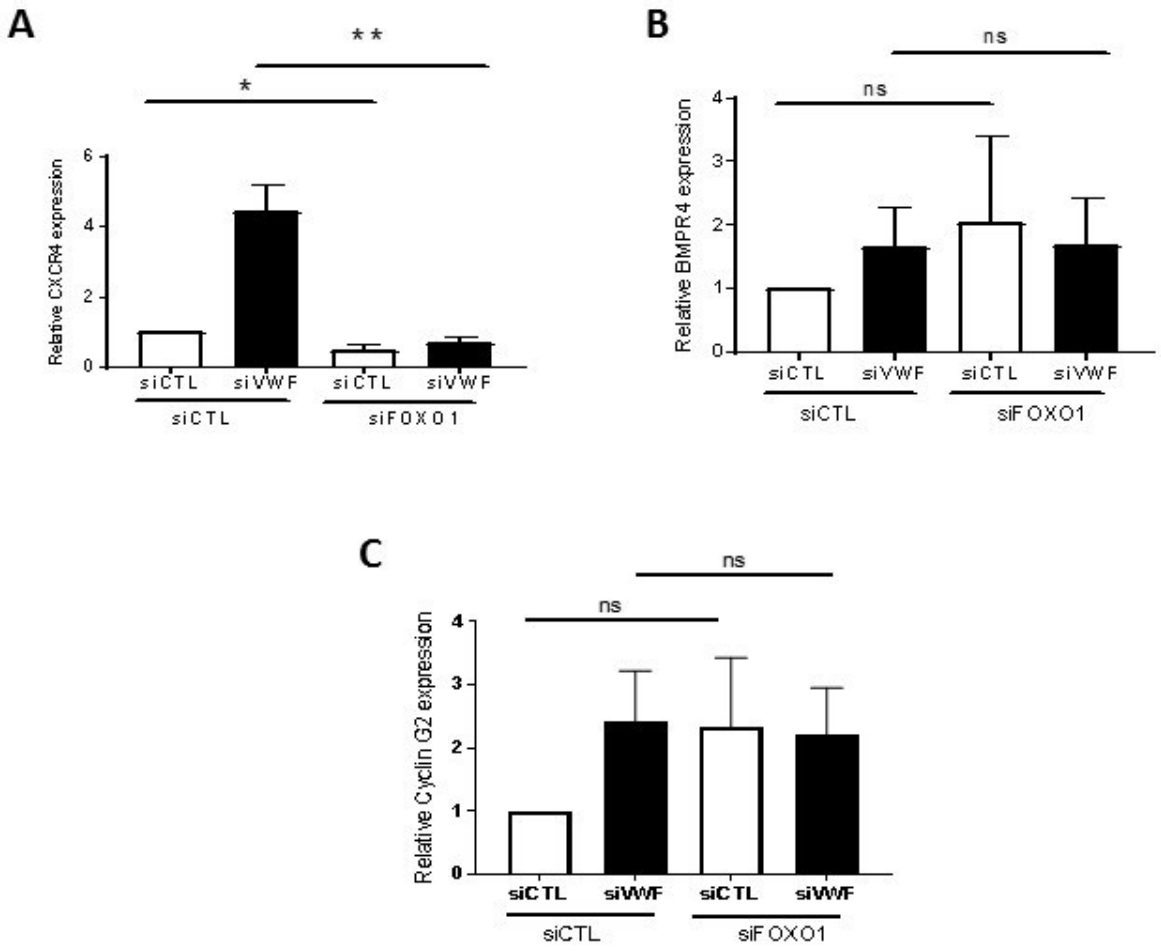

Figure 4.23 The effect of enhanced FOXO1 activity on drive targets of FOXO1 in VWF-deficient cells. HUVEC were co-transfected with control and VWF siRNA sequences for 48 hours. (A) CXCR4, (B) BMPR4 and (C) Cyclin G2 expression was measured by qRT-PCR. Data was normalised to GAPDH. Values were expressed relative to siCTL treated cells. Data presented as mean ± SEM of n =3. Statistical significance determined by student's t-test. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$.

4.1.13.2 Inhibition of VWF expression controls cell size and mTORC1 signalling endothelial cells

Enhanced FOXO1 activity an increases endothelial cell size (Dharaneeswaran et al., 2014). To test whether VWF-deficient cells showed increased cell size, VWF expression was inhibited using siRNA for 48 hours. Mean cell size was determined by a Cellometer® counter. Inhibition of VWF expression in ECs resulted in a significant increase in cell size using two different siRNA sequences to VWF for 48 hours (figure 4.23A) and (figure 4.23B). Given the role of FOXO1 in regulating cell size, we speculated that enhanced FOXO1 activity in VWF-deficient cells could be linked to increased cell size.

Cell size is controlled primarily through regulation of mammalian target of rapamycin complex 1 (mTORC1) (Fingar et al., 2002). Moreover, Akt has been shown to enhance mTORC1 signalling when FOXO1 activity was increased (Dharaneeswaran et al., 2014) (illustrated in figure 4.24C). To determine the effect on mTORC1 signalling in VWF-deficient cells, immunoblotting was performed for downstream targets phosphorylated S6 ribosomal subunit (S6) and total S6 after siRNA treatment for 48 hours. Inhibition of VWF expression for 48 hours caused a significant decrease in pS6 compared to controls (figure 4.24D). This suggest that mTORC1 signalling may be decreased in VWF-deficient cells, despite the enhanced cell size. This suggests that inhibition of VWF expression causes increase cell size independent of mTORC1 signalling. Further experiments will be required to investigate this pathways.

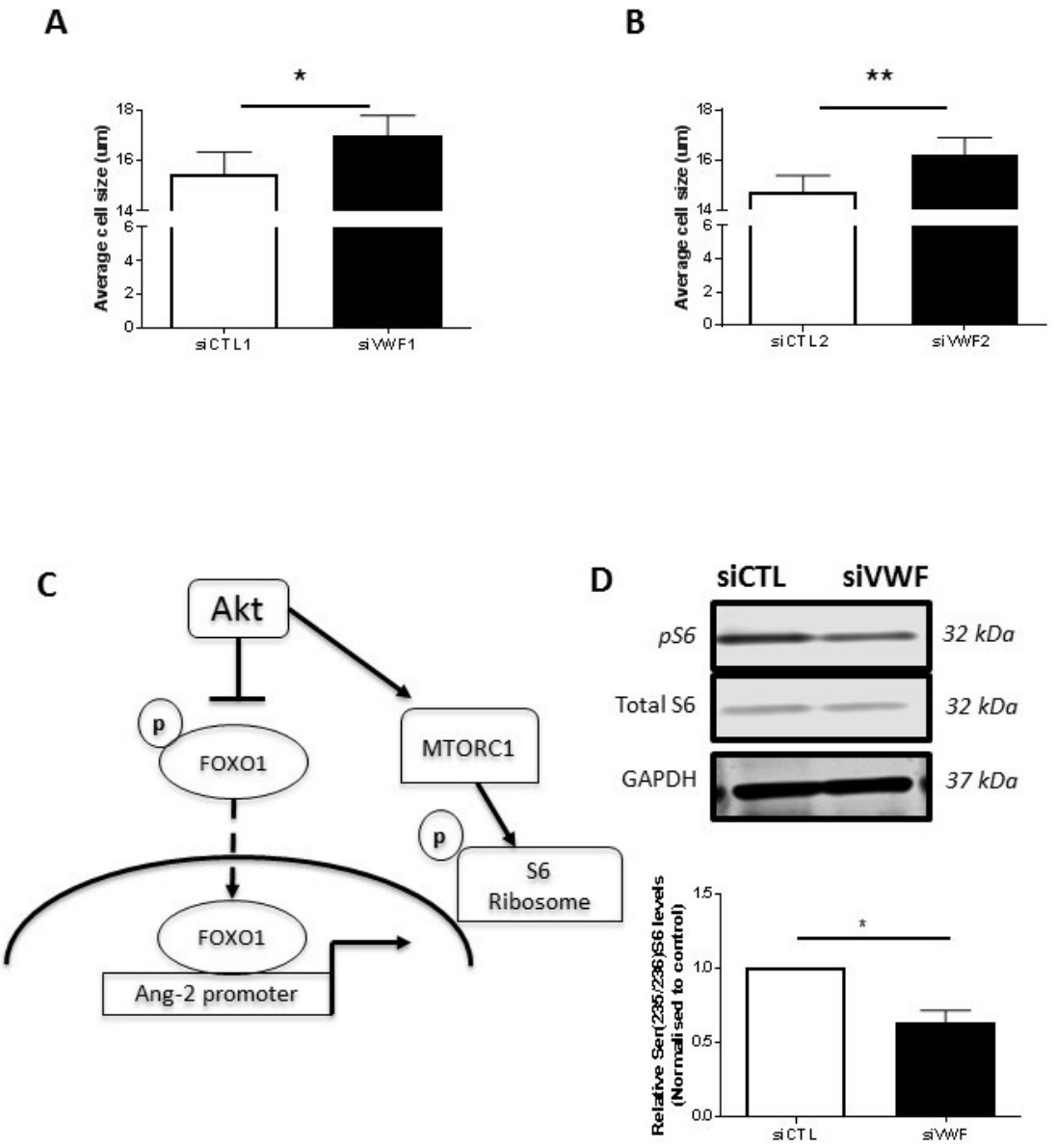

Figure 4.24 Enhanced cell size in VWF-deficient cells associated with decrease mTORC1 signaling. (A) Cell size in siCTL1 or siVWF1 transfected HUVEC and (B) siCTL2 or siVWF2 transfected HUVEC for 48 hours (C) Schematic representation of Akt signaling via FOXO1 and mTORC1. HUVEC were transfected with siRNA to VWF or siRNA control for 48 hours. (D) Representative Immunoblot of pS6, Total S6 and GAPDH. pS6 expression was quantified normalised to total S6 and expressed relative to siCTL. Data presented as mean ± SEM of n =3. Statistical significance determined by student's t-test. * p≤0.05, ** p≤0.01

4.1.14 High concentration Atorvastatin reduces Ang-2 mRNA levels in VWF-deficient cells

A recent report suggests that statins can induce nuclear exclusion of FOXO1 in endothelial cells and reduce Ang-2 synthesis (Ghosh et al., 2015). Statins have been reported to have biphasic effects during angiogenesis; inducing a pro-angiogenic response at low nM concentrations, while inhibiting angiogenesis at low μM concentrations (Weis et al., 2002). We therefore set out to investigate whether statins could reduce the VWF-dependent increase in proliferation, and if this was the case, then determine whether the effect was dependent on the ability of statins to control Ang-2 synthesis. Atorvastatin (ATV) treatment of control and VWF-deficient HUVEC at 0.01, 0.10 and 1 μM for 72 hours did not significantly reduce EC proliferation (figure 4.25A), in line with previous evidence (Dulak et al., 2005). However, treatment with higher dose of ATV, 5 μM for 72 hours, significantly reduced the VWF-dependent increase in proliferation compared to controls. To investigate whether this concentration could significantly reduce Ang-2 mRNA levels in VWF-deficient cells, HUVEC were treated with siRNA to VWF or control then ATV 5 μM for 24 hours. Ang-2 mRNA levels were significantly reduced in control and VWF-deficient cells in the presence of ATV, compared to controls (figure 4.25B). 5 μM ATV for 24 hours did not cause obvious changes change in cell morphology or cell death (appendix 6), in line with previously reported data(Feng et al., 2004). To determine whether the reduction in Ang-2 mRNA was due to the effect of the statin on FOXO1, immunoblotting was performed on cells treated with siRNA control or VWF sequences for 24 hours the Atorvastatin at 5 μM for a further 24 hours. As expected, inhibition of VWF expression in EC caused decrease in FOXO1 phosphorylation. Surprisingly, treatment of control HUVEC with 5 μM ATV significantly reduced FOXO1 phosphorylation. No significant change in FOXO1 phosphorylation was observed in VWF-deficient cells (figure 4.25C). These data show that Atorvastatin at high concentration can significantly reduce the VWF-dependent increase in proliferation. Additionally, it suggests that Ang-2 may be involved in this pathway and implicates FOXO1 regulation in the control of Ang-2 in ECs.

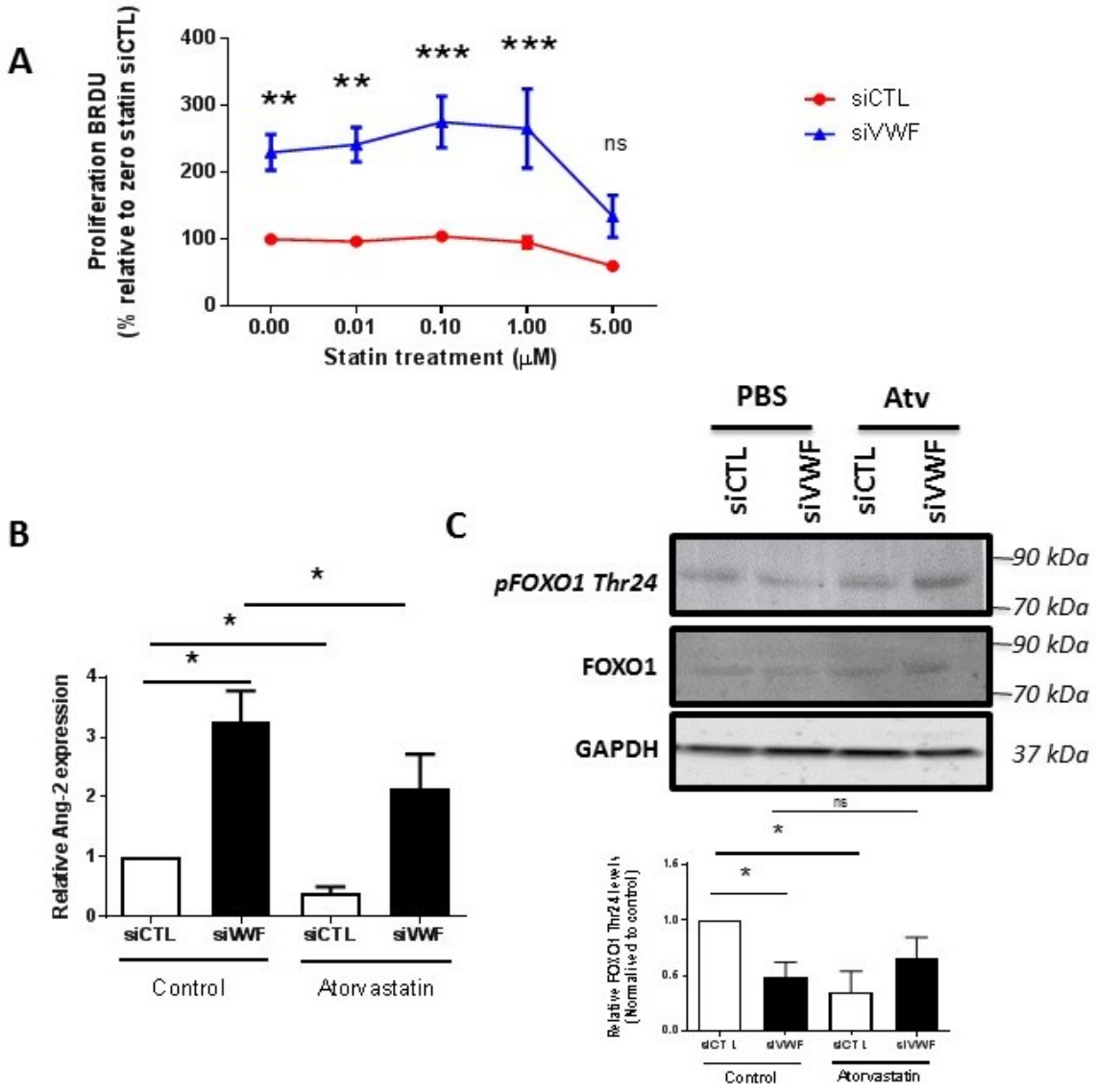

Figure 4.25 Pharmacological activation of Akt reduces Ang-2 levels in VWF-deficient cells. (A) HUVEC were treated with control or VWF siRNA sequences for 24 hrs then reseeded to 96-well plates in the presence of Atorvastatin at 0.01, 0.10, 1 or 5 µM. BrDU incorporation assay was performed after 72 hours. (B) Ang-2 expression was measured by qRT-PCR in control or VWF siRNA treated HUVEC after 48 hours. Cells were treated with ATV at 5 µM 24 hours before end of experiment (Experiment performed by Flavia Ribezzo). (C) Representative immunoblot and quantification of PFOXO1 Thr-24, total FOXO1 and GAPH after inhibition of VWF expression in HUVEC using siRNA for 24 hours then treatment with ATV at 5 µM for a further 24 hrs. Data presented as mean ± SEM of n =3. Statistical significance determined by student's t-test or two way ANOVA with Sidak post hoc test. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.15 Enhanced FOXO1 mRNA levels in VWF-deficient mouse heart but not lungs

The data described so far show that FOXO1 activity is increased in VWF-deficient HUVEC. As mentioned section 4.1.6, FOXO1 is able to increases FOXO1 mRNA expression in a positive feedback loop, both *in vitro* and *in vivo* (Dharaneeswaran et al., 2014, Essaghir et al., 2009). Moreover, i*n vitro*, inhibition of VWF expression in EC increases FOXO1 mRNA levels (figure 4.20A). Given that enhanced Ang-2 mRNA levels were found in the heart (but not the lungs) of VWF-deficient mice, we next investigated the whether the mRNA levels of FOXO1 were also elevated in the VWF mouse heart. Heart and lung samples were collected from mice aged between 8-12 weeks and processed for RNA. FOXO1 mRNA levels were increased in the VWF-deficient mouse, selectively in the heart (figure 4.26A) and not the lungs (figure 4.26B), in line with Ang-2 expression. These data show that VWF regulates FOXO1 activity in vivo and that like Ang-2, FOXO1 regulation by VWF is tissue specific.

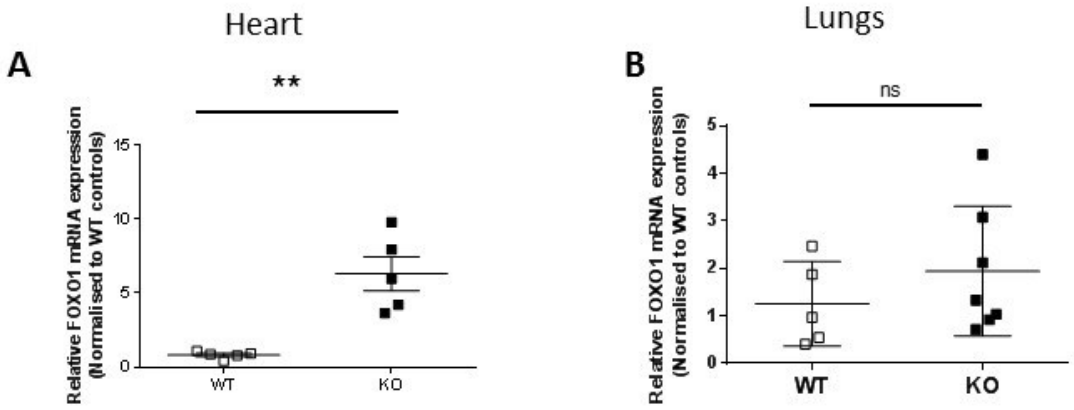

Figure 4.26 Enhanced FOXO1 expression in VWF-deficient mouse tissue. Whole tissue lysates were collected from 5 WT and 6 VWF-deficient mice. Ang-2 expression in Heart (A) and Lungs (B) were measured by qRT-PCR normalized to 18S. Data presented as mean ± SEM. Statistical significance determined by student's t-test. ns $p>0.05$, ** $p\leq0.01$

4.3 Discussion and Future Work

VWF regulates gene synthesis in endothelial cells

In chapter 3 of this thesis inhibition of VWF expression in ECs was found to increase Ang-2 mRNA levels. In the current chapter, we show that inhibition of VWF expression did not regulate Ang-2 mRNA stability in EC, suggesting VWF regulates the transcriptional activity of Ang-2. In line with this hypothesis, inhibition of VWF expression in EC induced transactivation of a region of Ang-2 promoter 4427 upstream and 476 downstream of the TSS (reported in Hegen et al, 2004). In this study, Hegen and colleagues demonstrated that the region -4427/-2270 contains a repressive element, as enhanced luciferase activity was observed after deletion of this fragment of the promoter. In this study, they demonstrated that the region -2004 to + 476 bp from the TSS could respond to the growth factors VEGF and bFGF and more specifically -109 to +476 contained an ETS binding site responsible for this response. Given the findings of our study, it is tempting to speculate that VWF regulates pathways which suppress Ang-2 expression. To our knowledge, little data exists on the TF responsible for Ang-2 repression in EC. Bioinformatic analysis of this -4427 to -2004 region would provide novel insight into which TFs VWF may act through to suppress Ang-2 expression.

Since our observations of enhanced Ang-2 mRNA levels occurred in VWF-deficient EC under quiescent conditions, we tested whether Ang-2 mRNA levels are augmented following EC activation in VWF-deficient mouse. We used a potent activator of Ang-2 synthesis, LPS, to induce EC activation (Menden et al., 2015, Mofarrahi et al., 2008, Zeng et al., 2016, Ziegler et al., 2013). We found, in the lungs of WT and VWF-deficient mice, a similar Ang-2 upregulation in response to LPS. We have not previously observed increase Ang-2 mRNA levels in the lungs of the VWF-deficient mice at baseline and in chapter 3 we proposed that under inflammatory challenge this may be observed. Given our findings above, this argued against this hypothesis showing that even in the absence of VWF, LPS was not able to augment Ang-2 mRNA levels over WT mice. Additionally, in the heart of the VWF-deficient mouse where we found enhanced Ang-2 mRNA levels at baseline, LPS did not augment the expression of Ang-2 above baseline levels. Since LPS did not augment Ang-2 mRNA levels in WT mice, it is possible any effects in the VWF-deficient mice may be missed in these conditions. To rule out whether our

effects on Ang-2 expression using LPS was a time dependent factor, we utilised an *in vitro* system and found in line with our findings in the heart that LPS did not augment the expression of Ang-2 in the absence of VWF. Remarkably, the fold change increase in Ang-2 mRNA induced by LPS was similar to that seen with siRNA to VWF; however the significance of this observation are unclear.

Since a number of signalling pathways could be involved in the VWF-dependent increase in Ang-2, we investigated Ang-2 mRNA levels in the presence of another potent inducer of Ang-2. In these experiments, we found that VEGF was able to induce the same degree of Ang-2 expression in both control and VWF-deficient cells. Interestingly, since serum deprivation normalised the levels of Ang-2 in VWF-deficient cells compared to controls (figure 4.4B). These experiments suggests an effect of GF on the VWF-dependent regulation of Ang-2, however further studies are required to confirm this hypothesis and identify the specific mechanism.

To investigate the pathways leading to increased Ang-2 expression in VWF-deficient cells a phospho-kinase array analysis was performed in the lab by Dr. Richard Starke. In the absence of VWF expression, increased ERK phosphorylation was found (appendix 3). These studies were validated using immunoblotting demonstrating that inhibition of VWF expression in ECs causes increased ERK1/2 phosphorylation (Starke and Randi, unpublished). Additionally, the VWF-dependent increase in angiogenesis has been shown to be VEGFR2 dependent (Starke et al., 2011). VEGF signalling is a potent inducer of Ang-2 mRNA levels (Hegen et al., 2004, Mandriota and Pepper, 1998). We tested whether the VWF-dependent increase in Ang-2 mRNA was VEGFR2 dependent. We found that inhibition of VEGFR2 signalling using the small molecular inhibitor SU4312 did not reduce the VWF-dependent increase in Ang-2 mRNA levels in normal growth conditions *in vitro*. However inhibition of VEGFR2 signalling did normalise ERK1/2 phosphorylation in VWF-deficient cells compared to controls. To specifically determine whether enhanced Ang-2 mRNA levels were due to VEGFR2 signalling, we addressed the question using specifically in the presence of VEGF. In these studies, we found that inhibition of VEGFR2 reduced VEGF dependent increase in Ang-2 mRNA in control but not VWF-deficient cells. More intriguingly, the increased ERK signalling observed in VWF-deficient cells was normalised in the presence of SU4312. This suggested that enhanced ERK

signalling in VWF-deficient cells is VEGFR2 dependent. While siRNA to ERK studies need to be performed, we believe this shows that increase VEGFR2-ERK signalling was not responsible for the increased Ang-2 mRNA levels in VWF-deficient cells.

Decreased expression of integrin β3 has been shown to cause enhanced VEGFR2 signalling leading to VEGF dependent angiogenesis (Reynolds et al., 2009, Reynolds et al., 2004, Reynolds et al., 2002). This may be due to the ability of the integrin αvβ3 limits the interaction between VEGFR2 and its co-receptor NRP-1 (Robinson et al., 2009). Recent data from the Augustin lab demonstrated that NRP-1 can signal independent of VEGFR2 activation to increase vascular permeability (Roth et al., 2016). While the exact signalling pathway remains unknown, activation of NRP-1 did not activate ERK phosphorylation which is activated by VEGF (Olsson et al., 2006). In our study we find that inhibition of VEGFR2 reduced ERK phosphorylation in the absence of VWF expression, however, Ang-2 mRNA levels remained increase. While NRP-1 siRNA studies are needed to confirm its involvement in the VWF-dependent increase in Ang-2, given its potent role in permeability, it is tempting to speculate a role for NRP-1 in the VWF dependent control of Ang-2 mRNA. Collectively the results described demonstrate that inhibition of VWF expression in EC induces the transcription of Ang-2.

VWF regulates β3 integrin signalling via FAK and Akt

Previous work from our lab has shown that inhibition of VWF expression in ECs causes decreased expression of integrin β3 as well as increased internalisation (Starke et al., 2011). In the current chapter we show that inhibition of VWF expression in ECs decreased the phosphorylation of FAK Y397 and Akt S473. Given that integrin activation predominately involves tyrosine phosphorylation at 397 residue of FAK (Parsons, 2003, Mitra et al., 2005), these data point to a decrease in integrin signalling after inhibition of VWF. To understand whether extracellular VWF could rescue β3 integrin expression, control and VWF-deficient cells were adhered to two immobilised forms of plasma derived VWF. The in-house purified VWF was purified from Haemate P, a plasma-derived production containing VWF and coagulation FVIII (Groner, 2008), whereas commercial VWF used in this case was a higher

purity plasma-derived VWF, directly supplied by the manufacturer covered by MTA. Immobilised VWF rescued the levels of integrin β3 in VWF-deficient cells using hVWF but not cVWF. While both forms of VWF originate from plasma derived sources, the difference in purity may explain their ability to regulate β3 expression, however this hypothesis remains to be explored.

While the effect of immobilised VWF on β3 expression in this study was clear, the effects on Ang-2 transcription are less clear. In one analysis, VWF-deficient HUVEC adhesion to cVWF appeared to have caused a normalisation of Ang-2 mRNA levels. On the other hand, adhesion to cVWF appeared to significantly increase Ang-2 mRNA levels in control cells. This could explain why the difference between control and VWF-deficient cells on immobilised cVWF was no longer significant. It has been reported that extracellular VWF can activate ERK1/2 in HUVEC (Potapova et al., 2010); this pathway could drive Ang-2 expression in EC via activation of the transcription factor ETS-1 (Hegen et al., 2004, Hasegawa et al., 2004), . However, we found that stimulation of HUVEC with VWF in the supernatant did not increase Ang-2 mRNA levels. This suggested that regulation of Ang-2 expression, at least in control cells, only occurred when VWF was immobilised. Follow up studies using VWF both immobilised and in supernatants, focusing on analysing both Akt-FOXO1 axis and ERK-ETS-1 axis will define which pathway are activated by VWF interaction with EC.

Adhesion of control and VWF-deficient cells on immobilised VWF normalised FAK phosphorylation. VWF binds to the integrin αvβ3 on EC (Cheresh and Spiro, 1987, Dejana et al., 1989, Huang et al., 2009). In the present study we have not confirmed whether the effect of immobilised VWF on HUVEC was integrin dependent. Confirmation using integrin blocking antibodies or more specifically blockade of VWF-integrin interaction using RGD mutants may address this specific question. In addition, these future studies are important clarify the interpretation of whether VWF does regulate Ang-2 via signalling via integrin αvβ3.

To confirm the involvement of the FAK-Akt axis in Ang-2 expression, we utilised a constitutively active Akt adenovirus to rescue Ang-2 expression. We confirm that the increase Ang-2 mRNA levels were significantly reduced in the presence of CA-Akt. Our data

demonstrates that Akt is a critical regulator of Ang-2 expression in VWF-deficient cells. Previous studies in ECs, identified that Ang-2 expression was significantly reduced by activation of PI3K-Akt pathway (Daly et al., 2004). Moreover, Daly and colleagues showed that the suppressive effects of Akt on Ang-2 were mediated by inhibition of FOXO1 activity. This highlighted that the Akt-FOXO1 axis may be perturbed in VWF-deficient cells. Together this demonstrates that the enhanced transcription of Ang-2 in VWF-deficient cells was dependent on Akt mediated signalling pathway.

VWF regulates the phosphorylation and activity of FOXO1 in endothelial cells

Akt phosphorylates FOXO1 inducing nuclear exclusion. In this study, we show via immunoblotting that FOXO1 phosphorylation is decreased in VWF-deficient cells. Furthermore, we show that in VWF-deficient cells this was associated with enhanced nuclear localisation of FOXO1. Enhanced FOXO1 nuclear localisation is associated with enhanced FOXO1 activity. In this study, similar to what has been described for Ang-2, we find a heterogeneous expression of FOXO1 in EC in culture. FOXO1 cytoplasmic localisation was strikingly reduced in VWF-deficient cells compared to controls. While most studies have focused on the activity of nuclear FOXO1, it is worth noting that FOXO1 in cytoplasm has been shown to regulate cellular processes such as autophagy (Zhao et al., 2010). In this study, the authors demonstrated that FOXO1 acetylation in the cytoplasm increased autophagy and played an important role in reducing tumour size *in vivo*. Forced expression of FOXO1 in the cytoplasmic caused increase protein degradation and the authors proposed serves as a vital response to cell stress (Zhao et al., 2010). Given these findings, this may suggest that inhibition of VWF could also regulate other processes such as autophagy in EC, however these studies remain to be explored.

Microarray analysis have identified FOXO1 targets in ECs using siRNA or expression of mutant active forms of FOXO1 (Daly et al., 2004, Dharaneeswaran et al., 2014, Potente et al., 2005). In this study, we selected 3 activated 2 repressed targets of FOXO1. We found that CXCR4, BMPR4 and Cyclin G2 were increased in VWF-deficient ECs, in line with FOXO1 activation. Surprisingly, we found that FOXO-1-repressed targets Elk-3 and eNOS were increased or unaffected, respectively, by VWF inhibition. Given these findings, we next investigated

whether the upregulation of the selected FOXO1-driven targets in VWF-deficient cells was dependent on FOXO1 and found that only CXCR4 was significantly decreased by FOXO1 siRNA. Of note, in these experiments we were only able to achieve a 50% reduction in FOXO1 expression in control and around 60% in VWF-deficient cells. This may skew some of the gene regulation data observed. In addition, studies from the Aird lab demonstrated that siRNA inhibition of FOXO1 was not sufficient to increase eNOS expression (Dharaneeswaran et al., 2014). This study identified that longer inhibition times using short hairpin RNA (shRNA) were needed to observe an effect on some FOXO1 targets. In my study, we find enhanced FOXO1 activity in VWF-deficient cells after 48 hours transfection with siRNA. Follow up studies examining longer time points may be required to determine the full extent of FOXO1 activity in the absence of VWF. Moreover, FOXO family members have been shown to have some degree of overlap. Particularly, FOXO3 and FOXO1 are both expressed in ECs, and have closely related gene targets (Potente et al., 2005). Further studies using siRNA to FOXO3 may be required to disassociate FOXO1 and FOXO3 dependent effects.

A recent study from the Aird group showed that ECs with enhanced FOXO1 activity had increased cell size (Dharaneeswaran et al., 2014). Since cell size is predominately controlled by mTOR, they showed increased mTORC1 activity due to increased Akt. Since Akt suppresses FOXO1 activity, the Authors proposed a feedback mechanism through which FOXO1 regulates Akt activity and mTORC1 activity (Dharaneeswaran et al., 2014). In the present chapter we show that VWF-deficient cells have decreased Akt phosphorylation, enhanced FOXO1 activity and increase in cell size. Unexpectedly, we found that VWF-deficient cells have decreased mTORC1 activity. It is possible that this decrease in activity is due to the Akt acting as an activator of mTORC1(Huang and Manning, 2009). Follow up studies using siRNA to FOXO1 are required to determine whether enhanced cell size in VWF-deficient cells is indeed a FOXO1 dependent phenomenon.

In vitro, the absence of VWF expression caused enhanced FOXO1 mRNA levels. Since enhanced FOXO1 activity causes an increase in FOXO1 mRNA levels (Essaghir et al., 2009) this provided another indication of enhanced FOXO1 activity in the absence of VWF. In Chapter 3 we showed increased Ang-2 mRNA in the hearts of VWF-deficient mice but not lungs. To determine if there was any evidence for increased FOXO1 activity *in vivo*, we measured

FOXO1 mRNA in hearts and lungs from VWF-deficient mice. In line with our findings of Ang-2 mRNA levels, FOXO1 mRNA was also increased in the heart but not the lungs of VWF-deficient mice compared to controls. To confirm that enhanced FOXO1 activity was responsible for increase Ang-2 expression in VWF-deficient cells we used siRNA to FOXO1. Here we demonstrated both a decrease in Ang-2 mRNA and protein release after inhibition of FOXO1 in VWF-deficient cells. In line with previous studies (Weis et al., 2002, Dulak et al., 2005), we also show that statins, in this case atorvastatin, has the ability to significantly reduce the VWF-dependent increase in proliferation. Two studies indicated the potential of statins to induce nuclear exclusion of FOXO1 thereby reducing activity and Ang-2 expression (Lee et al., 2013, Ghosh et al., 2015). We found that despite atorvastatin treatment of VWF-deficient HUVEC decreasing Ang-2 expression, FOXO1 phosphorylation was unchanged between control and VWF-deficient cells. Further studies in different models will be required to validate these findings.

We believe our findings may have important implications for patients with VWD-angiodysplasia. A recent study showed enhanced Ang-2 expression in patients with angiodysplasia (Holleran et al., 2015). In the current study, we demonstrate that activation of Akt or inhibition of FOXO1 was able to significantly reduce the VWF-dependent increase in Ang-2 expression. We show that, at least *in vitro*, atorvastatin could reduce Ang-2 expression in EC. It is worth noting that circulating levels of atorvastatin in healthy individuals on 10 - 80 mg daily is maintained around 0.002-0.2 µM (Cilla et al., 1996, Weis et al., 2002). In these studies, 10-80 mg of atorvastatin was well tolerated and no overt negative effects were observed. Moreover, in the study carried out by Weis et al., concentrations of statins around 0.05 µM started to inhibit angiogenesis *in vitro* (Weis et al., 2002). In our studies, only high concentration atorvastatin (1 µM and 5 µM) reduced both VWF-dependent increase in proliferation and Ang-2 expression. More recently it has been proposed that different statins, in this case pravastatin versus atorvastatin can have differential effects in angiogenesis (Lorenz et al., 2016). In particular while atorvastatin increased proliferation, pravastatin had no effect. The authors suggested that the hydrophilic/hydrophobic nature of the statin may be important in regulating activity.

Two reports have shown that statin therapy was able to correct GI bleeding that was refractory to VWF replacement in a severe type 1 and in a type 2A VWD patient, (Alikhan and Keeling, 2010, Sohal and Laffan, 2008). However, anecdotal evidence indicates that not all VWD patients respond to statins. A more recent report from the prophylaxis network demonstrated that GI bleeding around 50% of VWD patients do not respond to VWF replacement therapy (Abshire et al., 2013). This suggests that statin therapy may present as a novel therapeutic intervention in GI bleeding in these for some patients. In addition, the Ang-2 pathways may be differentially regulated in different VWD patients, where VWF storage is disrupted. BOEC studies may help understand this and may inform the therapeutic choice of stains in future.

In summary, in this Chapter we have demonstrated that VWF suppresses Ang-2 expression in endothelial cells through an Akt/FOXO1 dependent pathway. We show that VWF controls the cellular localisation and activity of the transcription factor FOXO1. We provide preliminary data to suggest that that VWF controls β3 integrin signalling of via FAK/AKT/FOXO1. The functional consequence of enhance FOXO1-Ang-2 activity both *in vitro* and *in vivo* in VWF-deficient cells will be the focus on the following chapter.

5 Chapter Five: The role of Ang-2 in VWF dependent blood vessel formation and function

5.1 Introduction

5.1.1 Ang-2 signalling in blood vessel formation

Studies have shown that both Ang-1 and Ang-2 are able to potentiate VEGF-induced angiogenesis *in vivo* (Augustin et al., 2009). Skin specific overexpression of Ang-1 caused hypervascularisation (Suri et al., 1998). In contrast, overexpression of Ang-2 causes vascularisation in the presence of VEGF(Lobov et al., 2002, Oshima et al., 2004), but vascular regression in the absence of VEGF expression (Oshima et al., 2005). Functionally, Ang-1 protects the vasculature from the effect of permeability inducing agents (Kim et al., 2001, Thurston et al., 2000, Thurston et al., 1999). In with its proposed destabilisation function, over expression of Ang-2 in the adult vasculature induces vascular leakage and pericyte loss (Hammes et al., 2004, Park et al., 2014, Ziegler et al., 2013). These studies *in vivo* highlight the role of the angiopoietin signalling in regulating not only blood vessel formation but function.

Unravelling the complex nature of angiopoietin signalling, Daly and colleagues showed that in EC where Ang-2 expression was induced via activation of FOXO1, Tie-2 phosphorylation increased (Daly et al., 2006). A subsequent study showed that high concentrations or prolonged exposure of cells to Ang-2 was able to induce Tie-2 phosphorylation (Yuan et al., 2009), further demonstrating the agonist properties of Ang-2. These data suggests an agonist ability of Ang-2, however the exact nature of which remains unknown. More recent studies have shown that Tie-2 signalling may be affected depending on cellular environment. The classic agonist Ang-1 induces Tie-2 phosphorylation and receptor localisation to the endothelial cell-cell junctions (Saharinen et al., 2008, Fukuhara et al., 2008). This is only apparent in confluent cell monolayers as the same stimulation under sparse cellular conditions results in Tie-2 localisation to the matrix in regions distinct from focal contacts. Interestingly Ang-1 has the ability to bind to the extra cellular matrix via its linker domain which does not happen with Ang-2 (Xu and Yu, 2001). These data suggests that in the quiescent endothelium, Tie-2 may be important for maintain cellular junctions via bridging through Ang-1. Since Ang-2 can antagonise Ang-1 effects, it could be proposed that interruption of junctional integrity is at least once mechanism through which Ang-2 could disrupt junctional integrity.

A mechanism controlling Ang-2 signalling is via regulation of receptor levels; in the mouse angiogenesis retina model, Tie-2 expression is lower in tip cells (Felcht et al., 2012). Interestingly, Ang-2 expression in these cells is high suggesting an alternative signalling pathway. Ang-2 has been shown to bind to integrins such as $\alpha v\beta 3$, highly expressed in angiogenic EC (Hodivala-Dilke, 2008), and through this to elicit signalling via FAK S910, which cause EC destabilisation and promotes angiogenesis (Thomas et al., 2010). Additionally Hakanpaa and colleagues demonstrated that in Tie-2 negative cells, Ang-2 but not Ang-1 was able to activate integrin $\beta 1$ (Hakanpaa et al., 2015). Thus, integrin-dependent pathways can mediate Ang-2 signals in the absence of Tie-2.

5.1.2 VWF regulation of blood vessel formation and function

Work from our lab using both *in vitro* and *in vivo* models of angiogenesis and vascularisation demonstrated that VWF controls blood vessel formation using both *in vitro* and *in vivo* model of angiogenesis and vascularization (Starke et al., 2011). In these studies, however, the functionality of these vessels was not assessed. As described in chapter 1, a number of studies have investigated the role of VWF in permeability. In studies examining the permeability of the blood brain barrier (BBB), results from Noubade et al., showed enhanced BBB permeability in VWF-deficient mice compared to controls during experimental allergic encephalomyelitis (EAE) (Noubade et al., 2008). In contrast, a study from Suidan and colleagues showed no difference in BBB permeability at baseline in VWF-deficient mouse but decrease in BBB permeability when subjected to hypoxia and reoxygenation model (Suidan et al., 2013). In a model of experimental cerebral malaria (eCM), loss of VWF appeared to cause increase survival (O'Regan et al., 2016). In addition, BBB permeability while not significantly different in VWF-deficient and wild type mice at baseline, VWF-deficient mice had significantly less permeability during eCM. Examination of the skin permeability in two models of contact dermatitis, the VWF-deficient mouse had decreased permeability, compared to controls (Hillgruber et al., 2014). These studies suggests that at baseline, the VWF-deficient mouse does not appear to have gross defects in permeability. However, in response to challenge, different responses have been observed in the VWF-deficient mouse which may reflect the nature of the challenge.

5.1.3 Hypothesis and Aims

We have shown that inhibition of VWF expression in HUVEC *in vitro* causes enhanced release of Ang-2 (Starke et al., 2011). The work described in chapter 3 shows that VWF also controls Ang-2 synthesis in ECs. I therefore hypothesised that Ang-2 mediates the VWF-dependent effects on blood vessel formation and vascular function.

The aims of the work described in this chapter are to:

- Investigate whether VWF bound to Ang-2 affects its interaction with ECs
- Determine the role of VWF in regulating Tie-2 signalling in VWF-deficient cells
- Determine whether Ang-2 mediates the VWF dependent effects in blood vessel formation
- Determine if Ang-2 mediates the VWF-dependent effects on vascular function, focusing on permeability

5.2 Results

5.2.1 Investigating the VWF-Ang-2 interaction using HUVEC binding assay

5.2.1.1 Optimising HUVEC binding assay

As described in chapter 1, Ang-2 bound to VWF in its A1 domain does not affect its interaction with Tie-2 (McKinnon et al., Submitted). However, VWF can bind integrins (Cheresh and Spiro, 1987, Dejana et al., 1989, Huang et al., 2009), which have been proposed as an alternative receptor for Ang-2 in the absence of Tie-2 (Felcht et al., 2012). To test whether VWF-Ang-2 complex regulate Ang-2 interaction with ECs an *in vitro* model of HUVEC binding was utilised. Since VWF and Ang-2 are complexed together in circulation, we first tested whether in-house purified VWF (hVWF) contained Ang-2. Recombinant Ang-2 (rhAng-2) and hVWF were immobilised on 96 well plates along with BSA control. BSA blocking was performed to reduce non-specific binding to the exposed plate. An Ang-2 ELISA was performed, which revealed no detectable Ang-2 in wells coated with hVWF (figure 5.1A). To next optimise cell detection, HUVEC were stained with Vybrant dye ® and seeded at 10,000 (figure 5.1B), 36,000 (figure 5.1C) and 50,000 cells per well (figure 5.1D) on plates pre-coated with 1, 6 or 10 µg/ml rhAng-2. Non adherent cells were washed away after 45 mins and fluorescence measured as outlined in chapter 2. As shown in figure 5.1C, only 36,000 cells per well was able to differentiate BSA and rhAng-2 at two different concentrations. To confirm these findings, HUVEC were seeded at 36,000 cells per well on plates pre-coated with rhAng-2, VWF or gelatin. BSA was used as negative control. As shown in figure 5.1E, HUVEC showed increased binding to rhAng-2, gelatin and VWF compared to BSA. These data showed that 36,000 cells per well allowed a good assessment of HUVEC binding in these *in vitro* assays.

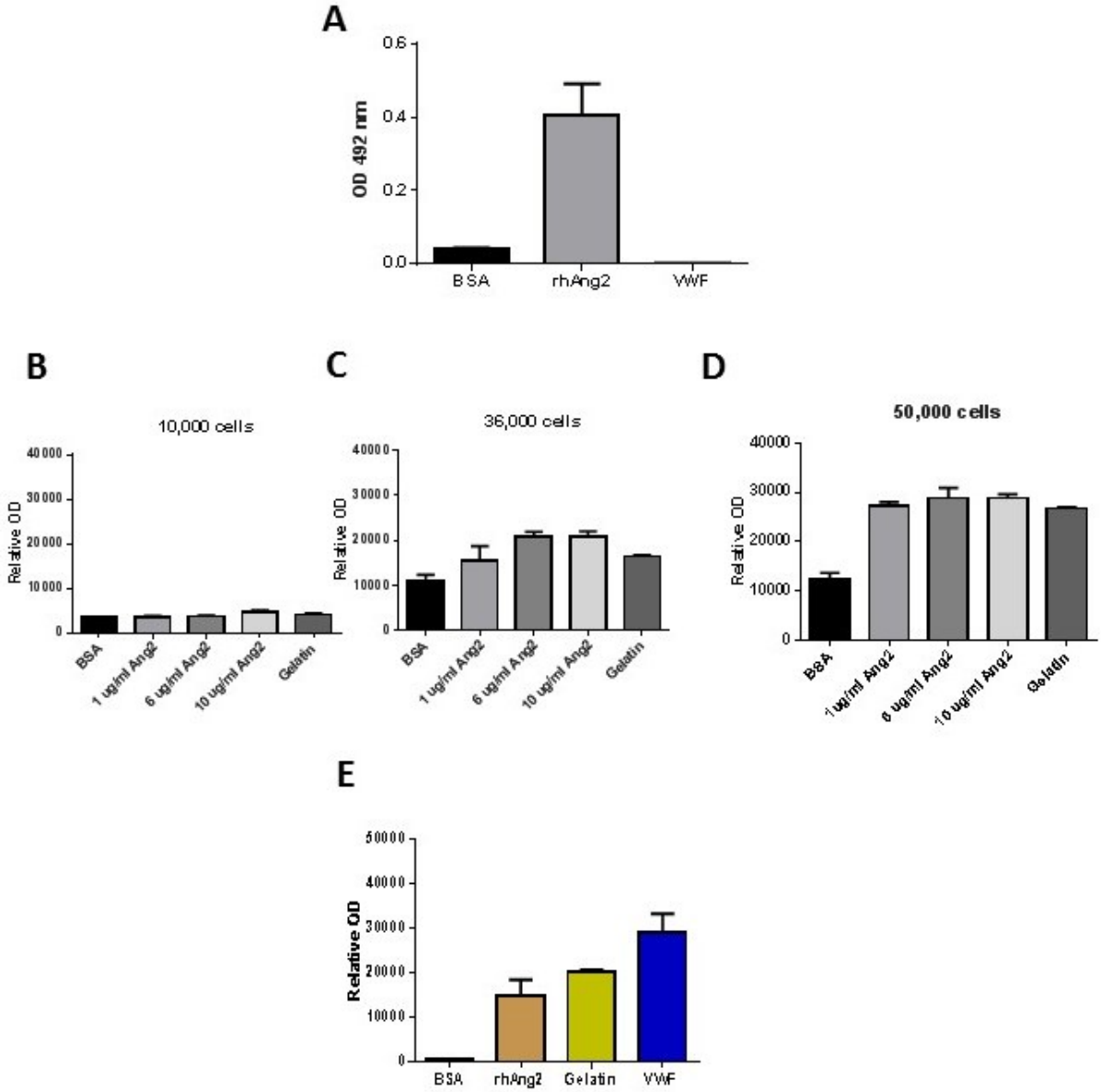

Figure 5.1 Optimization of VWF and Ang-2 binding assay. 96 well plates were coated with coating shown for 2 hours then blocked with BSA. HUVEC were then stained with Vybrant Dye and plated at cell concentration shown. After 1 hour OD was measured. (A) Ang-2 ELISA was performed on coated plates and expressed as raw OD. Cells plated on rhAng-2 at different concentrations at 10,000 cells per well (B), 36,000 cells per well (C) or 50,000 cells per well (D). Data shown as raw OD. (E) 30,000 cells were plated onto BSA, rhAng-2 (1µg/ml), 1% Gelatin or plasma derived VWF at 10 nM. Data expressed as raw OD. Data shown as mean ± SD of technical replicates n=1.

5.2.1.2 Ang-2 impairs VWF interaction with EC

Full length VWF had been shown to bind to EC via integrin $\alpha v\beta 3$ through its RGD site in the C terminal region (Cheresh and Spiro, 1987). Moreover, Ang-2 interacts with VWF in its A1 domain towards the N-terminal region (see figure 1.5). This would suggest that Ang-2 should not interfere with VWF interaction with HUVEC. To test this hypothesis, rhAng-2 or rhAng-2 bound to VWF were immobilised to 96 well plates. Background binding was determined with BSA coated well and fluorescence subtracted from all values. As shown in figure 5.2A, HUVEC showed increased binding to rhAng-2-hVWF complex compared to rhAng-2 alone. Wells coated with hVWF alone were used as control for VWF ability to bind ECs. Surprisingly these experiments showed that while HUVEC bound to rhAng-2-hVWF complex was increased over rhAng-2, this complex appeared to impede VWF binding to ECs. These data confirm that rhAng-2-hVWF complex interacts with HUVEC. In addition they reveal that while the rhAng-2-hVWF increases the ECs interaction compared to rhAng-2, it impairs VWF binding to ECs.

VWF binds integrins on ECs surface (Cheresh and Spiro, 1987). Ang-2 on the other hand binds Tie-2 as well as integrins (Felcht et al., 2012). In order to elucidate whether the Tie-2 receptor was involved in the Ang-2-VWF interaction with EC, Tie-2 expression was silenced using siRNA. In all experiments, Tie-2 siRNA caused a 90% reduction in Tie-2. After inhibition of Tie-2 expression for 48 hours HUVEC binding to Ang-2 showed a ≈40% reduction compared to control cells (Figure 5.2B), although not significant this was greater than the effect seen by Felcht and colleagues. In the presence of Tie-2 siRNA, the Ang-2-VWF complex showed reduced binding. To test whether this effect was due to VWF-Ang-2 interaction (which occurs predominantly via the VWF A1 domain) or through other regions of VWF (possibly the integrin-binding RGD sequence), the A1 domain fragment of VWF was used. The Ang-2-A1 domain complex sustained adhesion in a similar manner to Ang-2 alone. Surprisingly, the A1 domain itself appeared to sustain EC binding which suggests that this domain could contain a region which is able to engage with HUVEC. Given the variability in these experiments, no statistically significant results could be detected. Since Tie-2 and integrins share functional cross talk (Dalton et al., 2016), it is possible that silencing of Tie-2 could affect integrin expression, however further studies are needed to explore this hypothesis. Together these

data suggests that the VWF-Ang-2 complex can bind other receptors on HUVEC in the absence of Tie-2.

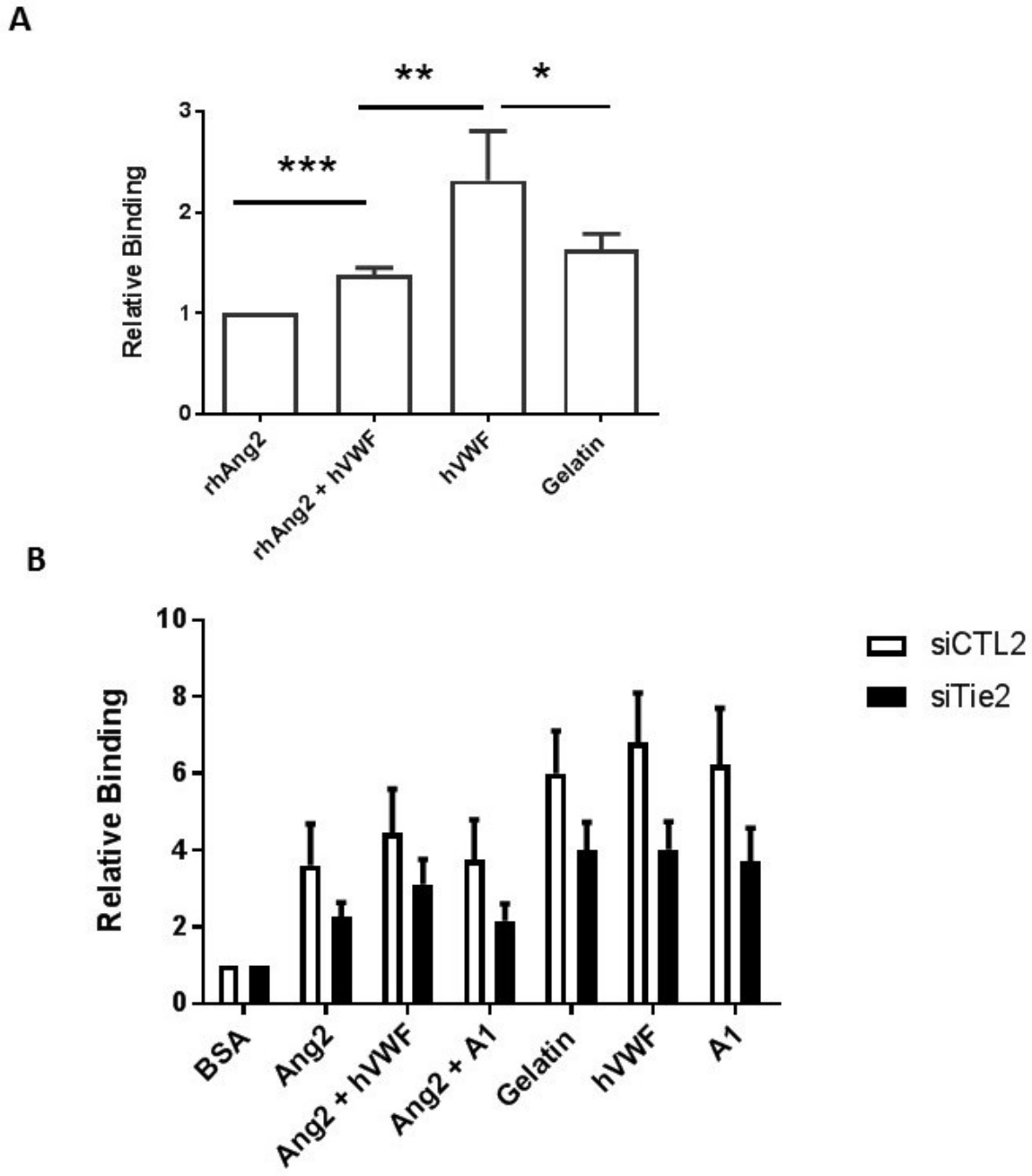

Figure 5.2 Differential binding of HUVEC to Ang-2-VWF complex is partly Tie 2 mediated. (A) 96 well plates were coated with coating shown for 2 hours then blocked with BSA. HUVEC were then stained with Vybrant Dye and plated. After 1 hour OD was measured. n=4. (B) HUVEC were treated with siCTL and siTIE2 for 47 hours then stained with Vybrant dye and reseeded into 96 well plates coated with matrices as indicated. After 1 hours OD was measured. n=3. Data shown as mean ± SEM. rhAng-2 (1µg/ml), 1% Gelatin or plasma derived VWF or A1 domain at 10 nM. Statistical significance determined by student's t-test. *p≤0.05 ** P ≤ 0.01, *** p ≤ 0.001

5.2.2 Tie-2 signalling in VWF-deficient cells

5.2.2.1 Inhibition of VWF expression does not affect protein levels of Tie-2

The presence of Tie-2 appears an important determinant of Ang-2 signalling in the endothelium (Felcht et al., 2012, Hakanpaa et al., 2015). To determine whether the absence of VWF affected the levels of Tie-2, VWF expression was inhibited for 48 hours using two different siRNA sequences. RNA was collected and analysed by qRT-PCR. Compared to control cells both VWF siRNA sequences showed Tie-2 mRNA levels (figure 5.3A and B). To determine whether VWF affected the protein expression of Tie-2, total cell lysates were collected from control and VWF siRNA treated HUVEC after 48 hours. Immunoblotting for Tie-2 and GAPDH revealed no significant difference between Tie-2 levels in control and VWF-deficient cells with siRNA sequence 1 (figure 5.3C) or siRNA sequence 2 (figure 5.3D). These data show that while inhibition of VWF expression caused increased Tie-2 mRNA levels, this was not translated into a difference in Tie-2 total protein levels.

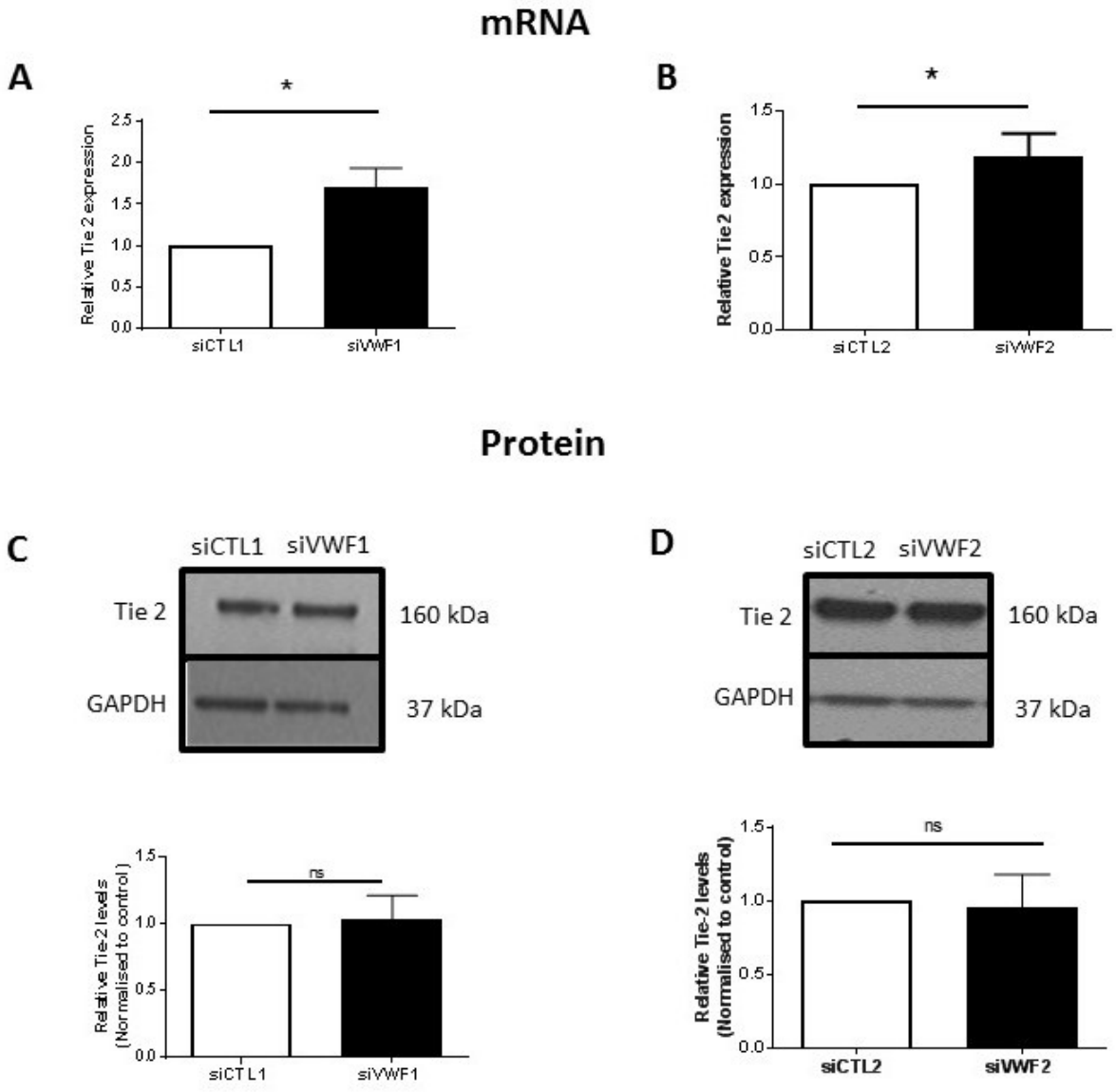

Figure 5.3 Enhanced Tie 2 mRNA but not protein in VWF-deficient HUVEC. HUVEC were transfected with either siRNA to VWF (siVWF) or corresponding siRNA control sequences (siCTL). After 48 hours Tie 2 expression (A -B) was measured by qRT-PCR. Data normalized to GAPDH and expressed relative to siCTL. (C-D) Representative immunoblot of Tie 2 and GAPDH in siCTL and siVWF treated HUVEC after 48 hours. Quantification of Tie 2 expression normalized to GAPDH and expressed relative to siCTL. Data shown as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$.

5.2.2.2 Optimisation of Tie-2 phosphorylation using confocal microscopy

To establish Tie-2 phosphorylation state in the absence of VWF, previous work in the lab used an immunoprecipitation approach; however, these experiments failed to obtain conclusive results (Elder JM and Randi AM unpublished). In addition, (Fukuhara et al., 2008, Saharinen et al., 2008) have shown that Ang-1 induces robust phosphorylation of Tie-2 at EC-EC junctions. To determine whether inhibition of VWF affects Tie-2 phosphorylation, we utilised immunofluorescence in control and VWF staining cells. To optimise staining, immunofluorescence was performed on HUVEC stimulated with cartilage oligomeric matrix protein (COMP)-Ang-1, a more stable and potent form of native Ang-1, to induce pTie-2 (Cho et al., 2004). In line with previous studies from both Fukuhara and Saharinen, we found that COMP-Ang-1 produced strong pTie-2 staining at regions of the cells similar to junctional staining (figure 5.4) (Fukuhara et al., 2008).

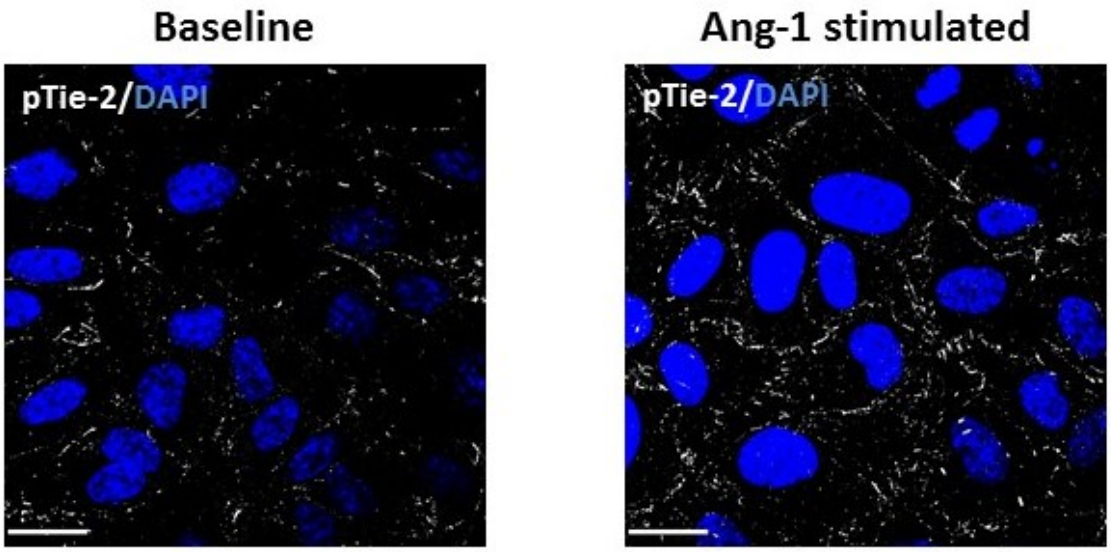

Figure 5.4 Optimization of Tie 2 staining in HUVEC. HUVEC were serum starved for 6 hours then treated with PBS control (left panel) or Ang-1, 250 ng/ml (right panel) for 6 hours. Cell were then stained for pTie -2 and DAPI for nuclei. Scale bar represents 20 µm.

5.2.2.3 Decreased Tie-2 phosphorylation in VWF-deficient cells

To determine the effect of inhibition of VWF on Tie-2 phosphorylation, HUVEC were treated with control or VWF siRNA for 48 hours. Cells were then fixed and immunofluorescence performed. Inhibition of VWF expression was confirmed by staining of separate coverslips from the same experiments for VWF and DAPI (figure 5.5A) due to species cross reactivity of VWF and pTie-2 antibodies. As shown in figure 5.5B, Tie-2 phosphorylation appeared at classical EC-EC contacts in control cells. After inhibition of VWF expression for 48 hours, Tie-2 phosphorylation was significantly reduced (quantified in figure 5.5C). Having shown that inhibition of VWF caused decrease in Tie-2 phosphorylation, we next wanted to confirm whether inhibition of VWF sequestered Tie-2 phosphorylation away from EC-EC junctions. To identify EC-EC contacts the junctional marker VE-Cadherin was used. HUVEC treated with control or VWF siRNA for 48 hours showed in line with our previous findings, decrease in Tie-2 phosphorylation. VE-Cadherin staining demonstrated that after inhibition of VWF, EC-EC junctions were largely intact. The majority of pTie-2 appeared to occur at the junctions with reduced expression but also in punctate clusters at regions of the cells not associated with these regions (Figure 5.6). Collectively these data demonstrated that inhibition of VWF expression significantly reduced Tie-2 phosphorylation at EC-EC junctions.

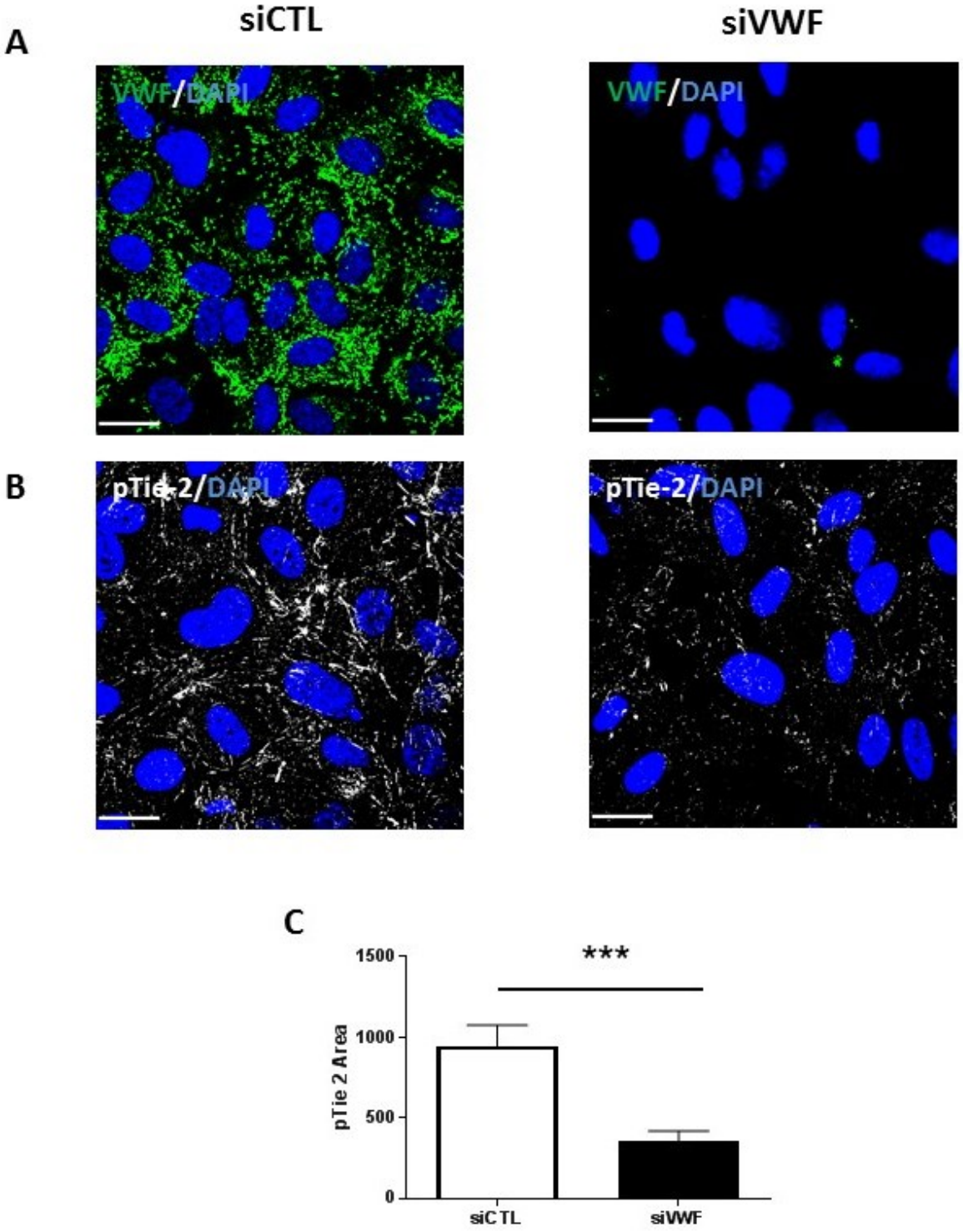

Figure 5.5 Less Tie 2 phosphorylation in VWF-deficient cells. HUVEC were transfected with siCTL (left panel) or siVWF (right panel) for 48 hours. (A) Representative images of cells stained for VWF (green) and DAPI (blue) (B) Representative images of cells stained for pTie -2 (white) and DAPI for nuclei (blue). Scale bar represents 20 µm. (C) Quantification of pTie 2 coverage in siCTL and siVWF treated cells of 3 x20 fields per condition. Data presented as mean ± SEM of n=3 experiments. Statistical significance determined by student's t-test. *** p≤0.001

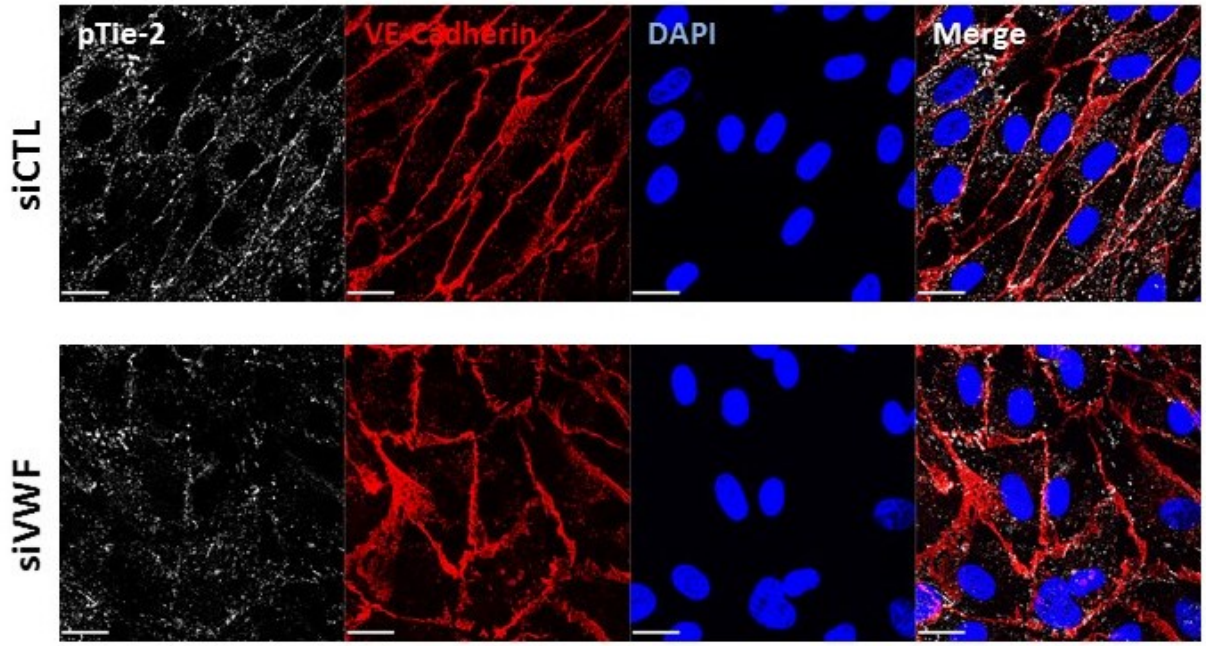

Figure 5.6 Reduced Tie-2 phosphorylation at EC-EC junctions after inhibition of VWF expression. HUVEC were treated with siRNA to VWF or control for 48 hours. Cells were then stained for pTie-2 (White), VE-Cadherin (Red) and DAPI (blue). Representative images of control (top panel) and VWF-deficient cells (lower panel). Scale bar represents 20 μm.

5.2.3 Functional consequence of enhanced Ang-2 levels during EC proliferation

5.2.3.1 No synergy between Ang-2 and VEGF during EC proliferation

Since Ang-2 is a context-dependent antagonist of Tie-2, we assessed whether Ang-2 alone could stimulate proliferation. In addition, since VEGF stimulates both proliferation and Ang-2 synthesis, we investigated whether synergy between VEGF and Ang-2 would be observed. HUVEC were seeded at 2000 cells per well of 96 well plate then stimulated with Ang-2 at different concentrations in the absence or presence of VEGF 100 ng/ml. BrDU incorporation was performed after 72 hours after treatments. VEGF alone was used as a positive control. As expected, in the presence of VEGF alone a significant increase in EC proliferation was observed. Ang-2, across a dose range of 10 – 250 ng/ml, did not significantly enhance proliferation (figure 5.7). This demonstrated that Ang-2 alone was not sufficient to increase EC proliferation. When VEGF was used in combination with Ang-2, a low dose of Ang-2 (10 ng/ml) increased proliferation ≈1.2 fold, however this was not significant. At higher concentrations of Ang-2 no increase in proliferation over VEGF alone was observed. These data suggested that in the 72 hours proliferation assay, Ang-2 alone does not induce proliferation and also that VEGF and Ang-2 do not synergise to increase proliferation.

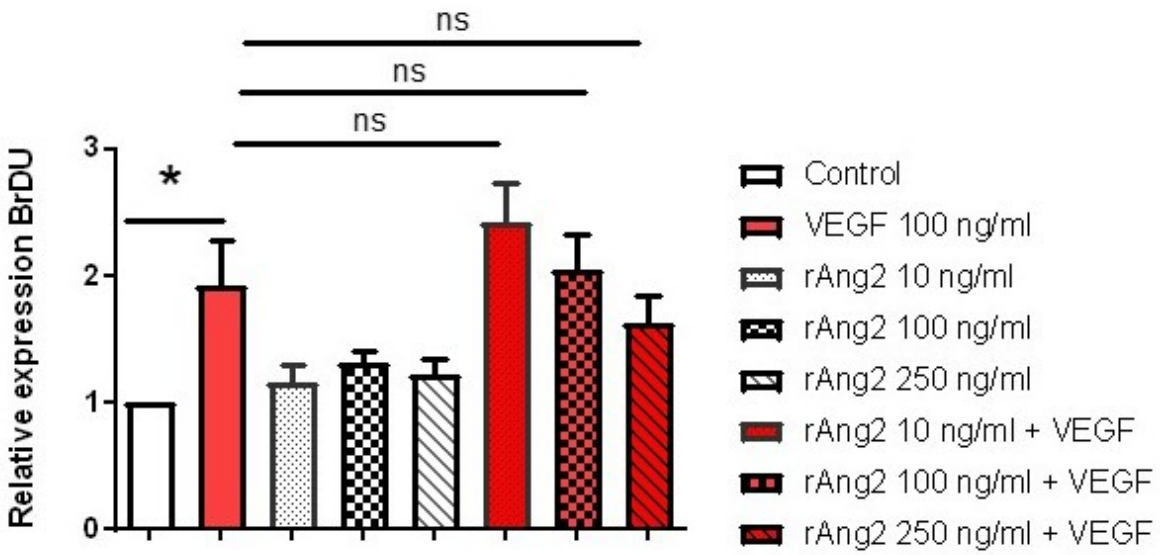

Figure 5.7 Optimising in vitro proliferation assay in the presence of Ang-2 and VEGF. HUVEC were serum starved for 6 hours in M199 supplemented with 1% FCS. Cells were then reseeded onto 96 well plates pre coated with 1% Gelatin and stimulated as indicated above. After 72 hours cell counts were determined by BrDU incorporation. Data presented as mean ± SEM of n=4 experiments. Statistical significance determined by student's t-test. ns $p>0.05$ * $p\leq0.05$

5.2.3.2 Inhibition of Ang-2 function does not affect the VWF-dependent control of EC proliferation

As described in chapter 3, inhibition of VWF expression for 48 hours caused enhanced Ang-2 release. In 2011, work from our lab showed that inhibition of VWF expression in high serum conditions increased EC proliferation (Starke et al., 2011). To test whether Ang-2 could mediate the VWF-dependent increase in proliferation HUVEC were transfected with control or VWF siRNA sequences for 24 hours, serum deprived for 6 hours then reseeded for 72 hours. RNA was collected for analysis to confirm that inhibition of VWF expression in these conditions still caused a significant increase in Ang-2 expression. As shown in figure 5.8A, inhibition of VWF expression in high serum still caused increased Ang-2 mRNA levels. To determine whether Ang-2 mediated these effects, HUVEC were treated with control or VWF siRNA for 24 hours and then for a further 72 hours with an Ang-2 neutralising antibody (AZ 3.19.3) or IgG control at 5nM. 72 hours after stimulating control and VWF-deficient cells with PBS control or VEGF in the presence or absence of Ang-2 neutralising antibody or IgG control the BrDu assay was performed. As shown in figure 5.8B, inhibition of VWF induced a 2.5-fold increase in proliferation. VEGF was able to significantly increase control and VWF-deficient cell proliferation above. In the presence of the Ang-2 neutralising antibody proliferation was significantly decreased in control cells at baseline but not VEGF induced proliferation. In VWF-deficient cells, the Ang-2 neutralising antibody did not normalise the enhanced proliferation at baseline or VEGF stimulated. These data suggests that VWF is a context dependent regulator of EC proliferation and that Ang-2 does not mediate the VWF-dependent effects in proliferation.

In Chapter 4, inhibition of VWF expression caused enhanced Ang-2 expression via a FOXO1 mechanism. Moreover, FOXO1 has been shown to have a context dependent role in EC proliferation (Dharaneeswaran et al., 2014, Potente et al., 2005, Potente et al., 2003). To determine the effect of enhanced FOXO1 activity in VWF dependent increase in proliferation, FOXO1 expression was inhibited using siRNA in HUVEC. BrDU incorporation was measured as previously described after 72 hours. As shown in figure 5.8C, inhibition of FOXO1 expression did not affect proliferation in control cells. In the absence of VWF, a trend towards increased

in proliferation was observed however this was not significant. These data, suggests that FOXO1 does not mediate the VWF dependent increase in EC proliferation.

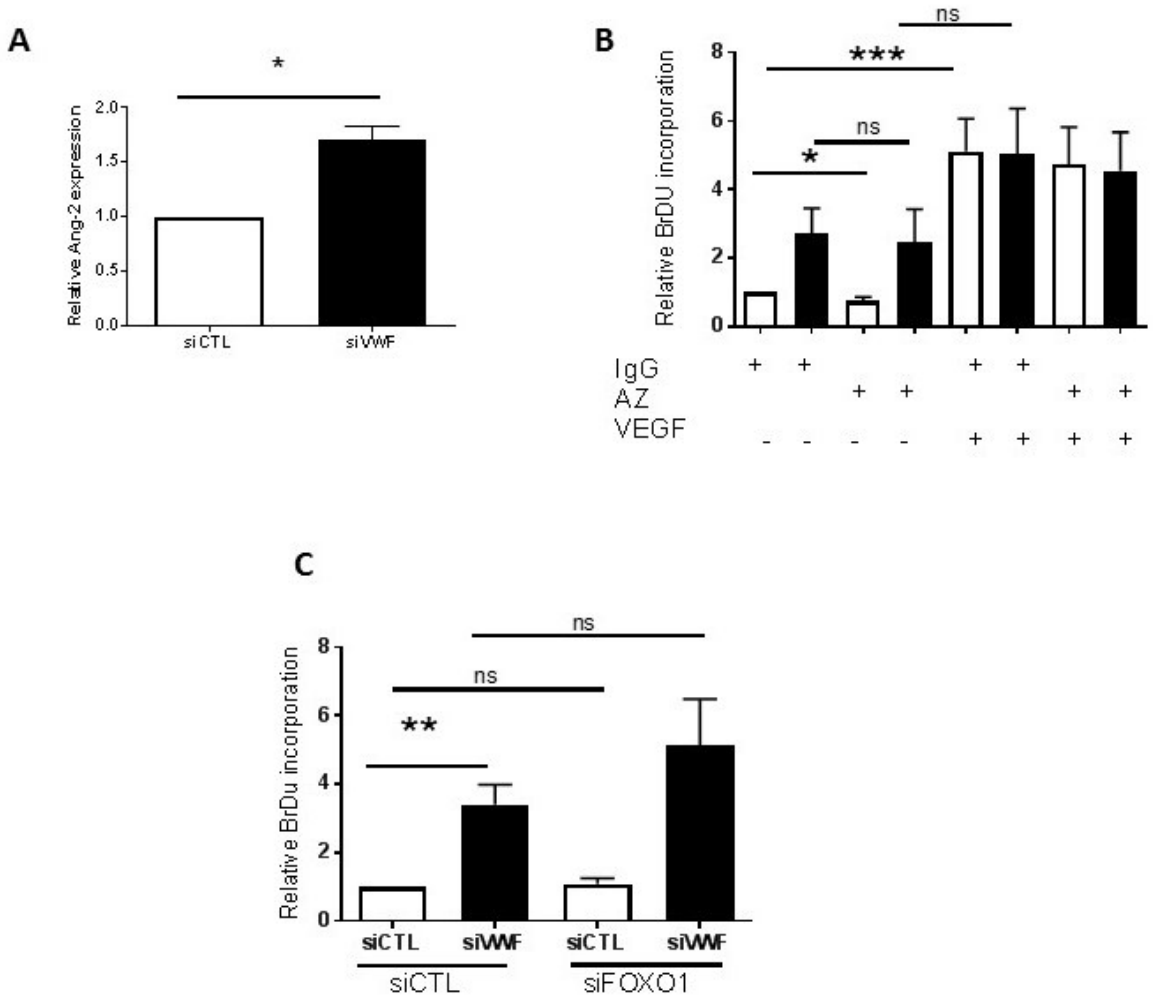

Figure 5.8 Inhibition of Ang-2 does not affected the VWF dependent in vitro proliferation. HUVEC were treated with control or VWF siRNA for 24 hours then reseeded onto gelatin at 2,000 cells per well of 96 well plate for 72 hours (A) Ang-2 expression analyzed by qRT-PCR in HUVEC treated with siRNA control or siVWF in proliferation assay conditions (B) BrDU expression in control and VWF-deficient cells treated with Ang-2 neutralizing antibody (AZ) for 72 hours or IgG control in the absence or presence of VEGF 100 ng/ml; N=8 (C) BrDU expression in control and VWF-deficient cells treated with siFOXO1 or control for 72 hours. n=3. Data normalised to siCTL control cells. Data presented as mean ± SEM. Statistical significance determined by student's t-test. ns $p>0.05$ * $p\leq0.05$,** $p\leq0.05$, *** $p\leq0.001$

5.2.4 Investigating the role of Ang-2 in VWF-dependent Matrigel tube formation *in vitro*

5.2.4.1 Ang-2 treatment alone does not stimulate Matrigel tube formation

The ability of HUVEC to form tubes in Matrigel is one of the hallmarks of *in vitro* angiogenesis. To determine whether Ang-2 stimulation of HUVEC alone could enhance tube formation HUVEC were treated with 250 ng/ml on growth factor reduced Matrigel for 24 hours. Representative images shown in figure 5.9A. Quantification of total number of completed Matrigel networks (figure 5.9B) or total tube length (figure 5.9C) after 24 hours did not show any changes in Ang-2 stimulated conditions compared to control. In this assay VEGF in combination with Ang-2 at 250 ng/ml was able to significantly increase total networks as well as increase in total tube lengths. These data shows that rhAng-2 treatment alone does not stimulate tube formation.

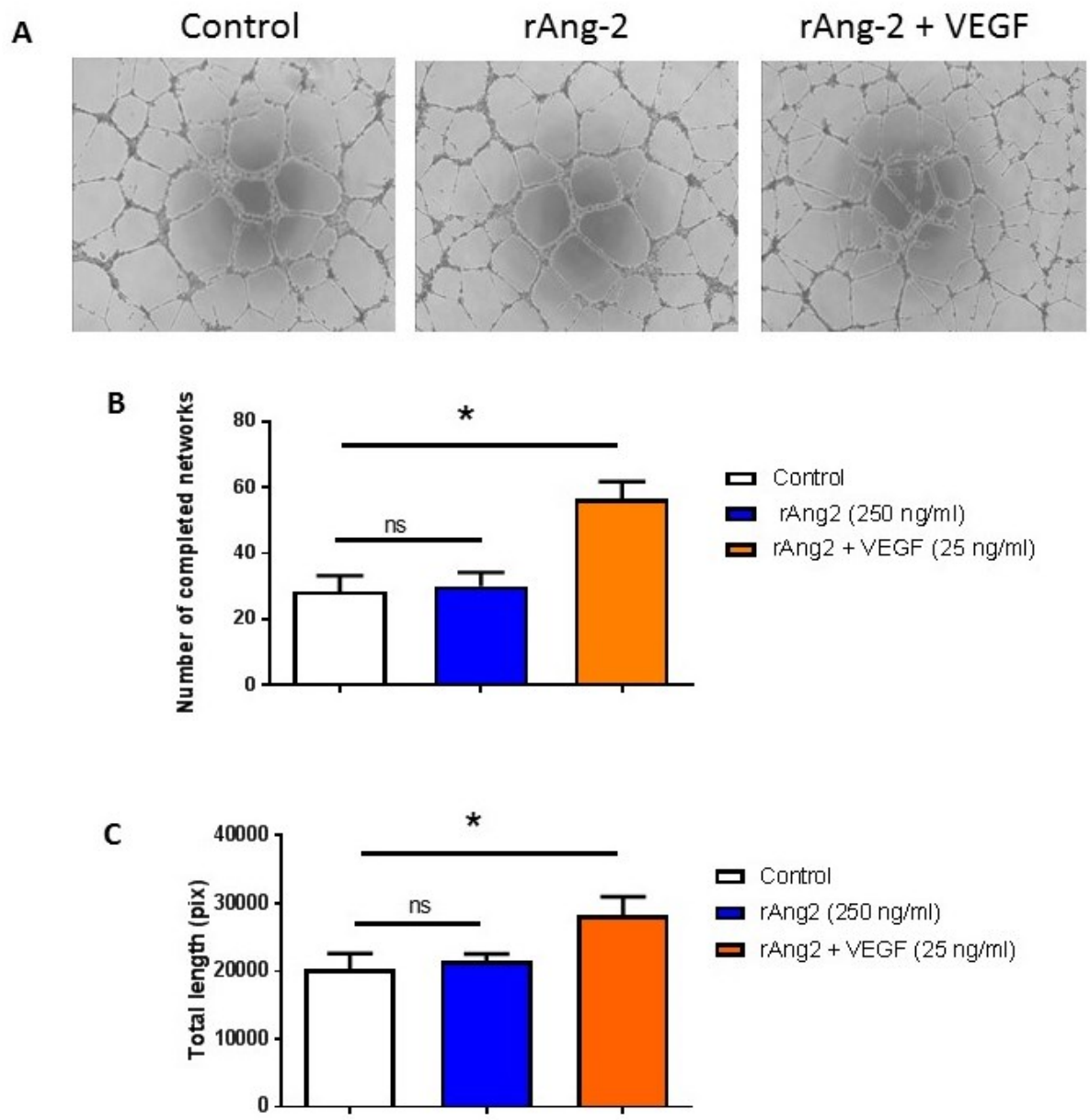

Figure 5.9 Optimising in vitro Matrigel tube formation in the presence of Ang-2 and VEGF. HUVEC were serum starved for 6 hours in M199 supplemented with 1% FCS. Cells were then reseeded onto growth factor reduced Matrigel. Cells were stimulated as indicated above. (A) Representative images after 24 hours. (B) Number of completed networks and (C) total length of tubes were quantified using Image J Neuron J Plugin. Data presented as mean ± SEM of n=3 experiments. Statistical significance determined by student's t-test. ns $p>0.05$ * $p\leq0.05$

5.2.4.2 Inhibition of Ang-2 function does not mediate the VWF-dependent increase in in vitro Matrigel tube formation

While Ang-2 alone did not stimulate tube formation it is possible that Ang-2 synergises with other pathways to regulate tube formation in the absence of VWF. To test this hypothesis, HUVEC were treated with control and VWF siRNA for 24 hours then reseeded onto growth factor reduced Matrigel as previously described in (Starke et al., 2011). Cells were treated with Ang-2 neutralizing antibody at 5 nM or IgG control to determine the role of Ang-2. After 24 hours, length of tubes were determined by Image J Neuron J plugin. In line with the findings of Starke et al, inhibition of VWF caused increase Matrigel tube formation (Starke et al., 2011). Inhibition of Ang-2 using a functional blocking antibody in control cells caused a significant reduction in total tube length. In VWF-deficient cells however inhibition of Ang-2 did not reduce normalise Matrigel tube formation compared to controls (Figure 5.10 B). These data suggests that at least *in vitro*, inhibition of Ang-2 does not mediate the VWF dependent increase in Matrigel tube formation.

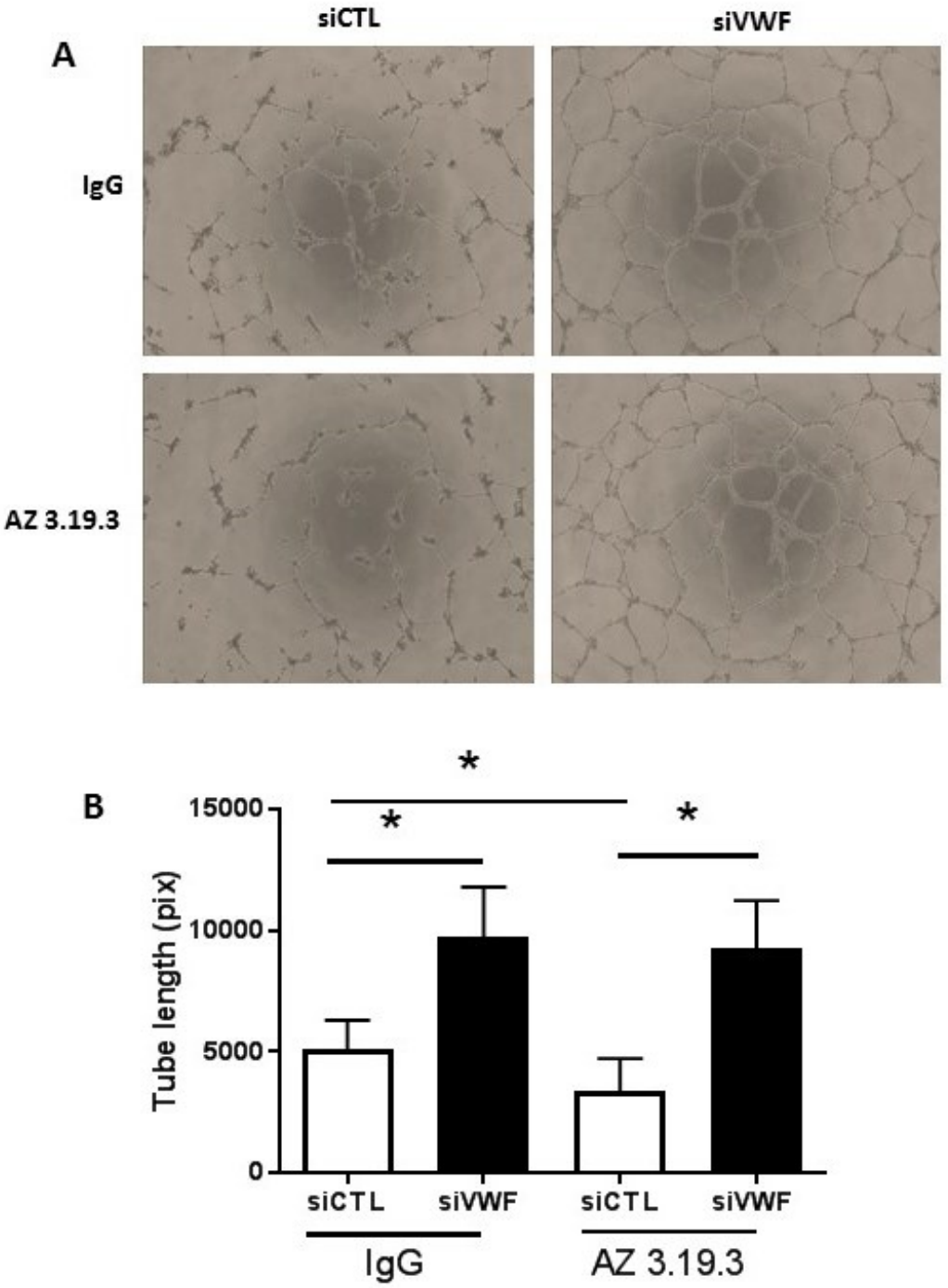

Figure 5.10 Inhibition of Ang-2 does not restore enhanced VWF dependent increase in Matrigel tube formation. HUVEC were transfected with siCTL or siVWF for 24 hours then serum starved for 6 hours in M199 supplemented with 1% FCS. Cells were then reseeded onto growth factor reduced Matrigel. Cells were stimulated with AZ 3.19.3 antibody at 5 nM or IgG control. (A) Representative images after 24 hours. (B) Total length of tubes were quantified using Image J Neuron J Plugin. Data presented as mean ± SEM of n=4 experiments. Statistical significance determined by student's t-test. ns $p>0.05$ * $p\leq0.05$

5.2.5 Ang-2 mediates the VWF-dependent increase in EC sprouting *in vitro*

Unlike the proliferation and Matrigel tube formation assays, the *in vitro* fibrin bead assay is a robust model for capturing more complex aspects of angiogenesis with resultant structures more closely mimicking the *in vivo* structure (Nakatsu et al., 2007, Nakatsu et al., 2003). The data described so far suggest that Ang-2 does not mediate the VWF-dependent control of proliferation or Matrigel tube formation. Previous reports suggests an important role for the Ang-Tie-2 axis in endothelial sprouting(Newman et al., 2011). To determine whether VWF regulates EC sprouting through this pathway, HUVEC were treated with control or VWF siRNA for 24 hours then coated onto cytodex beads and embedded in a fibrin marix with a fibroblast feeder layer. At day 5 inhibition of VWF induces enhanced number of sprouts compared to control treated cells (figure 5.11 A). To determine whether the Ang-Tie-2 axis mediates this effect cells were treated with a Tie-2-fc chimera at 2.5 μg/ml, to block the Angiopoietin/Tie-2 axis. As shown in figure 5.11B, Tie-2-fc reduced EC sprouting in control cells by ≈50%, although not significant. This was in line previous studies that demonstrated the Tie-2 axis was important for endothelial cell sprouting in this model (Newman et al., 2011). After inhibition of VWF expression, the enhanced EC sprouting was significantly reduced after treatment with Tie-2-fc. This suggests that the Tie-2 axis played an important role in the VWF dependent increase in sprouting.

To determine whether Ang-2 was responsible for the role of Tie-2 in the VWF dependent increase in sprouting, an Ang-2 functional blocking antibody (AZ 3.19.3) was used. Previous pharmacokinetic studies have shown that at 5 nM, AZ 3.19.3 specifically targets Ang-2 but not Ang-2 (Brown et al., 2010). HUVEC were treated as previously described above. In addition, HUVEC were treated with 5 nM AZ 3.19.3 or IgG control for 5 days. Representative images shown in figure 5.12A. Quantification of the number of sprouts on day 5 revealed that inhibition of VWF expression caused a significant increase in EC sprouting, as expected. Functional blockade of Ang-2 did not affect sprouting in control siRNA treated cells, however reduced the VWF-dependent increase in sprouting (Figure 5.12B). These data demonstrated that in this assay, the Tie-2 ligand Ang-2 was responsible for the VWF dependent control of EC sprouting after inhibition of VWF.

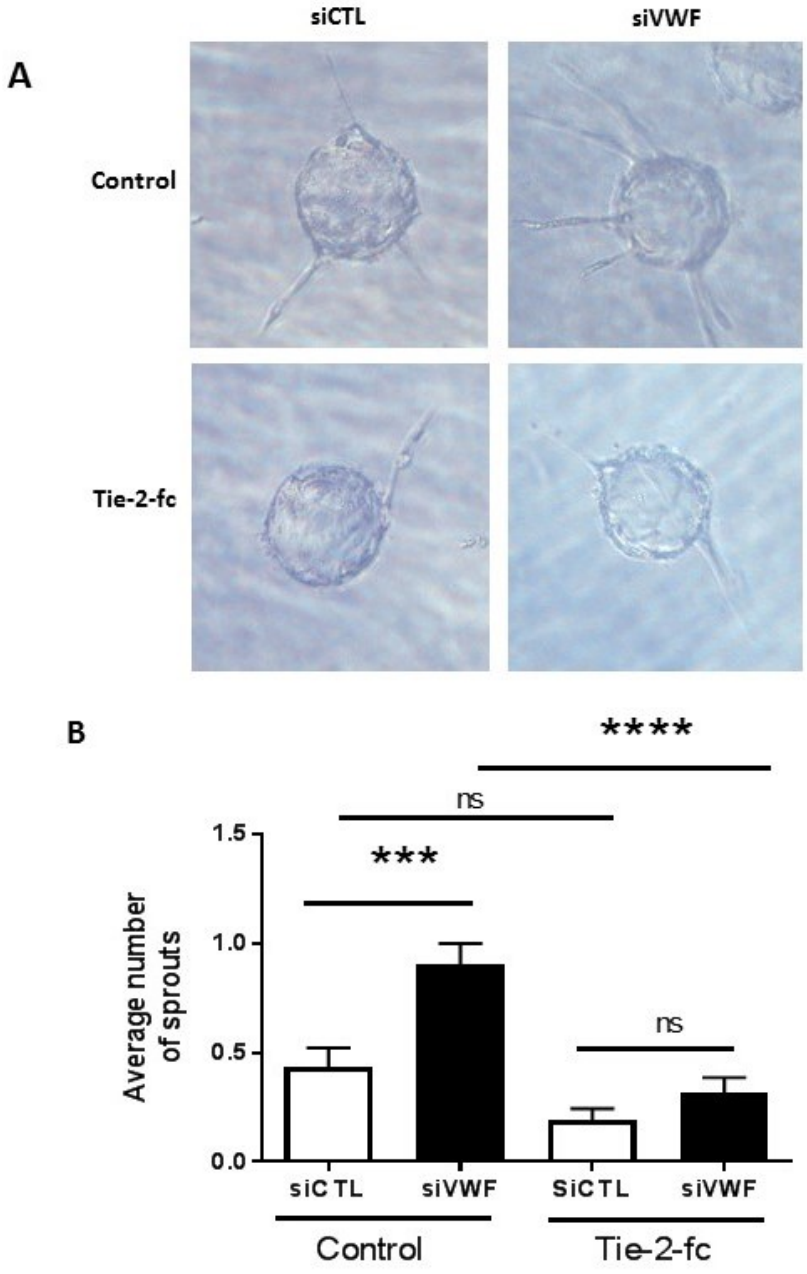

Figure 5.11 Inhibition of Ang-1/Ang-2 axis normalizes VWF dependent sprouting in vitro. HUVEC were transfected with siCTL or siVWF for 24 hours then coated onto Cytodex beads for 24 hours. Cells were then embedded in a fibrin matrix with a fibroblast feeder layer. Cells were stimulated with control or Tie-2-fc at 2.5 µg/ml. (A) Representative images after 5 days of co-culture. (B) Quantification of 30 beads per conditions. Data presented as mean ± SEM of n=3 experiments. Statistical significance determined by one-way ANOVA with Bonferroni's post hoc test. ns $p>0.05$,*** $p\leq0.001$,**** $p\leq0.0001$

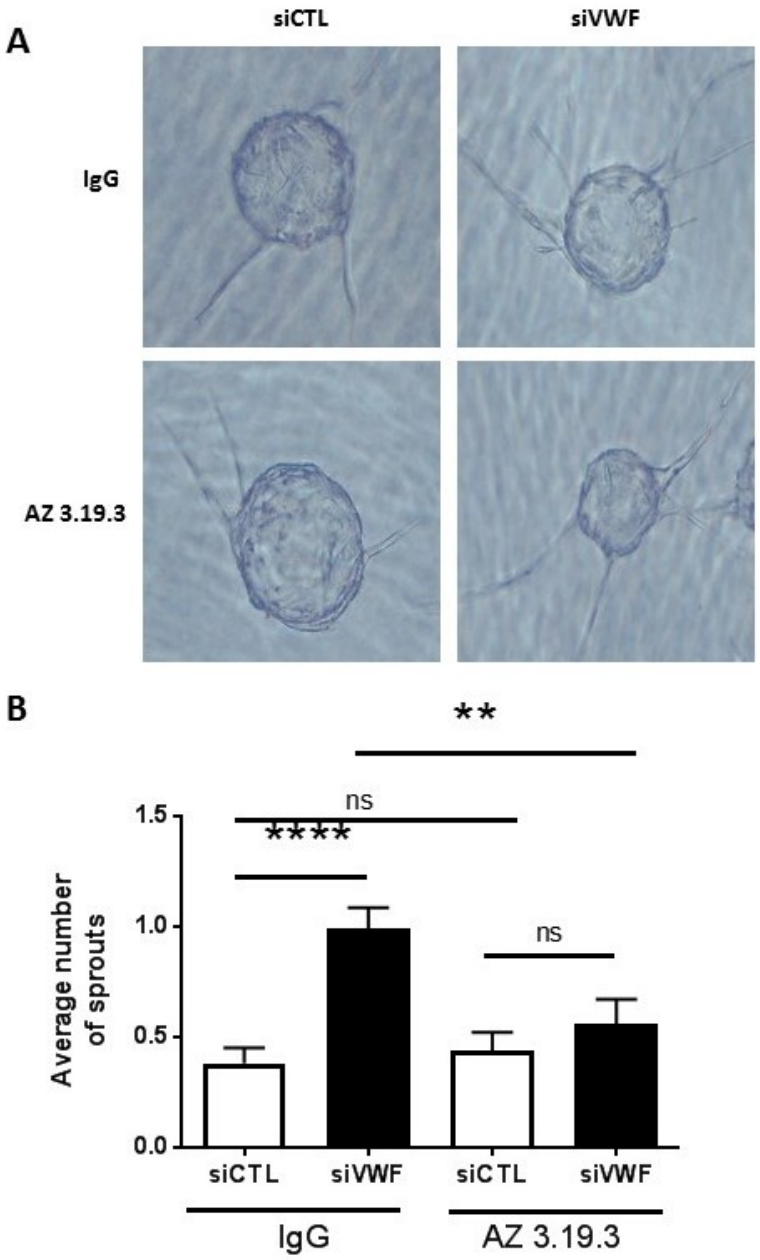

Figure 5.12 Inhibition of Ang-2 normalizes VWF dependent sprouting in vitro. HUVEC were transfected with siCTL or siVWF for 24 hours then coated onto Cytodex beads for 24 hours. Cells were then embedded in a fibrin matrix with a fibroblast feeder layer. Cells were stimulated with AZ 3.19.3 antibody at 5 nM or IgG control. (A) Representative images after 5 days of co-culture. (B) Quantification of 30 beads per conditions. Data presented as mean ± SEM of n=3 experiments. Statistical significance determined by one-way ANOVA with Bonferroni's post hoc test. ns $p>0.05$, ** $p\leq0.01$ **** $p\leq0.0001$

5.2.6 The VWF-Ang-2 pathway *in vivo*: regulation and functional consequences.

5.2.6.1 Gross profiling of VWF-deficient mouse

Results described so far suggests that VWF control Ang-2 levels and that Ang-2 mediates the VWF dependent increase in sprouting angiogenesis. *In vivo*, global overexpression of Ang-2 in the adult caused increase mouse size from resultant vascular leak (Ziegler et al., 2013). VWF-deficient mice on the other hand have no gross abnormalities (figure 5.13A) and have normal body weight (figure 5.13B). Despite enhanced Ang-2 expression in the heart no difference in heart weight was observed (figure 5.13C). In the liver where no enhanced Ang-2 expression was found (figure 5.13D) no difference in weight was observed compared to controls. Overall, these data suggests the VWF-deficient mice had no apparent oedema nor gross abnormal physical characteristics compared to littermate controls.

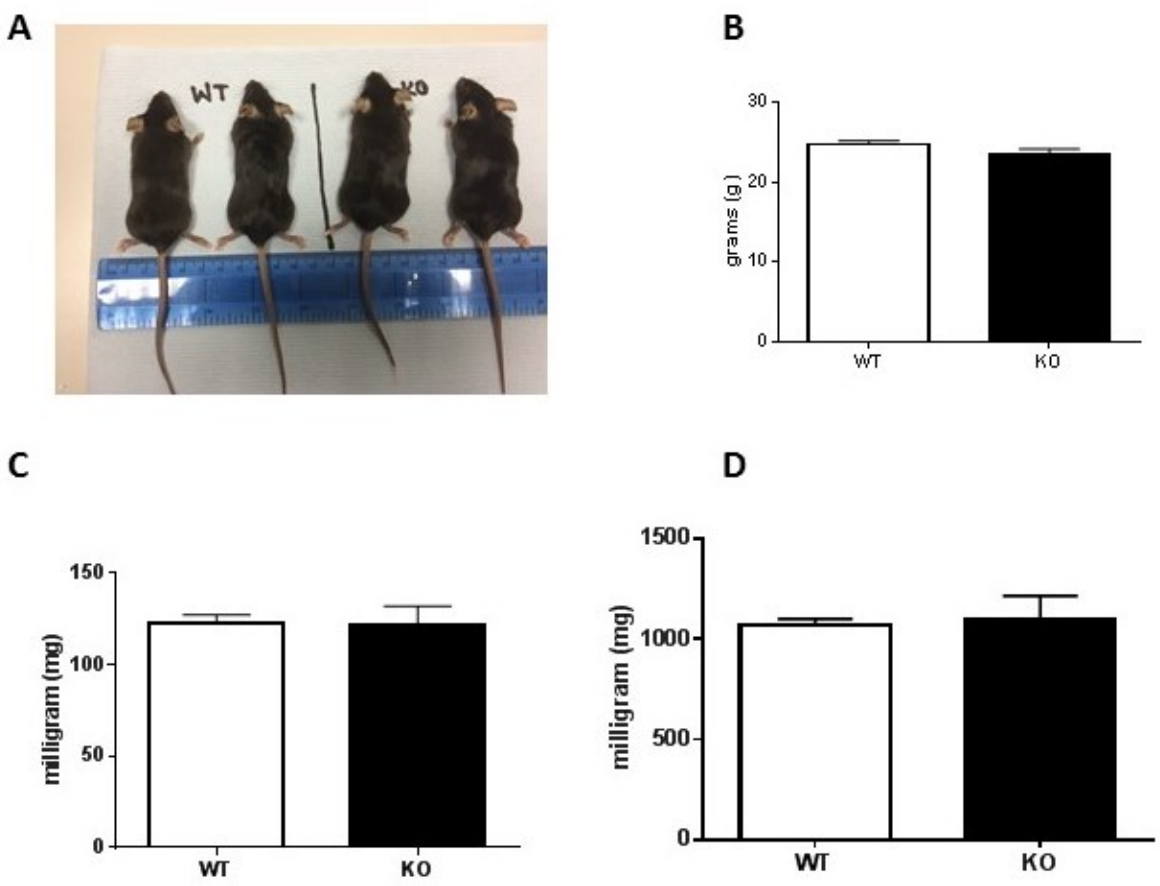

Figure 5.13 No gross abnormalities in VWF-deficient mice. (A) Representative images of WT and VWF KO mice aged 6 weeks. (B) Gross weight (C) heart weights and (D) Liver weights of WT and KO sex and aged matched mice. Data presented as mean ± SEM of 4 mice per group.

5.2.6.2 VWF affects the VEGF-induced cellular infiltration into matrigel plug in vivo

Ang-2 is important for postnatal angiogenesis (Gale et al., 2002). To test whether Ang-2 affects postnatal vascularisation in the VWF-deficient mouse the Matrigel plug model was utilised. Control and VWF-deficient mice were injected subcutaneously with two 250 µl Matrigel plugs supplemented with PBS or VEGF (100 ng/ml) as described in (Birdsey et al., 2008). After 7 days mice were sacrificed, plugs collected and analysed for vascularisation. Sections were stained for vascular marker endomucin (white) and nuclear marker DRAQV (purple) by immunofluorescence. Imaging of plugs revealed a very heterogeneous distribution of vessels in Matrigel plugs (representative images shown in figure 5.14A). Quantification of endomucin (figure 5.15A) could not detect a difference between VEGF and control treated plugs in these experiments. In previous studies, cellular infiltrate has been shown to precede vascularisation in Matrigel plugs (Anghelina et al., 2004, Tigges et al., 2008). We have previously shown that VWF-deficient mice had basal increase in cellular infiltrate (Starke et al., 2011). Using DRAQV quantification we found that VEGF stimulation of plugs caused a significant increase in Matrigel cellular infiltrate, measured by DRAQV (figure 5.15B). Surprisingly, in the presence of VEGF, VWF-deficient mice did not show increased cellular infiltrates, suggesting that the response to VEGF may be defective in these mice. However, all experiments showed a lack of robust vascularisation of the mouse plug, suggesting technical issues.

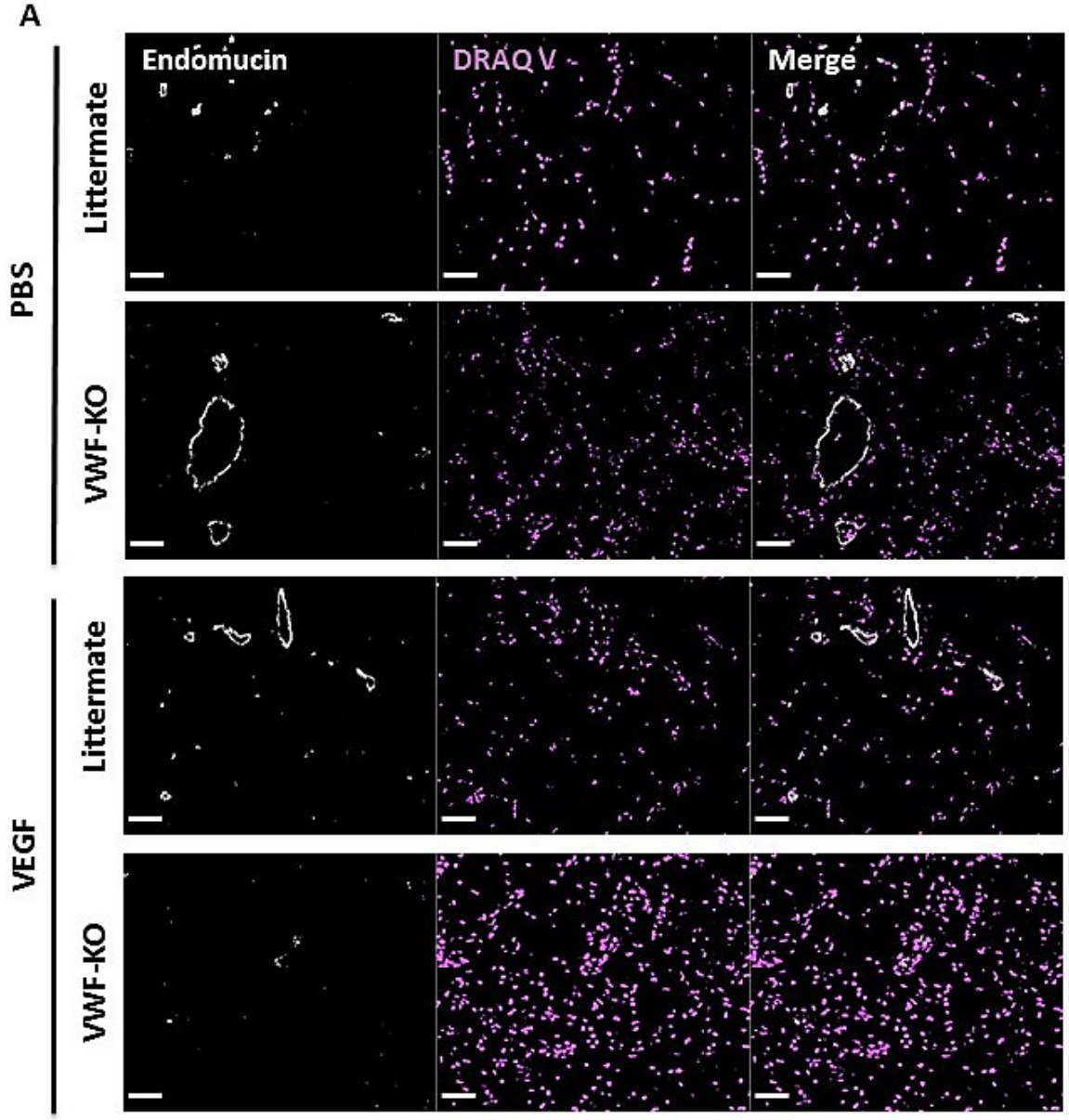

Figure 5.14 Optimization of in vivo Matrigel plug (A) Representative Images of Littermate or VWF-deficient mice Matrigel plug after 7 days implantation with PBS or VEGF 100 ng/ml incorporated. Plugs were sectioned and stained for Endomucin (white) and DRAQ V (blue). Scale bar represents 50 µm.

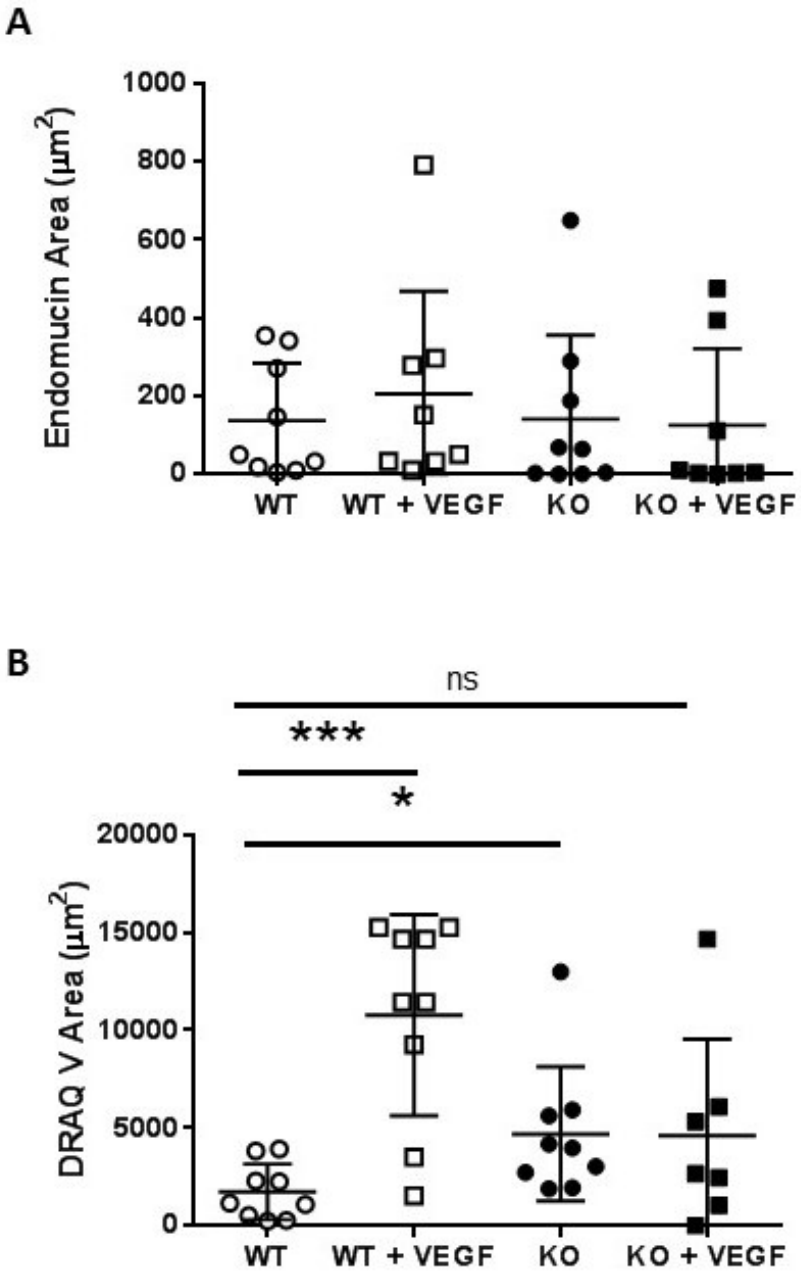

Figure 5.15 Quantification of DRAQV (cellular infiltrate) in Matrigel plugs 7 days after implantations. (A) Quantification of Endomucin coverage and (B) DRAQV coverage in Matrigel plugs 7 days after implantations. 3 fields per plug per mouse. Data presented as mean ± SEM of 3 fields per plug of 3 WT and 4 KO mice. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, *** $p\leq0.001$

5.2.6.3 Increase Ang-2 expression in mouse heart after 12 hours LPS treatment

Work described in this thesis showed that VWF regulated Ang-2 expression specifically in the heart. Moreover, LPS can upregulate Ang-2 expression in the lungs but not heart of mice at 6 hours (Chapter 4). Although, limited data exists on Ang-2 expression in the heart after LPS treatment, LPS has been shown to cause cardiac dysfunction around 12 hours in rodent models (Hu et al., 2015). In addition, over expression of Ang-2 in the EC has been reported to be associated with defects in cardiac output and stroke volume (Ziegler et al., 2013).To determine whether Ang-2 levels were upregulated in the mouse heart after 12 hours a pilot study was performed. 12 week old male mice were injected with 50 µg LPS i.p for 12 hours. Hearts were collected and Ang-2 mRNA measured by qRT-PCR. In this study, LPS for 12 hours caused an increase in Ang-2 expression in 2 out of 3 treated mice (Figure 5.16). This experiment showed that 12 hours LPS treatment was sufficient to upregulate Ang-2 expression in most samples of mouse heart.

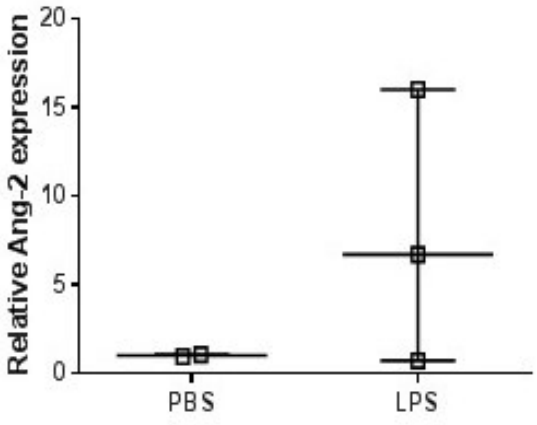

Figure 5.16 Regulation of Ang-2 levels in mouse heart after LPS challenge. Male C57BL6 mice between 8-12 weeks were injected with LPS 50 µg, i.p for 12 hours. Hearts were then collected and RNA extracted. Ang-2 expression was measured by RT-PCR and normalised to 18S. Data expressed relative to PBS control treated mice. Data expressed as mean ± SEM of 2-3 mice per group.

5.2.6.4 Regulation of cardiac permeability under stress in VWF-deficient mice

As mentioned above, increased Ang-2 expression in the ECs was associated with increase vascular leak and cardiac defects measured by cardiac output and stroke volume (Ziegler et al., 2013). Interestingly, the VWF-deficient mouse has been shown to have a baseline cardiac dysfunction and decrease survival in response to cardiac stress caused by aortic banding (Yuan et al., 2016). Since our pilot data showed that 12 hours was sufficient to increase Ang-2 expression in mouse heart, we tested whether VWF-deficient mice were more susceptible to cardiac vascular leak because of increased Ang-2 expression. To test this hypothesis we used the Miles assay. Mice were injected with LPS at 50 µg ip for 11.5 hours then anaesthetised and injected with 2% Evans Blue. Mice were recovered for 30 mins then perfused with PBS after culling. To determine the effect of LPS, mice were scored using a behaviour chart (shown in appendix 7). This monitoring revealed that in WT mice all mice showed sickness behaviour (figure 5.17A) with only 2/3 in VWF-deficient mice showing sickness response to LPS. Analysis of Evans Blue extravasation in the lungs revealed no difference in VWF KO mice compared to littermate controls at baseline (figure 5.17B). Surprisingly in the presence of LPS, WT mice showed reduced evans blue extravasation. A very variable response was observed in the VWF-deficient mouse during LPS treatment. Analysis of the mouse heart revealed no difference in evans blue extravasation in WT or KO in either PBS or LPS treated group (figure 5.17C). These data showed that 12 hours LPS caused a reduction in vascular permeability in WT but not VWF KO mice. These experiments could not identify gross vascular leak in the hearts of VWF-deficient mice; however, the unexpected lack of response in control mice suggests that this experimental design may not be suitable to conclusively address this question.

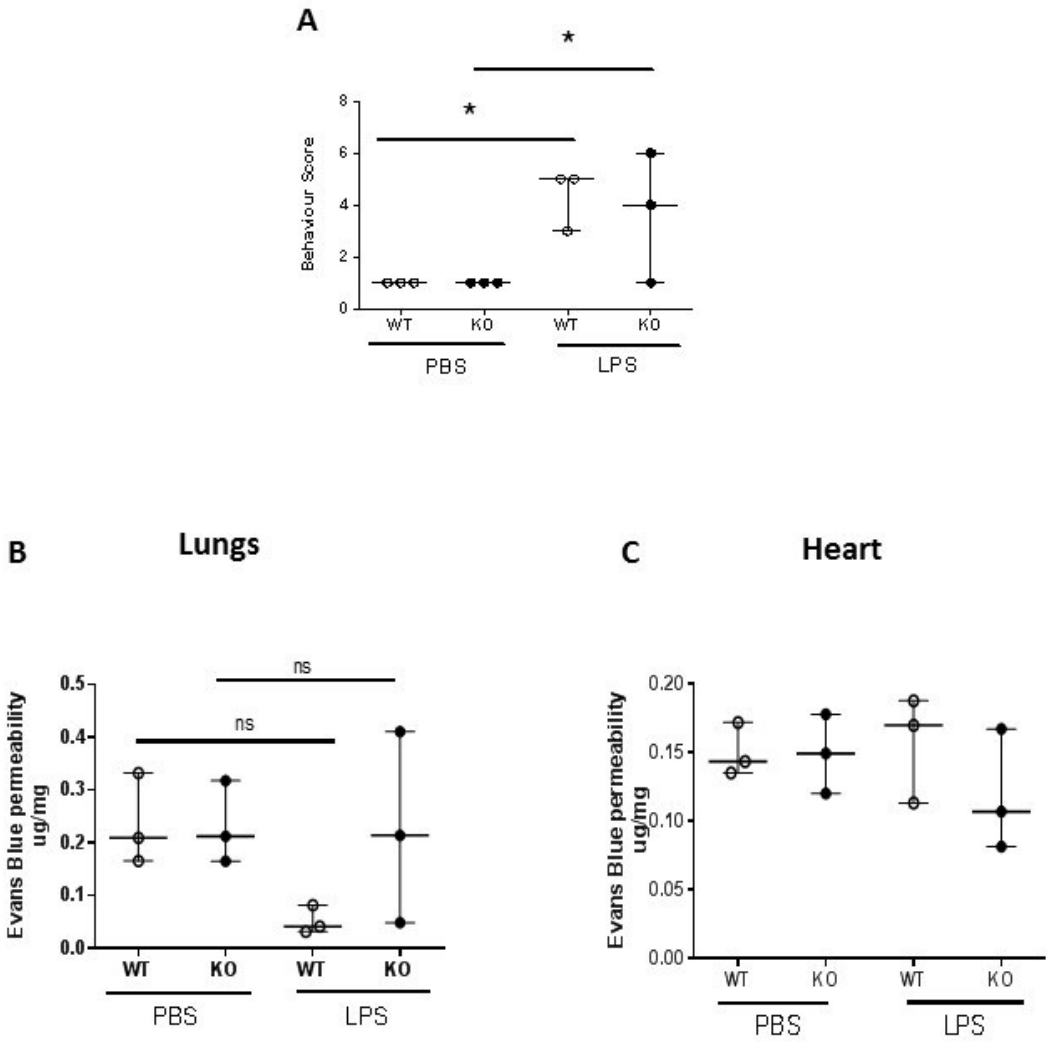

Figure 5.17 VWF-deficient mice do not have gross increased permeability at baseline or LPS induction. WT (open circles) and VWF-deficient mice (black circles) were treated with LPS 50 µg or PBS control for 11.5 hours. (A) Mice were scored every hour using behavior chart shown in Appendix 7. A score of 0 was considered normal however 1 was added to all scores to allow statistical analysis. Mice were then anaesthetized and injected with 2% Evans Blue and recovered for 30 mins. Evans blue was extracted using formamide. (B) lungs and (C) heart Evans blue quantification was measured in µg/ml and normalised to total mass in mg. Data presented as media ± IQR of n=3 mice per group. Significance was determined ns $p>0.05$, * $p\leq0.05$

5.2.6.5 VWF regulates cardiac vascularisation and microvascular permeability

5.2.6.5.1 Enhanced vascular perfusion in VWF-deficient mice

The data described above was inconclusive with respect to the cardiac permeability phenotype in VWF-deficient mice. Because Evans blue relies on the passage of albumin across the EC barrier and therefore subtle effects on permeability would not be detected, we used two molecular weight dextrans to further assess vascular function (Birdsey et al., 2015). Control and VWF-deficient mice were injected with 10 mg/ml of 1:1 2 x 10^6 molecular weight dextran FITC conjugated and 4.4 x10^3 molecular weight dextran TRITC conjugated for 15 mins intravenously. Mice were then sacrificed and tissues collected, paraffin fixed, wax embedded and cut into 4 µm section. To assess the vessel perfusion and vascularization tissues were stained for Endomucin (White), anti HMWD (Red) and DAPI (Blue) (representative images shown in figure 5.18A). Quantification with the image analysis software Volocity revealed enhanced perfusion of the HMWD in the VWF KO compared to littermate controls (figure 5.18B). These data demonstrated that VWF-deficient mice heart had enhanced perfusion of HMWD compared to littermate controls.

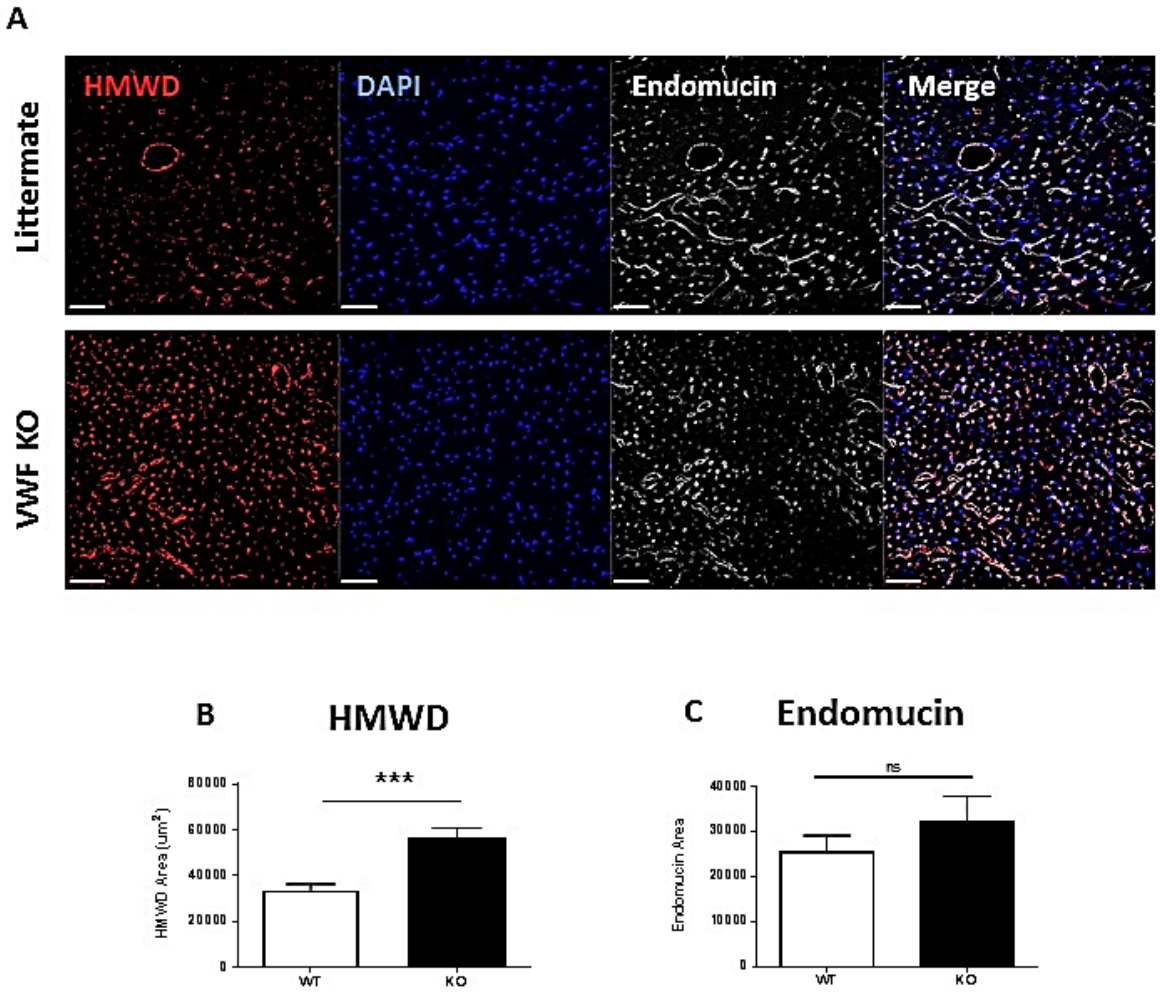

Figure 5.18 Enhanced perfusion in VWF-deficient mouse hearts. Mice aged between 6-8 weeks were injected with 10 mg/ml of 6 x 10^6 FITC conjugated Molecular Weight Dextran (HMWD) intravenously for 15 mins prior to being culled by cervical dislocation. (A) Representative images of Heart sections stained for Endomucin (white), anti-FITC dextran (Red), DAPI for nuclei. Scale bar represents 50 µm. (B) Quantification of HMWD coverage in littermate vs VWF-deficient mice. (C) Quantification of Endomucin. Data presented as mean ± SEM of 5 x20 fields per mouse of 3 WT and 4 KO mice. Statistical significance determined by student's t-test. ns $p>0.05$, *** $p\leq0.001$

5.2.6.5.2 Enhanced vascularisation in the VWF-deficient mice

Enhanced HMWD in the previous experiments could suggest enhanced perfusion or increased vascularisation in the VWF-deficient mouse compared to controls. Endomucin quantification surprisingly revealed no difference in vessel staining in VWF-deficient mice compared to littermate controls (figure 5.18C). Endomucin staining preferentially detects veins rather that all vessels(dela Paz and D'Amore, 2009), therefore the effects on smaller vessels such as capillaries would not be detected by endomucin. Since VWF-deficient mice have increase vascularisation, we first tested this hypothesis. Staining protocols were optimised using HMWD FITC conjected, SMA (red), Isolectin (white) and DAPI (blue) for nuclei. To determine the specificity of the HMWD-FITC conjugated signal, images were compared to tissue from control animals not injected. In mice injected with HMWD-FITC conjugated dextran fluorescence was detected in isotype (green, top panel figure 5.19). This signal was absent in control mice not injected with HMWD-FITC conjugated dextrans (bottom panel figure 5.19). To assess whether HMWD-FITC (green) signal caused interference with neighbouring red channel signal, smooth muscle actin (SMA; red) staining was performed where only larger SMA structures would be detected. Isolectin staining was performed which highlighted all structures which were HMWD positive as expected (middle panel figure 5.19). SMA staining and HMWD staining was only apparent in larger structures suggesting no interference of HMWD-FITC signal.

Previous studies have shown that SMA coverage of retinal vessels is delayed in VWF-deficient mice during developmental angiogenesis (Scheppke et al., 2012). To determine whether VWF-deficient mice vascularisation and vessel maturity were affected in the heart vasculature, heart sections from control (figure Top panel 5.20A) and VWF-deficient mice (bottom panel figure 5.20A) were stained for FITC conjugated HMWD (unstained), SMA (red), Isolectin (white) and nuclei (blue). Analysis of sections using volocity revealed no difference in SMA coverage in the VWF-deficient mice compared to control, suggesting that maturation of larger arterial vessels is unaffected by VWF deficiency (figure 5.20B). Analysis of Isolectin coverage revealed enhanced vascularisation in the VWF-deficient mice compared to controls (figure 5.20C). These Isolectin positive structures were also positive for HMWD suggesting they are

perfused. These data demonstrated increased vascularization and perfusion of structures in the heart of VWF-deficient mice.

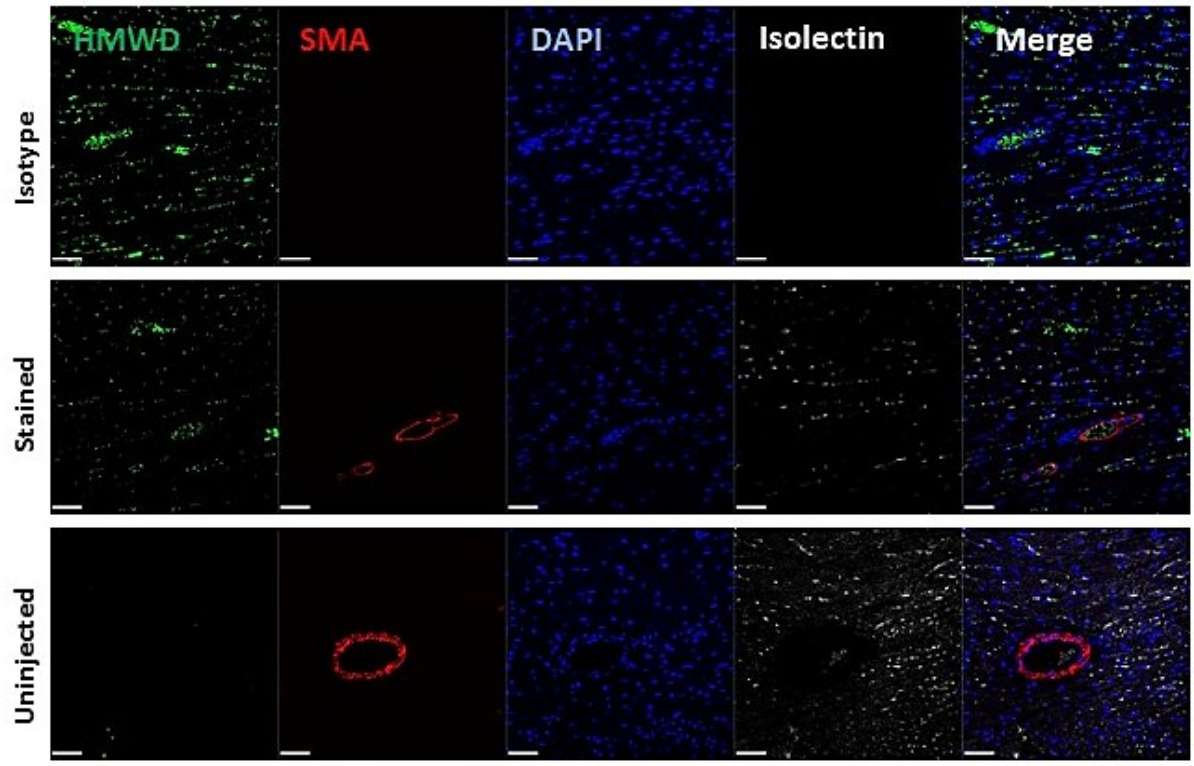

Figure 5.19 Optimization of TRITC and FITC conjugated dextran staining in mouse heart tissue. Mice aged between 6-8 weeks were injected with 10 mg/ml of 6 x 10^6 FITC conjugated Molecular Weight Dextran (HMWD) and 4.4 x 10^3 TRITC conjugated molecular weight dextrans intravenously for 15 mins prior to being culled by cervical dislocation. Sections were stained for anti SMA (Red), anti Isolectin (White), DAPI for nuclei and FITC dextran was detected as natural signal from injected material. Representative images of Isotype (top panel), stained section (middle) and mouse uninjected (bottom panel). Scale bar represents 50 µm

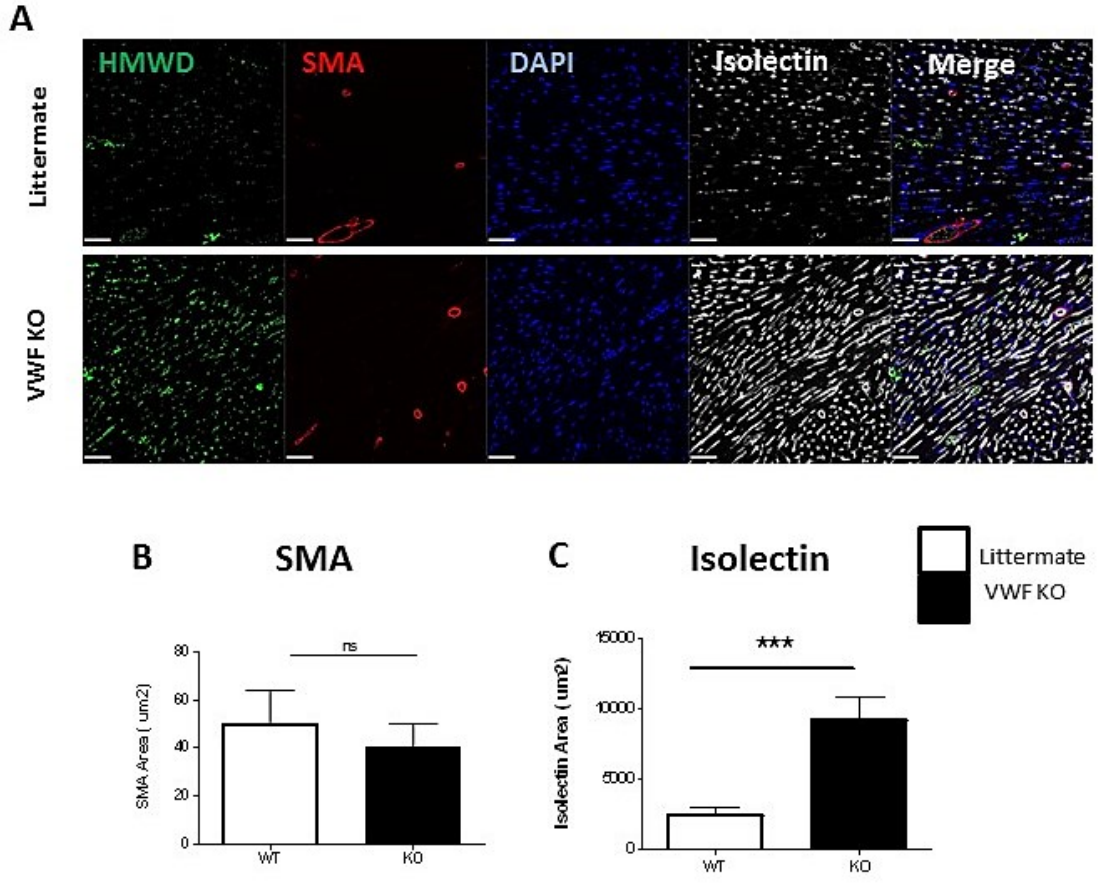

Figure 5.20 Enhanced vascularization in VWF-deficient mouse hearts. Mice aged between 6-8 weeks were injected with 10 mg/ml of 2 x 10^6 FITC conjugated high Molecular Weight Dextran (HMWD) intravenously for 15 mins prior to being culled by cervical dislocation. (A) Representative images of Heart sections stained for anti SMA (Red), Anti Isolectin (White) and DAPI (blue) for nuclei. HMWD was detected using natural signal from injected material. Scale bar represents 50 µm. (B) Quantification of SMA coverage in littermate vs VWF-deficient mice. (C) Quantification of Isolectin coverage in littermate vs VWF-deficient mice Data presented as mean ± SEM of 5 x20 fields per mouse of 3 WT and 4 KO mice. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, *** $p\leq0.001$

5.2.6.5.3 Enhanced micro vessel leakage in cardiac vasculature in VWF-deficient mice

The above work demonstrated no difference in the leakage of large molecules (Evans blue or 2 x 10^6 MW dextran) in VWF-deficient mice. The low molecular weight dextran (LMWD) can detect more subtle defects in vascular leakage. To determine the effect of loss of VWF on leakage of the small molecular weight dextran in the heart, heart sections were stained for LMWD (red), Isolectin (White) and DAPI (Blue) (representative images shown in figure 5.21A). Quantification by volocity ® demonstrated significant enhanced vascular leakage of the small molecular weight dextran in the hearts of VWF-deficient mice compared to littermate controls (figure 5.21B). These data demonstrate increased baseline permeability to small solutes in the heart vessels in VWF-deficient mice, indicating that VWF is required to maintain a tight barrier in the heart microvasculature.

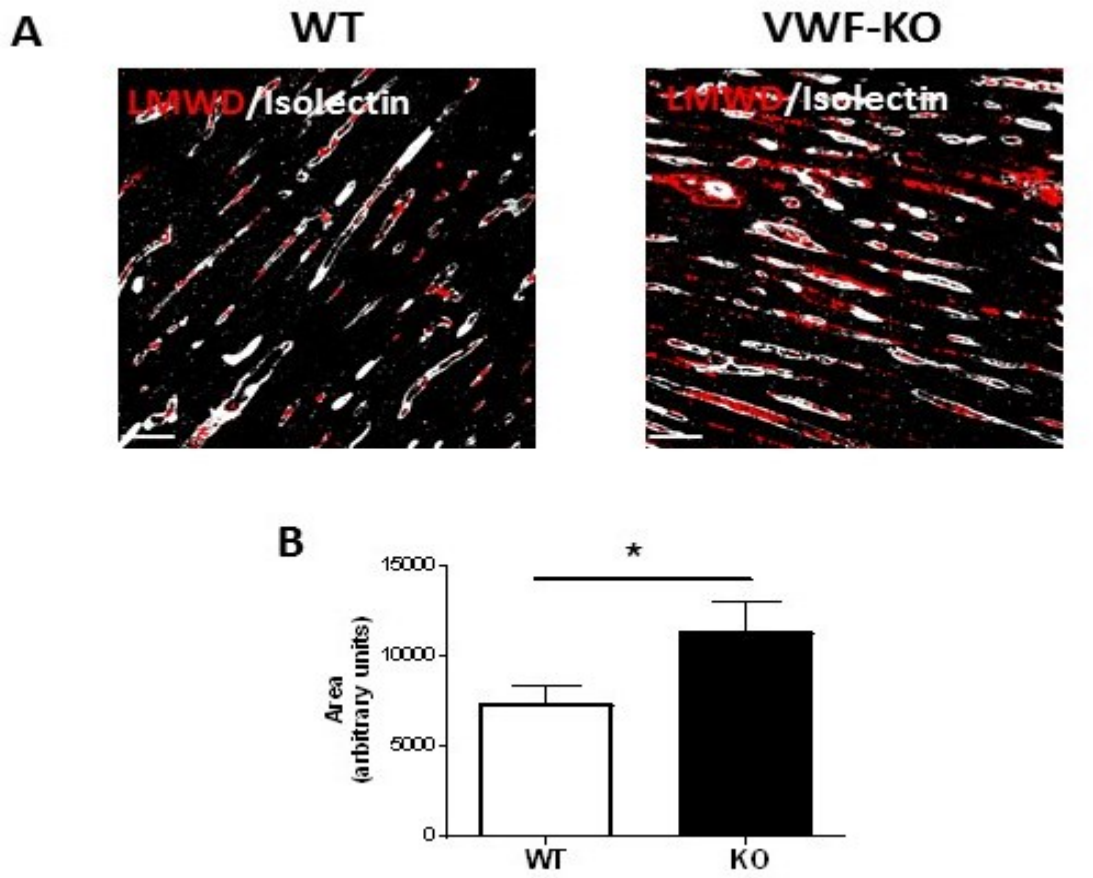

Figure 5.21 Enhanced extravasation of Low Molecular Weight dextrans from heart vasculature in VWF-deficient mice. Mice aged between 6-8 weeks were injected with 10mg/ml of 4.4 x 10^3 TRITC conjugated Molecular Weight Dextran (LMWD) intravenously for 15 mins prior to being culled by cervical dislocation. (A) Representative images of Heart sections stained for anti TRITC (Red) and Anti Isolectin (White). Scale bar represents 50 µm. (B) Quantification of extravasated TRITC dextrans in littermate controls VWF-deficient mice. Data presented as mean ± SEM of 5 x20 fields per mouse of 3 WT and 4 KO mice. Statistical significance determined by student's t-test.* p≤0.05

5.3 Discussion and Future Work

Ang-2-VWF complex regulates endothelial adhesion.

In this study we have utilised both *in vitro* and *in vivo* studies to elucidate the role of Ang-2 in VWF-dependent angiogenesis. A recent study carried out by Mckinnon and colleagues revealed that Ang-2 and VWF are bound together after release from EC (McKinnon et al., Submitted). This study also revealed that Ang-2 bound to HUVEC, did not interfere with the interaction with Tie-2. In this chapter we have demonstrated using EC adhesion Ang-2 and VWF-Ang-2 complex that VWF appears to enhance Ang-2 interaction with the endothelium. We speculated that this was due to the RGD site present in VWF; these findings were supported using just the A1 domain where Ang-2 has been shown to interact. Further studies using RGD mutants or inhibitors would be needed to confirm these findings. Surprisingly, further studies showed that both the A1 domain and full length VWF were able to sustain binding of HUVEC. In addition, the presence of Ang-2 reduced binding of cells to both the A1 domain and full length VWF to HUVEC. In order to understand this VWF interaction with the endothelium, we inhibited Tie-2 which is the main Ang-2 receptor; in the absence of Tie-2 Ang-2 has been shown to interact with integrins (Hu et al., 2006, Bezuidenhout et al., 2009, Carlson et al., 2001, Felcht et al., 2012). We confirmed that Ang-2 still maintained interaction with HUVEC when bound to the A1 domain. An interesting finding from these studies was the ability of the A1 domain to maintain the interaction with HUVEC in the absence of Tie-2. Follow up studies are needed to understand what mediates this interaction.

VWF binding to Ang-2 occurs in at pH 7.4, however optimal binding conditions appear at ≈pH 6.5 and high calcium (Mckinnon et al., submitted). In the current study although we have shown that purified VWF did not contain Ang-2, it is possible that the *in vitro* binding conditions may not be optimal. Future experiments may be needed to address this limitation. In particular, quantification of the relative amounts of VWF, Ang-2 and Ang-2 bound to VWF may be necessary for interpreting of the ability of Ang-2 or VWF to signal in a complex.

The binding of integrins and ligands has been shown to occur via the I (inserted) domain(Takagi, 2007). In particular, since Ang-2 does not have an RGD site, the interaction of

Ang-2 with integrins is thought to be I-domain mediated (Felcht et al., 2012). In particular, this interaction occurs through a metal-ion-dependent adhesion site (MIDAS) which is located at the centre of the I-domain of the β integrin. The I domain in integrins is known to be structurally similar to the A domain of VWF(Emsley et al., 1998). A possible hypothesis to VWF-A1 domain interacting with HUVEC was the involvement of a MIDAS, however studies have confirmed that this is not present in A1 domain (Emsley et al., 1998). In our study, we have not yet determined whether the interaction of VWF-A1 with HUVEC is integrin dependent. Inhibition of αvβ3, α5β1 and αvβ5 alone revealed that Ang-2 can bind all three integrins suggesting a compensatory role (Felcht et al., 2012). This presents an interesting potential for fully elucidating the role of VWF-Ang-2 complex in vascular function. In addition, the interaction of this complex with Tie-2 may help unravel some of the complex nature of Ang-2 interactions and signalling.

VWF regulates Tie-2 signalling in EC

Enhanced Tie-2 signalling is associated with vascular malformations (Limaye et al., 2009). Prolonged exposure to Ang-2 has been shown *in vitro* to activate Tie-2 receptor (Yuan et al., 2009) and *in vivo* to drive the increase in vascular diameter (Korhonen et al., 2016). In this study we investigated the phosphorylation of Tie-2 in VWF-deficient cells where we observe enhance Ang-2 expression. Surprisingly we found decrease Tie-2 signalling in VWF-deficient cells, which supports in part the role of Ang-2 as an antagonist. Ang-2 like Ang-1 possess the ability to also recruit Tie-2 to the junctions; however Ang-2 induced Tie-2-αvβ3 complex causes internalisation and degradation of the αvβ3 integrin (Thomas et al., 2010). The prevailing hypothesis therefore is that in the absence of Ang-1, Ang-2 promoted EC destabilisation despite the ability to activate Tie-2. It must be noted that our studies were performed in the absence of Ang-1; however, baseline Tie-2 phosphorylation was observed. It remains to be seen whether in *in vitro* assays the presence of Ang-1 can activate Tie-2 signalling in the absence of VWF.

One proposed explanation for these observations is that Ang-2 produced from control cells possessed the ability to bind to Tie-2 and promote basal phosphorylation. In support of this

hypothesis, studies have shown that Ang-2 circulates as a lower order oligomers (trimeric and tetrameric structures). These had high affinity for Tie-2 but limited ability to induce phosphorylation. Interestingly, Ang-1 normally exists as higher order multimers (Procopio et al., 1999, Davis et al., 2003, Augustin et al., 2009). When a COMP-pentameric form of-Ang-2 was generated, it possessed the same ability as COMP-Ang-1 in Tie-2 localisation and model of angiogenesis (Kim et al., 2009). Since in our studies we observe that inhibition of VWF was able to induce decrease Tie-2 phosphorylation, the question that remains is whether Ang-2 bound to VWF could help in maturation/multimerization of Ang-2 in the WPB. The functional consequence of VWF binding to Ang-2 remains to be uncovered, however another potential explanation could be that VWF regulates Ang-2 localisation. Structural analysis of Ang-2 showed that the hinge region of Ang-2 differed from Ang-1. This revealed that while Ang-1 can localise to EC matrix, Ang-2 is unable (Xu and Yu, 2001). This is important as studies have demonstrated that during angiogenesis and inflammation, Tie 2 is downregulated (Felcht et al., 2012, Ghosh et al., 2012). This allows Ang-2 to signal via integrins including β1 and β3 integrins to facilitate angiogenesis and endothelial destabilisation (Hakanpaa et al., 2015, Thomas et al., 2010). Revision of structural data to include VWF in the Ang-2 binding may be needed to further understand Tie-2 signalling.

VWF regulates blood vessel formation and leakage via Ang-2.

Previous work from our group has demonstrated that inhibition of VWF causes an increase in *in vitro* Matrigel tube formation and *in vitro* proliferation (Starke et al 2011). In the current study we demonstrated that inhibition of Ang-2 using a functional blocking antibody was unable to normalise the VWF dependent increase in Matrigel tube formation and proliferation. In part, the observation that Ang-2 did not mediate the VWF dependent increase in tube formation could be explained by growth conditions and Ang-2 levels. Assessing tube formation in this assay, we utilise growth factor reduced angiogenesis and low serum. However, as outlined in Chapter 4, serum deprivation for 24 hours was able to normalise Ang-2 expression in VWF-deficient cells. This does not hold true for proliferation, where in conditions where inhibition of VWF increase EC proliferation, Ang-2 levels remained increased in VWF-deficient cells. In addition, Ang-2 alone did not appear to induce EC Matrigel tube formation and proliferation. Surprisingly, in control cells, Inhibition of Ang-2 was able to

significantly reduce both proliferation and Matrigel tube formation. Since VWF controls WPB and has been shown to bind to other pro-inflammatory molecules we therefore cannot rule out their contribution to the proliferative and tube formation processes. This may suggest that the role of VWF in proliferation may be growth factor dependent.

While these models can recapitulate separate aspects of angiogenesis, because of the versatile nature of Ang-2, the absence of Ang-1 and the resultant effect on Tie-2 signalling the results obtained may be difficult to interpret. The more complex fibrin bead assay provided a better *in vitro* model for studying the effects of Ang-2 in the VWF dependent control of angiogenesis. Previous studies have identified an important role of the Ang/Tie-2 system in this model (Newman et al., 2011). Using this model we demonstrated for the first time that Ang-2 mediates the VWF dependent increase in sprouting. These results presents an interesting avenue for exploration in future *in vivo* studies.

The VWF-deficient mouse has enhanced vascularisation in the mouse ear demonstrating a role for VWF in embryonic vascular development. Surprisingly inhibition of Ang-2 *in vivo* demonstrates that Ang-2 is dispensable for embryonic vascular development and overexpression studies have shown a lethal phenotype. Since Ang-2 levels are only increased in the heart of the VWF-deficient mouse, we profiled the vascularisation. We did not observe a change in the venous marker endomucin (dela Paz and D'Amore, 2009) however using isolectin staining we identified an increase in vascularisation. These results are in line with previous findings in the VWF-deficient mouse, which showed enhanced vascularization in the ear.

We therefore focused our attention on the heart of the VWF-deficient mouse. The miles assay has been used to assess vascular permeability in numerous mouse models (Radu and Chernoff, 2013) including the VWF-deficient mouse (Hillgruber et al., 2014, O'Regan et al., 2016). While conflicting vascular permeability results have been reported in VWF-deficient mice in response to various stimuli, the suggestion was no difference in permeability at baseline. Studies have predominately focused on the blood brain barrier in the VWF-deficient mice at baseline but these reveal no difference between VWF deficiency and littermate controls. In this study, we have also demonstrated no difference in baseline permeability in

the heart or lungs of VWF-deficient mouse using the miles assay. These studies rely heavily on the ability of albumin to pass across the endothelial layer. The ability to detect permeability at baseline relies on a number of factors including pressures of vascular beds, surface area, and heart rate to name a few (Radu and Chernoff, 2013). Work from (Yuan et al., 2016) has demonstrated that VWF-deficient mice have decreased cardiac output at rest. The implication for the effect on permeability remains unknown. Since the VWF-deficient mice have no gross defects at baseline and are born in normal mendelian inheritance (Denis et al., 1998), we speculated that the use of a stressor would allow to identify an underlying defect. The premise of LPS as an inducer of permeability to reveal subtle differences in permeability in the VWF-deficient mouse was hindered by a lack of a time course in permeability in this model. Using a more sophisticated model of permeability assessment, we identify enhanced vascular permeability in the VWF KO mice compared to littermate control. Specifically, the translation of these studies remain to be seen.

To date, no reports exist for developmental defects in patients with VWD. However, micro vessels of the nail-bed of VWD patients have been reported to show enhance permeability and increased tortuosity (Blackburn, 1961, Koscielny et al., 2000). This would suggest that the GI tract may not be the only place for vascular malformations. Morever, Ang-2 expression has been recently reported to be increased with regions of the colon affected by angiodysplasia (Holleran et al., 2015). In the current study we provide a plausible hypothesis for how VWF may contribute to vascular malformations through regulation of Ang-2 expression.

In summary, data from this chapter demonstrates that inhibition of VWF does not affect Tie-2 levels but affects Tie-2 signalling. We provide new evidence to show that Ang-2 mediates the VWF dependent increase *in vitro* sprouting. While enhanced genetic deletion of VWF is associated with enhanced vascularisation in this tissue there is also an increase in the leakage of the small molecular weight dextrans suggesting microvascular defect. It remains to be seen whether Ang-2 mediate these effects. In Chapter 6, I propose how these can potentially be addressed in future work.

6 Chapter Six: Final Discussion and Future Work

6.1 Summary of findings

In Chapter 3 of this study, I showed that inhibition of VWF expression in HUVEC caused enhanced release of the proangiogenic protein Ang-2. In addition, inhibition of VWF expression caused reduced IL-8 protein levels in cell supernatant. Despite enhanced release of Ang-2, VWF-deficient cells did not show any difference in Ang-2 intracellular levels. In addition, VWF-deficient cells showed increased Ang-2 mRNA levels. IL-8 mRNA levels on the other hand remained unchanged with decreased protein in total cell lysates and decreased release after inhibition of VWF expression. In addition, in EC isolated from a type 3 VWD patient, enhanced Ang-2 synthesis and released were observed. Collectively, these data show that VWF regulates Ang-2 levels in ECs. *In vivo*, global deletion of VWF results in enhanced Ang-2 mRNA in the heart but not lungs or liver of the VWF-deficient mouse. Moreover, circulating levels of Ang-2 were similar in the VWF-deficient mouse compared to controls. In patients with both qualitative and quantitative defects in VWF, Ang-2 circulating levels were not significantly different compared to controls. These data show that Ang-2 levels in the circulating are not affected by the absence of VWF. Moreover, these data suggests that tissue-specific regulation of Ang-2 in the VWF-deficient mouse may contribute to the lack of difference in circulating levels.

Chapter 4 of this study examined the role of VWF in regulating Ang-2 mRNA levels. These experiments showed that inhibition of VWF regulates integrin β3 levels and signalling in EC via the adaptor molecular FAK. Downstream rescue of Akt and FOXO1 normalised the VWF-dependent increase in Ang-2 mRNA levels. This suggested that VWF regulated Ang-2 mRNA levels in an Akt-FOXO1 dependent manner. VWF from a commercial source was able to stabilise integrin β3 levels, but this was not observed in the presence of in house purified VWF. We speculate that purity of VWF may affect the ability of VWF to stabilise integrin β3. When immobilised, both in house and commercial VWF increased Ang-2 mRNA levels; however, in the absence of VWF, no effect was observed. Since commercial VWF did not regulate Ang-2 mRNA levels in the supernatant, we propose that VWF conformation may be important for its function in regulating EC gene expression. Interestingly, these studies also showed that atorvastatin appeared to significantly reduce Ang-2 mRNA levels in VWF-

deficient cells. Together these data suggests that VWF-dependent control of Ang-2 appears to be Akt-FOXO1 dependent.

Chapter 5 of this study focused on understanding the role of Ang-2 in the VWF-dependent control of blood vessel formation and function. VWF binding to Ang-2 appear to inhibit the ability of VWF to bind to ECs. In the absence of Tie-2, VWF bound to Ang-2 did not affect VWF binding to EC. These data may suggest a role for Tie-2 in VWF-dependent cell adhesion. Inhibition of Ang-2 in VWF-deficient cells did not affect all aspects of *in vitro* angiogenesis. In particular inhibition of Ang-2 function normalised only the VWF-dependent increase in sprouting angiogenesis. These data suggests that the VWF-dependent increase in endothelial sprouting is Ang-2 dependent. *In vivo*, cellular infiltrate a surrogate marker of vascularisation in the VWF-deficient mouse was not increased in the presence of VEGF. In addition, global deletion of VWF enhanced vascularisation of the mouse heart where Ang-2 mRNA levels are increased. Furthermore, enhanced leakage of small molecular weight dextrans from these vessels suggested increased permeability. These data points towards a potential role for Ang-2 in regulating cardiac vascular permeability in the VWF-deficient mouse.

6.2 BOEC as a tool for studying the cellular basis of von Willebrand Disease

The essential role of VWF in haemostasis is evident from qualitative or quantitative defects in the protein resulting in the bleeding disease Von Willebrand Disease. Identifying mutations in VWD patients has helped improve our understanding of structure-function of VWF. HUVEC with the use of gene silencing technology has provided a useful tool for assessing the effect of quantitative defects in VWF on EC biology. However, assessing qualitative defects in VWF in EC biology have proven a major limitation of HUVEC. One novel approach to not only provide gene silencing but mutagenesis is by the CRISPR/Cas9 system. This approach has however had limited use in primary cells such as HUVEC due to their poor replicative lifespan. A recent study, as a proof of concept, utilised BOEC to demonstrate their potential to overcome this barrier to gene manipulation in ECs using CRISPR/Cas9 (Abrahimi et al., 2015). Moreover, this provides a potential tool for correcting gene mutations in ECs directly from patients and as a model, studying specifically the effect of mutations on EC biology.

The use of VWF mutants in non-ECs has limited capacity to fully recapitulate the processing, maturation and storage of VWF Two recent studies on BOEC from VWD patients have demonstrated that these cells can be used to characterise the cellular defects in VWF processing, storage and release within the endothelial context (Starke et al., 2013, Wang et al., 2013). In addition, BOEC isolated from type 3 VWD patients have identified heterogeneity in VWF expression and processing within a cohort where plasma levels of VWF are absent, therefore believed to be linked to lack of synthesis (Casey et al., 2013). As described in chapter 1, these cells have been used to characterised unexplained clinical phenotypes in a patient with decreased FVIII levels when the observed mutation did not explain this result (Berber et al., 2009).

Work from our lab demonstrated an overall increase in a panel of *in vitro* angiogenesis in BOEC isolated from VWD patients, and although differences between subtypes was observed the numbers investigated were too small to be conclusive (Starke et al., 2011). A recent study showed a defect in in vitro angiogenesis only in type 3 VWD BOEC (Groeneveld et al., 2015). In this study, the authors identified that loss of angiogenic capacity was observed in BOEC from type 3 VWD patients earlier compared to controls. Work from our lab has identified enhanced Matrigel tube formation in type 3 VWD patient (Starke and Randi unpublished). In addition, these studies have identified that while differences in proliferation were observed between types 1 and type 2 patients, all BOEC had impaired directionality (Starke and Randi unpublished). Given the lack of standardisation of *in vitro* systems for angiogenesis across different labs, it is difficult for comparison of results between BOEC due to such heterogeneity in patients; larger studies will be required to define the specific characteristics of each subtype.

In this thesis, we show increased Ang-2 release from type 3 BOEC. In chapter 3 we show that inhibition of VWF does not regulate all WPB in the same manner. Future studies will investigate other WPB in BOEC from VWD patients. Given the heterogeneity in VWF processing and maturation in VWD BOEC, analysis of WPB proteins may provide novel insight into how VWF direct proteins towards WPB. In particular a study from the James' lab in Canada identified that Ang-2 release was increased in patients with type 2A and type 2B VWD (Casey, 2015). Since McKinnon and colleagues have identified that VWF binds to Ang-2 in the

A1 domain, this provided an interesting insight into VWF regulation of Ang-2. Important studies investigating the expression of WPB in BOEC are needed to fully understand the role of VWF in regulating WPB proteins.

Additionally, in this study we show that circulating Ang-2 levels are unaffected in a small cohort of patients with qualitative and quantitative defects in VWF. A recent study by Groeneveld et al identified some changes in Ang-2 plasma levels in a larger cohort of type 1, type 2 and type 3 VWD patients (Groeneveld, 2015). In type 1 and type 2 VWD patients, Groeneveld and colleagues reported a significant decrease in circulating Ang-2 levels. In the type 3 VWD patients measured the decreased levels of Ang-2 did not reach statistical significance. Interestingly, circulating Ang-2 levels are unaffected in the VWF-deficient mouse; this may be linked to the findings that VWF controls Ang-2 expression in a tissue specific manner. In order to conclude on the regulation of Ang-2 levels in patients and whether these are affected by VWF, it would be important to be able to characterise Ang-2 release from BOEC as well as plasma levels from the same patient. To date no study has provided such an extensive investigation. Using BOEC we may be able to identify underlying defects which may otherwise remain hidden given the multifunctional nature of VWF.

6.3 VWF regulation of blood vessel formation and function via Ang-2-Tie-2 pathway

In 2011, work from our lab revealed by analysis of the ear vascularisation by whole mount staining, increase density of blood vessels of VWF-deficient mouse compared to controls (Starke et al., 2011). More recent studies have shown enhanced remodelling and tip cell formation also in the retina vasculature of VWF-deficient new born mice (Starke and Randi, unpublished). These data demonstrate that VWF not only contributes to embryonic development but also post-natal angiogenesis. In the current study, analysis of the cardiac vasculature revealed that the VWF-deficient mouse heart has increased vascularisation, in line with evidence in the ear. In this study we proposed that enhanced Ang-2 in the mouse heart may have contributed to this effect; further studies including genetic deletion of Ang-2 on a VWF-deficient background will be required to investigate this hypothesis.

In contrast, global overexpression of Ang-2 in ECs results in embryonic lethality at E9.5 – 10.5 (Maisonpierre et al., 1997). In this study, the authors also highlight that Ang-2 unlike Ang-1 is not expressed in the cardiac vasculature during embryonic development around E9.5-10.5. It is therefore unlikely that increased Ang-2 expression alone in the heart would cause enhanced vascularisation of the VWF-deficient mouse. Moreover, forced expression of Ang-2 during adulthood has not been reported to cause enhanced angiogenesis. In part, these somewhat confusing observations may be explained that different responses of vascular beds to Ang-2 expression. In one study, increased expression of Ang-2 in the retinal vascular bed in the absence of VEGF induces vascular regression (Lobov et al., 2002). This would suggest a context dependent role of Ang-2 *in vivo*.

The data presented in this thesis confirms enhanced vascularisation in the mouse heart in the VWF-deficient mouse. Utilising a dextran method of assessing permeability, we identify increase leakage of these vessels which only occurs with the small molecular weight dextran. Since we also identify enhanced Ang-2 expression in the heart tissue of the VWF deficient mouse, it is possible that this could contribute to the vascular leak observed in the VWF-deficient mouse. Since we lack a model for angiodysplasia, assessment of vascular permeability in the VWF-deficient mouse using the dextran model may provide a method for assessing the effect of VWF on vascular function which may be relevant to understanding angiodysplasia.

Crucial to this study was work which demonstrated that inhibition of Ang-2 using a functional blocking antibody was able to normalise the enhanced *in vitro* endothelial sprouting caused by inhibition of VWF expression in HUVEC. We found that Ang-2 did not mediate the VWF dependent increase in *in vitro* proliferation nor Matrigel tube formation. While inhibition of Ang-2 using the Ang-2 neutralising antibody provides a great model for in vitro studies of the role of Ang-2, *in vivo* its use is someone complicated by a system which lacks active angiogenesis. In this study we have established a new model of assessing cardiac vascular permeability in the VWF deficient mouse which can be used to assess another function of Ang-2. In other reports, transient increase in Ang-2 has been shown to induce vascularisation of the retinal vessels (Oshima et al., 2005). Ongoing experiments in our lab are aimed at understanding whether the effects of Ang-2 are limited to developmental or postnatal

angiogenesis. Since the VWF-deficient mice have been reported to show cardiac defects at baseline an important question of further studies would be to establish to what extent Ang-2 may contribute to this phenotype.

Finally, one aspect of blood vessel formation unexplored in this study was the role of VWF-Ang-2 pathway in arteriogenesis. Recent work from our lab has shown that ligation of the hind limb in the VWF-deficient mouse causes increased angiogenesis but decrease arteriogenesis and collateral blood flow (Starke, Deindl and Randi unpublished). A previous study in the literature has linked enhanced Ang-2 expression to impaired collateral blood flow (Reiss et al., 2007). A more recent study has suggested that this effect of Ang-2 may be linked to the ability of Ang-2 to regulate macrophage polarisation. To what effect this may contribute to arteriogenesis remains unclear (Tan et al., 2016). This study however has provided us with enough background evidence for a clear role of Ang-2 in regulating arteriogenesis and blood flow after hind limb ischaemia in the VWF deficient mouse. Inhibition of Ang-2 using the Ang-2 functional blocking antibody may help to address how Ang-2 may contribute to this process in the VWF-deficient mouse.

6.4 Intracellular von Willebrand factor regulate pathways to control gene transcription in endothelial cells

In this study we identified that inhibition of VWF expression in EC regulated the transcription of a number of genes. In addition to showing increased Ang-2 mRNA levels we identify increase CXCR4, BMPR4, Cyclin G2 and Elk-3. In addition, Cyclin D1 and p21 were also increased after inhibition of VWF. To our knowledge this is the first study to demonstrate that inhibition of VWF expression can regulate the expression of genes in ECs. In this study we have not investigated whether extracellular VWF and intracellular VWF can activate differential signalling pathways in ECs. Given that we have previously found that inhibition of VWF expression cause reduced ICAM-1 expression (Smith, Starke and Randi unpublished), which is unaffected after stimulation of VWF (Potapova et al., 2010), it is tempting to speculate that this is indeed the case. In this study we show that stimulation of control HUVEC with VWF for 24 hours did not affect Ang-2 mRNA levels in VWF-deficient cells but increased Ang-2 expression in control cells, most notable in the presence of commercial VWF. It would

be worth exploring further the mechanisms into this. Since MAPK regulates Ang-2 expression, it could be suggested that Ang-2 mRNA are increased due to VWF stimulation of MAPK. Further examination of the effects of immobilised VWF vs inhibition of VWF using gene expression profiling may be necessary in order to really understand the role of VWF in regulating gene transcription.

Decreased Akt phosphorylation in VWF-deficient cells allowed us to identify at least one mechanism through which VWF can control Ang-2 expression. In particular, FOXO1 has been shown to regulate a number of EC genes and processes (Daly et al., 2004, Potente et al., 2005). Importantly, we have shown that FOXO1 activity in VWF deficient cells could be reduced by the use of atorvastatin treatment. This finding has important implications for patients with VWD associated angiodysplasia. Only isolated cases of VWD patients responding to statin therapy have been reported and larger trials are needed to elucidate efficacy in statin therapy. This therapeutic approach may bypass the extracellular effects of VWF inducing downstream suppression of Ang-2. Future studies to establish a working *in vivo* model of angiodysplasia may be required to test this hypothesis.

In this study since mTOR is the primary regulator of cell size (Fingar et al., 2002), we investigated whether mTORC1 activity would be enhanced in the absence of VWF. However, we found that mTORC1 activity appeared to be decreased. Enhanced FOXO1 activity has been demonstrated to decrease mTORC1 levels via activation of sestrin 3 (Chen et al., 2010). Interestingly, increase FOXO1 activity induces the expression of Rictor which is a key activator of mTORC2 complex(Chen et al., 2010). Importantly for this work, recent data has implicated mTORC2 activity in regulation of endothelial sprouting (Wang et al., 2015, Farhan et al., 2015). This presents a potential new avenue for exploration in VWF deficient cells and could suggest a novel pathway through which VWF could regulate angiogenesis.

In this study I have shown that VWF regulates the levels of integrin $\beta 3$ and phosphorylation of FAK and Akt. We have also identified that inhibition of VWF causes increased FOXO1 activity which drives the expression of Ang-2. Ang-2 does not mediate all the effects of VWF on angiogenesis; however, *in vivo* evidence suggests enhanced Ang-2 is associated with increased permeability in the heart of the VWF-deficient mouse (summarised in Figure 6.1).

It remains to be see whether this contributes to cardiac defects observed in this mice. Finally, while it remains to be validated, Ang-2 may eventually be considered a novel therapeutic target in VWD-associated angiodysplasia.

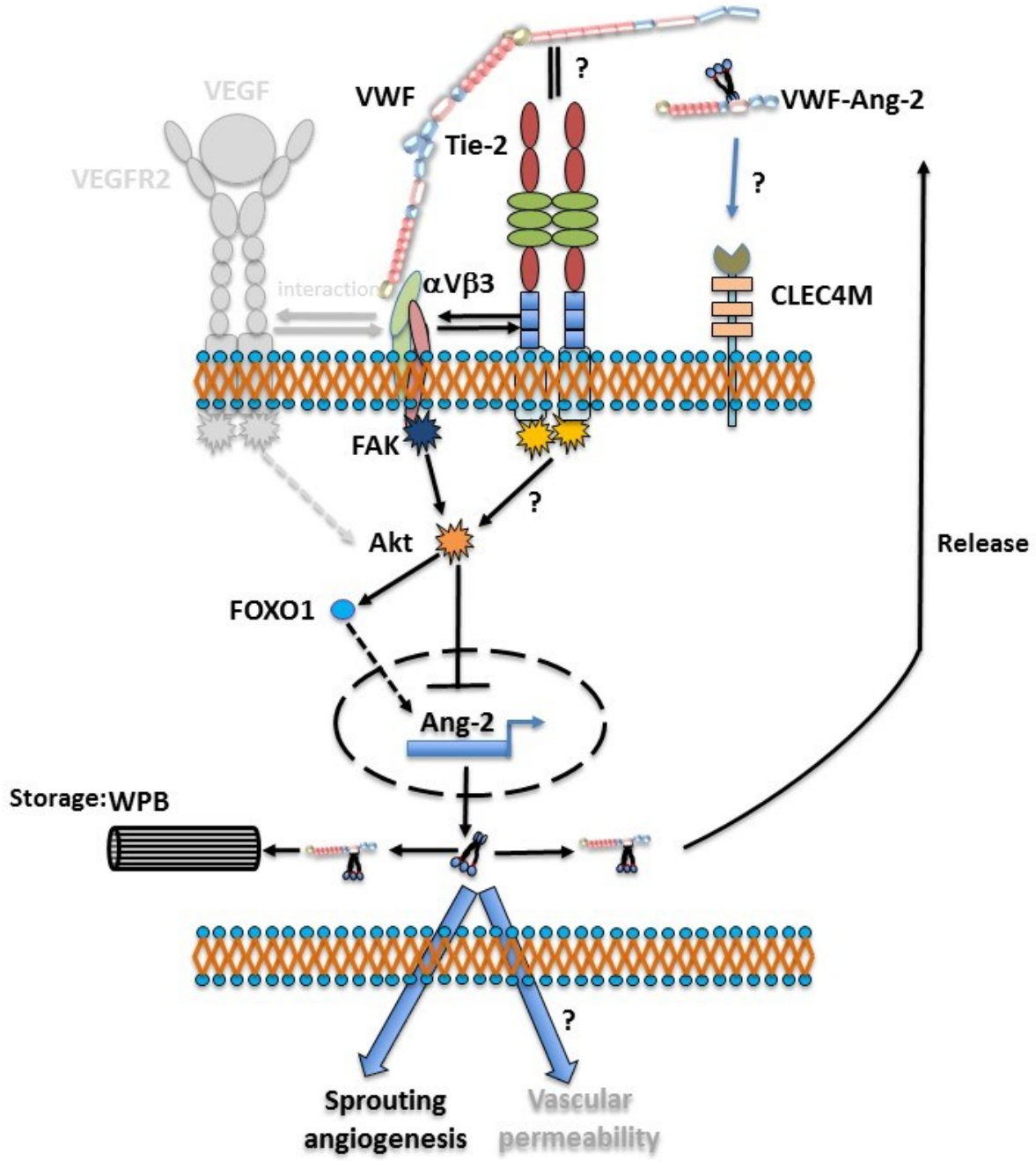

Figure 6.1 Model of VWF control of blood vessel formation and function via Ang-2. On the cell surface VWF interacts with a number of cell surface molecules. VWF binds to the integrin β3 and regulates signalling. Integrin signalling via FAK and Akt, suppresses Ang-2 expression via the control of the transcription factor FOXO1. Intracellular VWF controls the formation of WPBs where Ang-2 and VWF physically interact and are stored. Ang-2 is bound to VWF in the circulation and may be regulated by similar clearance mechanisms. Inhibition of VWF expression results in enhanced Ang-2 transcription via enhanced FOXO1 activity. In addition loss of the storage organelle WPB causes increase Ang-2 release in VWF-deficient ECs causing increase sprouting angiogenesis and potentially vascular permeability.

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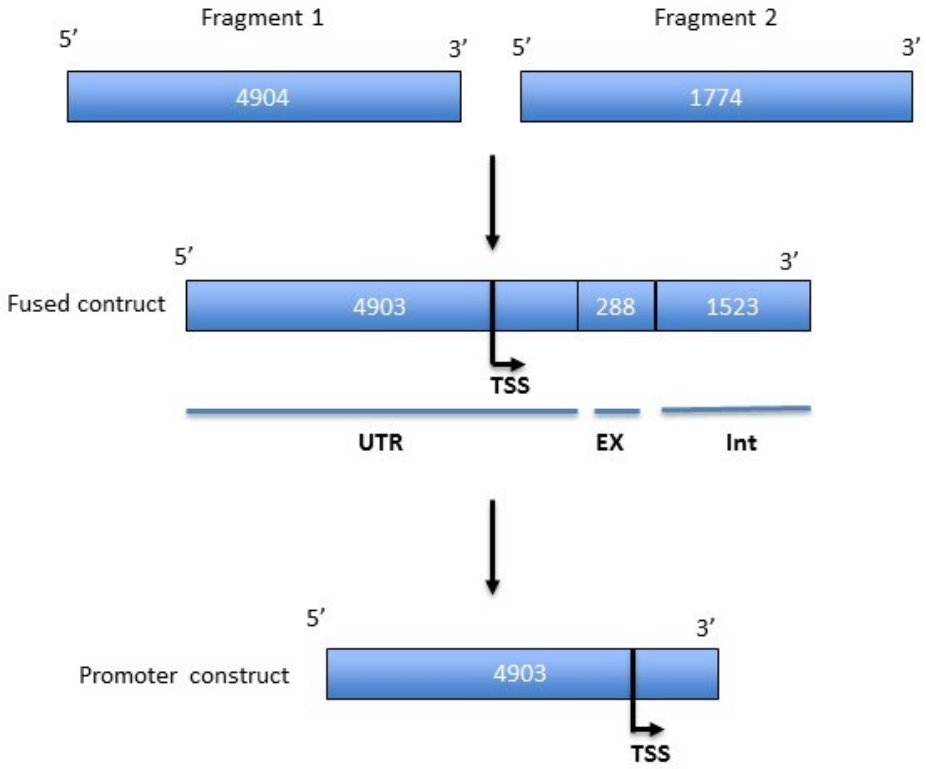
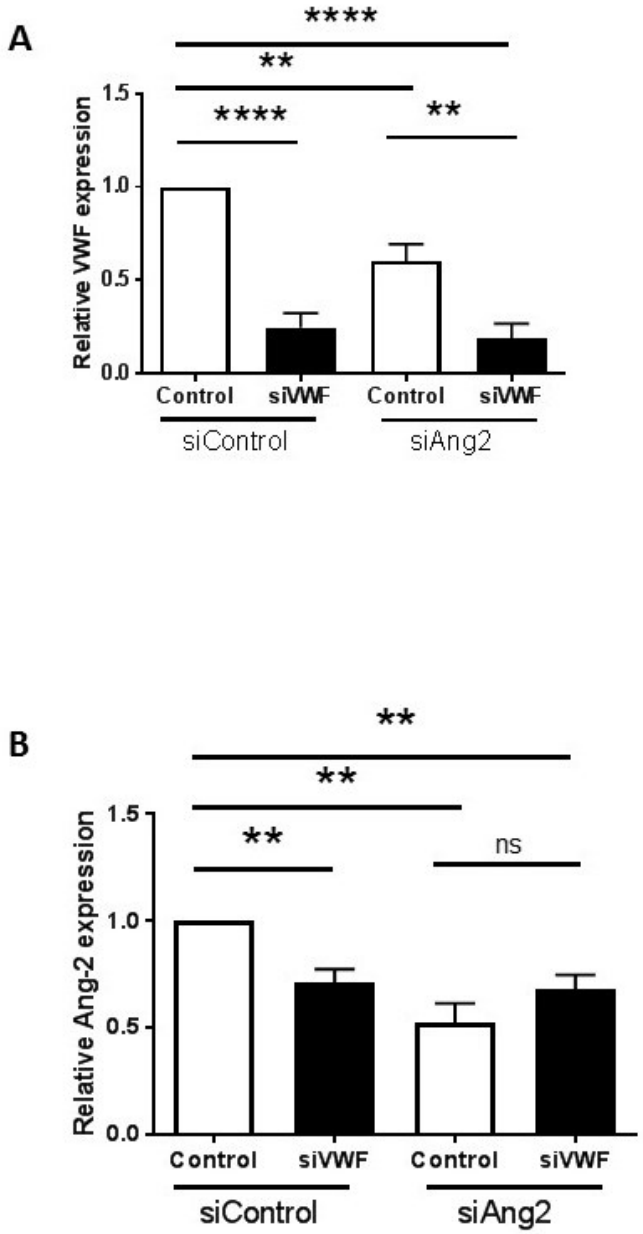
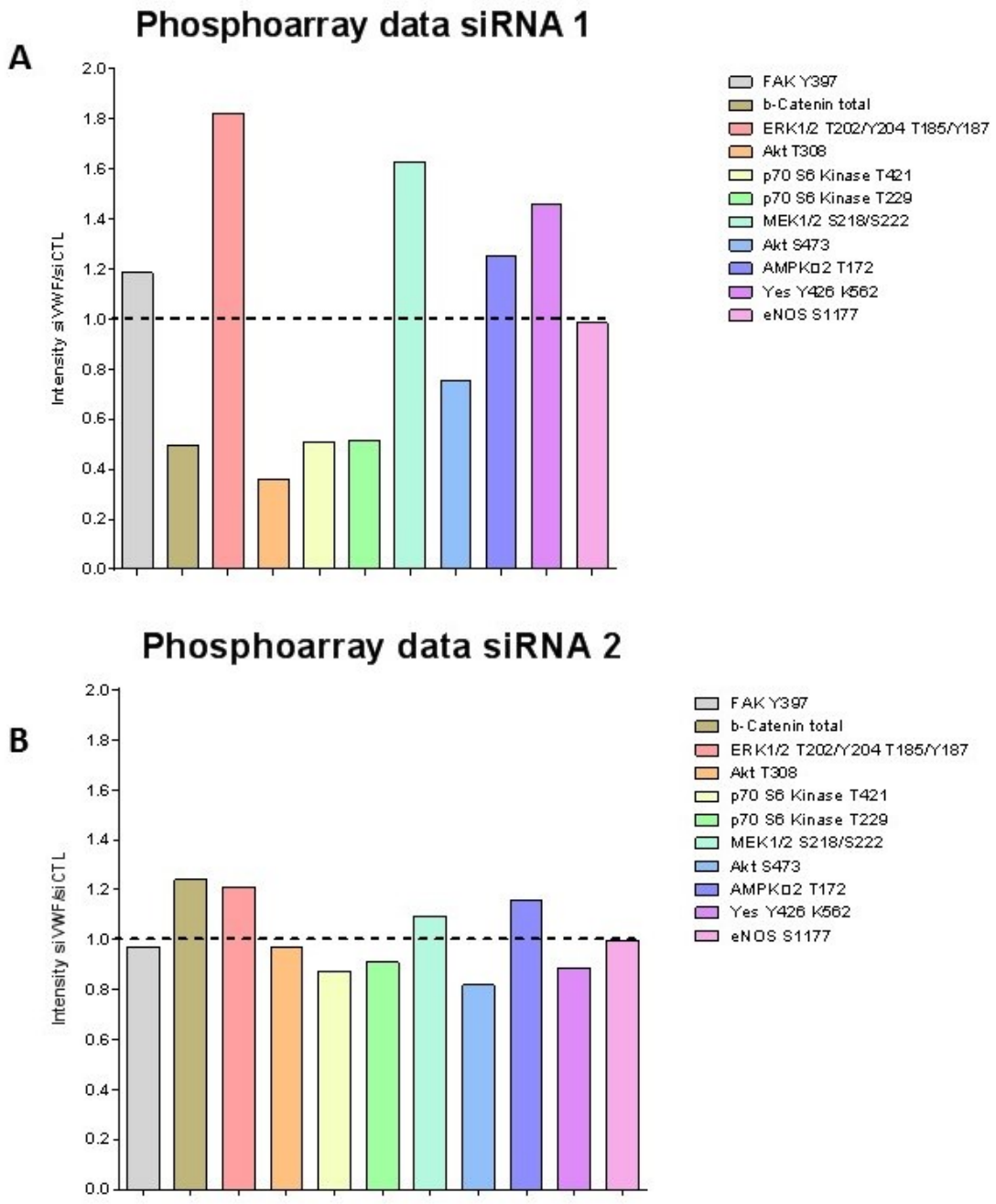
Appendicies



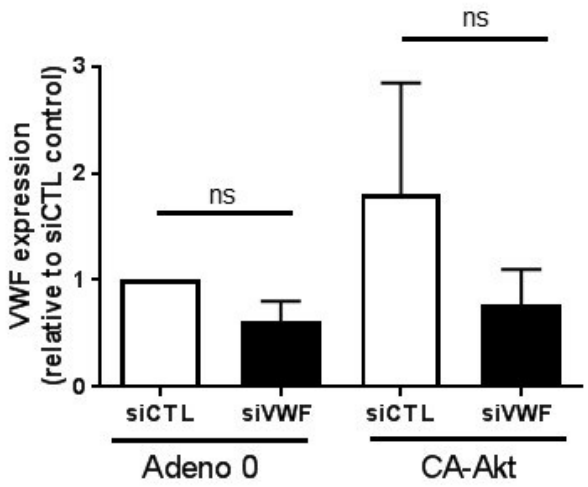
Appendix 1 Cloning of Ang-2 gene and promoter sequence from Hegen et al 2004. Two fragments of the Ang-2 gene were generated after cloning from phage plaques after using a genomic library from Hela cells. Fragment 1 was generated by digestion with BamH1 and HindIII and fragment 2 was digested from HindIII. The two fragments were fused to contain a 4903 untranslated 5' region of the Ang-2 promoter (UTR), coding 288 bp region (Ex) and the first intron of 1523 (Int). The Ang-2 promoter was identified in the 5' 4903 region and cloned into a Pgl3 vector.



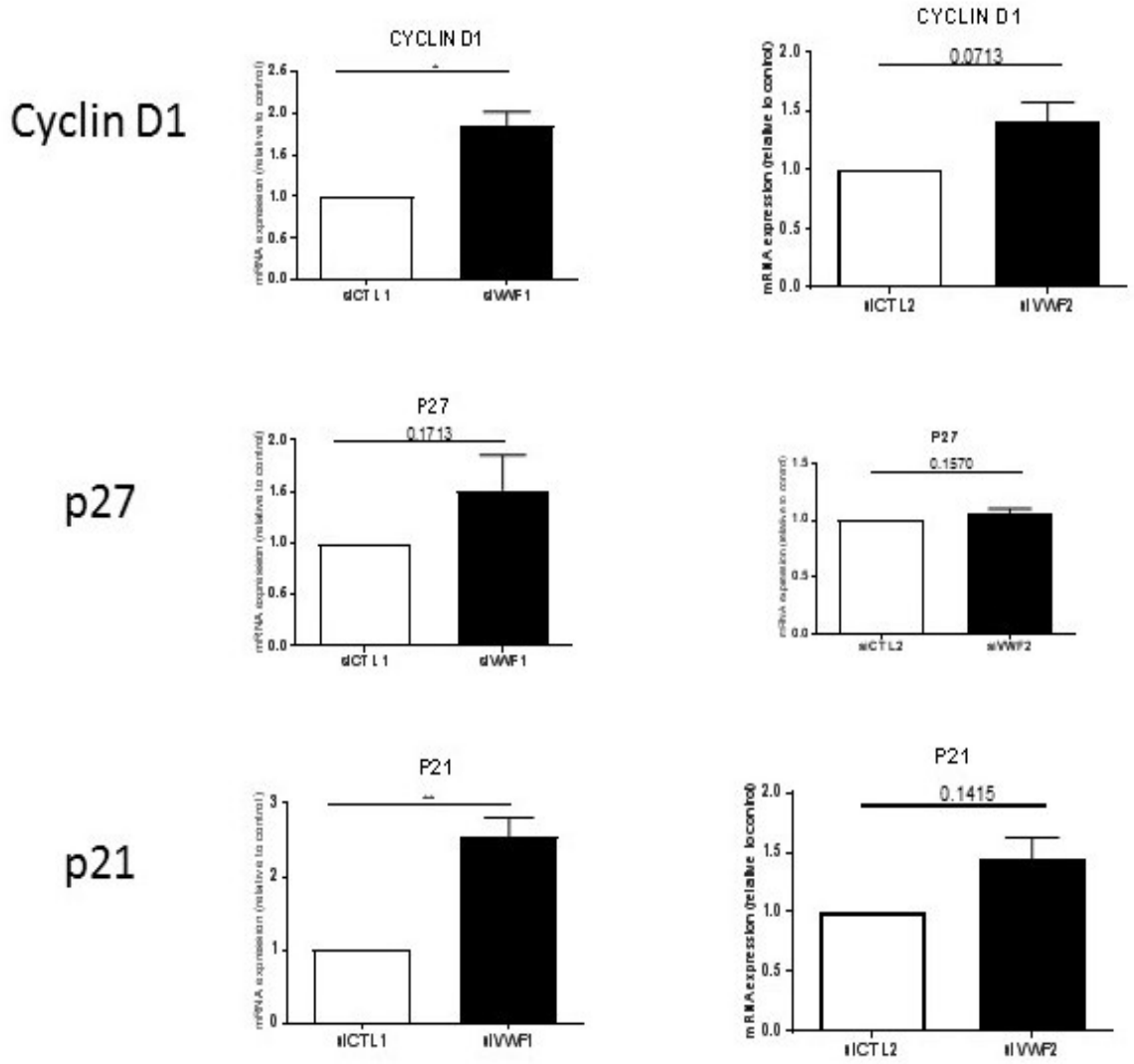
Appendix 2 Optimising Ang-2 siRNA and VWF siRNA transfection in HUVEC. HUVEC were transfected with control or VWF siRNA sequence for 48 hours combined with control or Ang-2 siRNA sequence. (A) VWF expression and (B) Ang-2 expression were measured by qRT-PCR. Data presented as mean ± SEM of n=3. Statistical significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, **** $p\leq0.0001$



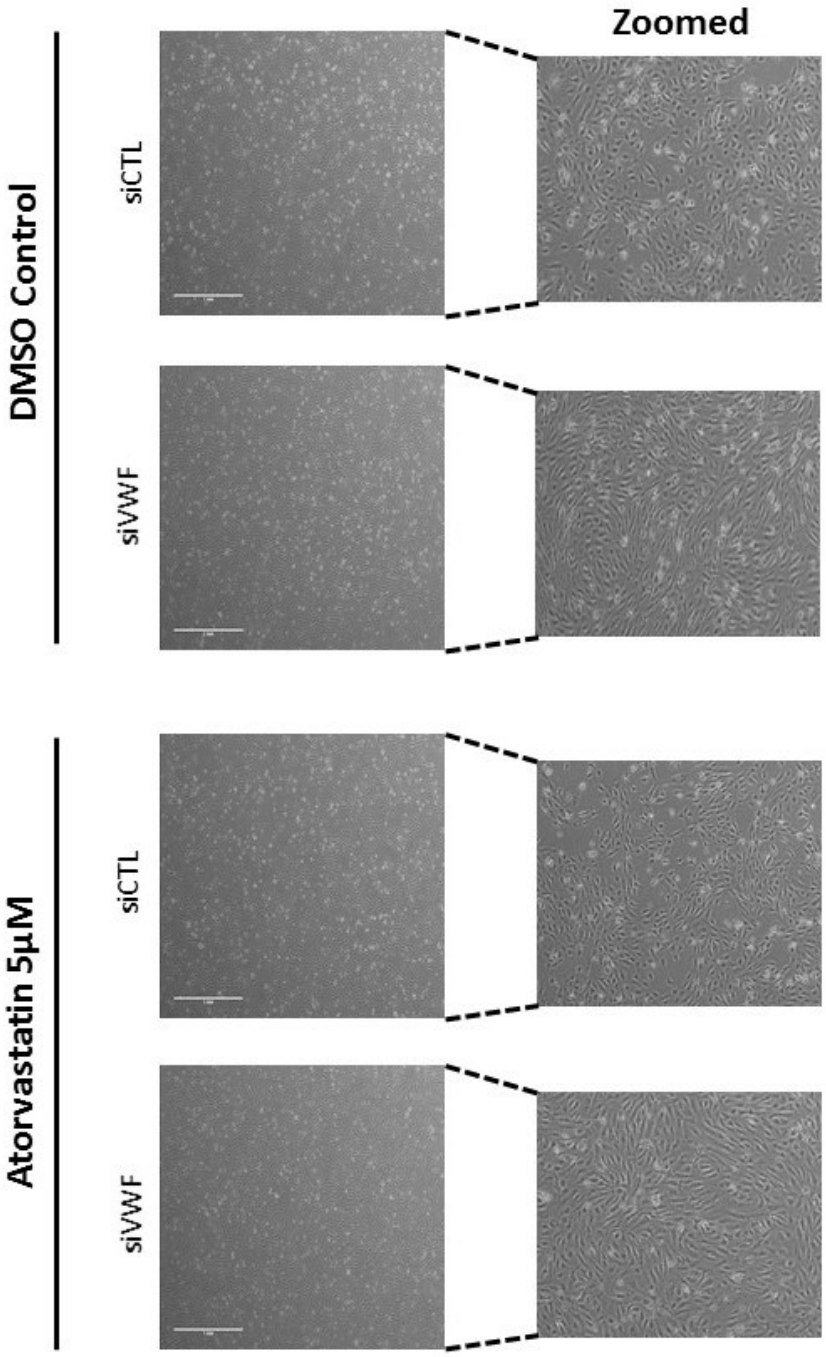
Appendix 3: Phosphokinase array of siCTL and siVWF treated HUVEC. Phosphokinase array was performed in siCTL1 and siVWF1 treated HUVEC (A) and siCTL2 and siVWF2 treated HUVEC (B). Data was normalised to siCTL expression. Experiment performed by Dr. Richard Starke



Appendix 4 Optimising Akt Adenoviral transduction in HUVEC. HUVEC were transfected with control or VWF siRNA sequence for 24 hours. HUVEC were then transduced with Akt adeno virus or Adeno0 for 24 hours. RNA was collected and VWF expression was measured by qRT-PCR. Data presented as mean ± SEM of n=3. Statistical analysis performed by student's t-test. ns $p>0.05$



Appendix 5 Screen of FOXO1 cell cycle targets in VWF-deficient cells. HUVEC were treated for 48 hours with siCTL1 or siVWF1 (Left panel) and siCTL2 and siVWF2 (Right panel). RNA was collected and Cyclin D1, p27 and p21 was measured by qRT-PCR. Data presented as mean ± SEM of n ≥ 3. Significance determined by student's t-test. ns $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$ (qRT-PCR analysis performed by Elizabeth Beales).



Appendix 6 HUVEC treated with Atorvastatin 5 µM. HUVEC were treated with siRNA control and VWF sequences for 24 hours. Cells were then treated with Atorvastatin at 5 µM for a further 24 hour. Images were captured using an Olympus camera using X4 objective. Scale bar represents 1 mm.

MOUSE MONITORING SCORE SHEET				
	0	A	B	C
Coat/Appearance	Normal	Rough/Lack of grooming/Piloerection	Unkempt, thin, wounds Staring coat	Severe unkempt appearance
Behaviour	Normal	Quiet, but responsive and alert	Quiet, less responsive but alert Unprovoked and abnormal vocalisation	Complete inactivity even when provoked
Posture & Mobility	Normal	Mildly hunched but standing Reduced mobility	Moderately hunched but standing Reluctant to move	Hunched and lying in recumbancy
Breathing	Normal	Rapid, shallow	Rapid, abdominal	Laboured, blue
Movement	Normal	Slight incoordination/ abnormality	Uncoordinated, walking of toes, reluctant to move	Staggering, paralysis, limb dragging
Eating/Drinking	Normal	Increased/Decreased for 24 hrs (Only if food intake measured specifically)	Increased/decreased for 48 hrs	Increased/decreased >48 hours
Condition	Normal	Thin	Loss of fat, no growth	Loss of muscle
Dehydration	Nil	Skin less elastic	Skin tents	Skin tents, eyes sunken
Faeces/Urine	Normal	Moist faeces	Diarrhoea or dry faeces, abnormal urine (volume/colour)	Uncontrolled diarrhoea, blood in faeces. Nil urine or incontinent
Temperature	Normal	<36.5°C or >37.5°C	<30°C or >39°C	<25°C or >40°C
Body Weight	Normal	Markedly reduced growth (e.g. severe runting) 5% over 24 hours	Weight loss >15% (or up to 10% in 24 hours)	Weight loss >10% over 24 hours or >20% over time
Surgery Site/Wound	Normal, healing	Discharge	Discharge ++ or gap forming	Bleeding heavily, wound reopened, red
Other Information	If surgery has taken place, please indicate time, date and surgeon			
	Specific requests/comments regarding mice/strains should be recorded on the database and made by email, these forms are ONLY for daily monitoring			

Scoring was obtained from counting categories at end of experiment and 1 added for statistical analysis
Score 0: Normal behaviour
Score A: 1
Score B: 2
Score C: 3

Appendix 7 Mouse behaviour scoring sheet (Courtesy of Dr. James Leiper). Mice were scored 1 for A category behaviours, 2 for B category, and 3 for C category. Mice obtaining a score of 2-3 from category A or a score in category B or C, advise to continue experiment was obtained from Named Animal Care and Welfare Officers (NACWO) or Advanced animal technicians. For statistical purposed a score of 1 was added to all mice at the end of experiments.

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