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The Role of WT1 in Ischaemic Angiogenesis

Robert James Ogley

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Declaration

The thesis herein is solely my own work. Apart from where stated the experiments were performed entirely by me. I confirm that this work has not been previously submitted for any other degree.

Acknowledgements

I am not the same scientist, or indeed person, on completion of this PhD as I was when commencing it. This was by no means a solo effort, however, and I am in no doubt that I would never have made it to this point without the invaluable help of a number of people. For this I could not be more grateful.

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Abstract

Ischaemia causes irreversible tissue damage in cardiovascular disease. Since regenerative angiogenesis fails to consistently induce sufficient reperfusion to facilitate repair, targeted manipulation of angiogenesis is clinically desirable. The Wilms' tumour suppressor (*Wt1*) is a transcription factor which regulates numerous genes and cellular processes, including many intrinsic to angiogenesis. We hypothesise that WT1 in the endothelium influences the angiogenic function of endothelial cells.

WT1 was identified in endothelial and non-endothelial cells comprising vessel outgrowths generated by cultured aortic rings from WT1-GFP reporter mice. Inducible deletion of WT1 from the endothelium (VE-Wt1 KO) significantly delayed angiogenesis in this assay ($p<0.05$ relative to controls).

In vivo, WT1 expression was evident in vascular endothelial and perivascular cells of the hindlimb as early as 3 days following femoral artery ligation to induce ischaemia, often in cells expressing epithelial and mesenchymal markers simultaneously. However, VE-Wt1 KO had no effect on hindlimb reperfusion (laser Doppler; days 0-28) or on vessel density (day 28). Similarly, VE-Wt1 KO had no effect on vessel density or expression of angiogenic factors (qRT-PCR) in sponges inserted subcutaneously in mice (20 days). To further understand the role of WT1 in angiogenesis, transcriptomic RNA expression analysis was performed in $WT1^+$ and $WT1^-$ cells isolated (FACs) from sponges after implantation in WT1-GFP mice. $WT1^+$ cells exhibited higher expression of genes involved in a number of processes relevant to tissue repair, including angiogenesis ($p=3.11 \times 10^{-8}$), wound healing ($p=3.45 \times 10^{-7}$) and epithelial-to-mesenchymal transition (EMT) ($p=5.86 \times 10^{-4}$).

These results shed new light on the role of WT1 in ischaemic angiogenesis. In concurrence with previously published work, we show that deletion of endothelial WT1 can delay angiogenesis however, WT1 is not just instrumental in endothelial cells in this context. WT1 has a broader role in tissue repair in ischaemia, in part through regulation of cell transition (EMT). This work has improved our understanding of the regulatory role of WT1 in angiogenesis and repair, while revealing a number of novel insights into the

function of WT1. This highlights WT1 as a potentially beneficial therapeutic target to facilitate regeneration in cardiovascular disease.

Lay Summary

In cardiovascular disease, blockage of blood vessels results in some tissues becoming starved of oxygen. Angiogenesis – the formation of new blood vessels from existing vasculature – occurs in response, but often fails to completely resupply blood to these damaged tissues. It is important, therefore, to establish new ways to improve this process. One gene of interest, the Wilms' tumour suppressor (WT1) is a transcription factor and so effectively acts as a 'master switch' to activate a number of other genes. We hypothesise that WT1 acts in blood vessels to regulate the process of angiogenesis and so could be a promising target to improve regeneration of damaged tissues in cardiovascular disease.

WT1 was found to be expressed in blood vessels in two models of angiogenesis simulated in mice; implantation of sponges under the skin and a model of critical limb ischaemia, simulated by tying off the femoral artery. However, WT1 was also expressed in a number of other parts of damaged tissue, including in damaged muscle and immune cells. While reducing the expression of WT1 in the inner cell layer of blood vessels did not seem to reduce angiogenesis in these models, it did reduce angiogenesis in a simpler model. In this model, aortic blood vessels would spontaneously form new blood vessels by angiogenesis when removed from the mouse and grown in a petri dish.

Using a mouse which expresses a green fluorescent protein when WT1 is expressed, we were able to separate out cells which expressed WT1 from those which did not and screen for the expression of thousands of genes to understand what WT1 expressing cells do differently from non-expressing cells. This revealed that WT1 expressing cells were not only involved in the process of angiogenesis, but generally influenced the healing process. These cells were potentially able to change from one specific cell type to another to facilitate regeneration.

This work sheds new light on the role of WT1 in angiogenesis and cardiovascular disease. We show that WT1 is indeed an important factor in angiogenesis and may be able to regulate angiogenesis to improve repair. Intriguingly though, WT1 seems to have a more general role in enabling regeneration and repair of damaged tissues. In summation, WT1 appears to be a promising therapeutic target in the context of cardiovascular disease.

List of Abbreviations

$^{+/-}$KTS – $^{+/-}$ Lysine, Threonine and Serine

5-HT – 5-hydroxytrytamine (Serotonin)

AA – Amino acid

Ach – Acetylcholine

APC – Adipose progenitor cell

CC – *VE-Cadherin CreER*^{T2} control

CD31 – Cluster of differentiation 31

CHIP-seq – Chromatin immune-precipitation sequencing

CVD – Cardiovascular disease

DAPI - 4′,6-diamidino-2-phenylindole

EC_{50} – Effective concentration for 50% contraction

Emax – Pharmacological efficacy

EMT – Epithelial-to-mesenchymal transition

EndMT – Endothelial-to-mesenchymal transition

EPC – endothelial progenitor cell

FACs – Fluorescence-activated cell sorting

FCS – Foetal calf serum

GFP – Green fluorescent protein

HCl – Hydrochloric acid

HIF – Hypoxia induced factor

HLI – Hindlimb ischaemia

HSC – Haematopoietic stem cell

HUVEC – Human umbilical vein endothelial cell

IF – Immunofluorescence

IHC – Immunohistochemistry

IL – Interleukin

KO – knock-out

KO – Vascular endothelial *Wt1* KO (kindly provided by Kay and Nicole Wagner)

KPSS – Physiological saline solution + K^+

MET – Mesenchymal-to-epithelial transition

miRNA – Micro ribonucleic acid

mRNA – Messenger ribonucleic acid

MSC – Mesenchymal stem cell

NA – Noradrenaline

NO – Nitric oxide

PBS – Phosphate-buffed saline

PCNA – Proliferating cell nuclear antigen

PCR – Polymerase chain reaction

PD_2 – $^{-}log^{10}$ EC_{50}

PDGF – Platelet derived growth factor

PDGFR – Platelet derived growth factor receptor

PSS – Physiological saline solution

qRT PCR – Quantitative reverse transcriptase polymerase chain reaction

RA – Retinoic acid

shRNA – Short hairpin ribonucleic acid

siRNA – Short interfering ribonucleic acid

SNP – Serotonin

SSI – Subcutaneous sponge implantation

UTR – Untranslated region

VC – Vehicle control

VEGF – Vascular endothelial growth factor

VE-Wt1 KO – Vascular endothelial *Wt1* knock-out

Wt1 - Wilms' tumour 1 gene

WT1 - Wilms' tumour 1 protein

αSMA – Alpha smooth muscle actin

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CHAPTER 1:

Introduction

Current Understanding of Wt1 and its Role in Cardiovascular Disease

The Wilms' Tumour Suppressor gene (*Wt1*), first identified in 1990 (Call *et al.*, 1990; Gessler *et al.*, 1990), was originally recognised as a gene whose mutation causes the eponymous renal paediatric cancer, Wilms' tumour (Haber *et al.*, 1990). Since then, the number of contexts identified in which WT1 is intrinsic has steadily increased and it is now accepted to be an important regulator in a range of cancers, in embryogenesis, in reproductive tissue and in kidney function, amongst others. On a cellular level, WT1 is known to influence cell growth, cell proliferation, apoptosis, generation of mesenchymal progenitors and crucially, cell differentiation by epithelial-to-mesenchymal transition (EMT) and the reverse process mesenchymal-to-epithelial (MET) (Hastie, 2017).

To imagine a single gene having such diverse functions may seem confounding. However, WT1's far-reaching impact is attributable to its role as a transcription factor; essentially acting as a master switch for expression of other genes, some of which may themselves be transcription factors. Mapping to chromosome 11p13, WT1 is a Krüppel type zinc finger protein (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992). It is primarily involved in transcriptional regulation of DNA, but has also been found to have a role in post-transcriptional RNA processing (Burdach *et al.*, 2012).

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 4 million deaths a year across Europe, 45% of all mortality (Townsend *et al.*, 2016). CVDs such as myocardial infarction and stroke occur when arterial occlusion disrupts the blood supply to crucial organs, resulting in downstream oxygen deprivation and ischaemia. Prolonged ischaemia causes irreversible tissue damage; in CVD this increases mortality and detrimentally impacts quality of life. Angiogenesis, the process of neovascularisation from pre-existing blood vessels may act to resupply blood to ischaemic tissues in CVD, but, regenerative angiogenesis fails to consistently induce sufficient reperfusion to facilitate repair. Therefore, targeted therapeutic manipulation of angiogenesis is clinically desirable.

WT1 is important for regulating vascular progenitors and some developing vasculature, but is absent from adult, quiescent blood vessels (Scholz,

Wagner and Wagner, 2009; Duim *et al.*, 2016). There is increasing evidence that WT1 may have a role in angiogenesis in the adult, both in cancer and in CVD. Following induction of myocardial infarction, WT1 is expressed in the coronary vasculature proximal to the site of infarction (K. Wagner *et al.*, 2002). There is also growing evidence that WT1 is regulated by hypoxia inducible factor 1 alpha (HIF-1α) and, in turn, transcriptionally regulates vascular endothelial growth factor (VEGF), both of which are major angiogenic factors (Graham *et al.* 2006; Hosen *et al.* 2007; Amin *et al.* 2011; McCarty *et al.* 2011; Iranparast *et al.* 2014). Despite recent studies into WT1 in angiogenesis (Wagner *et al.*, 2014; Duim *et al.*, 2015), the role of WT1 in angiogenesis and cardiovascular disease is imperfectly understood.

The work described in this thesis is primarily concerned with the role of WT1 in cardiovascular disease, specifically in the process of angiogenesis (new blood vessel formation from existing vasculature). In order to contextualise the work contained herein, this introduction will summarise the current understanding of the field, to date. First, WT1 will be discussed in more detail, encompassing its molecular biology and the numerous biological processes in which it functions. Then, the response to ischaemia in cardiovascular diseases will be discussed, with a specific focus on angiogenesis; its molecular processes and importance in cardiovascular disease. Finally, these topics will be brought together in a summary of what is known about WT1 in cardiovascular diseases and ischaemic angiogenesis.

1.1 The ***Wt1*** Gene

Wt1 has a high degree of evolutionary conservation in its sequence homology between humans, mice, rats, birds, amphibians and fish (Miles *et al.*, 1998; Carmona *et al.*, 2001; Ladomery *et al.*, 2003; Yamamura *et al.*, 2005; Eisermann *et al.*, 2008). This demonstrates a degree of indispensability of WT1 on a molecular level, which is reflected on a phenotypic level. Mutations in the *Wt1* gene lead to development of Wilms' tumour, a renal paediatric cancer, due to the essential role of WT1 in kidney development (Dong, Pietsch and Englert, 2015). In mice, homozygous deletion of *Wt1* during development results in complete absence of gonads, kidneys and spleen, with death mid-

gestation due to heart failure (Kreidberg *et al.*, 1993; Herzer *et al.*, 1999). Even in adults, where *Wt1* is only expressed in a small proportion of cells, *Wt1* deletion undermines normal tissue homeostasis, resulting in multiple organ failure (Chau *et al.*, 2011). In a number of diseases, WT1 expression is upregulated and dependent on context, contributes to pathological and regenerative processes. It would not, therefore, be unwarranted to consider WT1 a major transcriptional regulator of physiological and pathophysiological function.

1.1.1 Molecular Genetics of WT1

The mammalian *Wt1* gene is roughly 50kb in length and spans 10 exons on chromosome 11p13 (Hohenstein and Hastie, 2006). *Wt1* encodes a zinc-finger transcription factor with four C-terminus C2H2 Krüppel-type zinc-fingers (Figure 1.1). In this sense, WT1 is a relatively typical zinc-finger transcription factor, with a high specificity for GC-rich regions and each zinc finger capable of binding to three base pairs (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992). It incorporates a transcriptional repression domain between residues 71-180 (Wang *et al.*, 1993) and a transcriptional activation domain between residues 180-250 (Madden, Cook and Rauscher, 1993). Therefore, WT1 is capable of both activating and repressing gene expression.

Combinations of alternative transcription start sites, RNA editing and RNA splicing give rise to somewhere between 24 and 36 human WT1 protein isoforms (Hohenstein and Hastie, 2006; Toska and Roberts, 2014). Four of the most widely studied and functionally important isoforms arise from alternative splicing at two sites; exon 5 and exon 9. In exon 5, there is a possible insertion event of 17 amino acids (+/- 17AA) between the proline/glutamine-rich N-terminus and zinc finger domain of the C-terminal. In exon 9, there is a possible insertion of three amino acids, (lysine, threonine and serine (+/-KTS)) into the linker sequence between the third and fourth zinc fingers. These four major isoforms (+17AA/+KTS, +17AA/-KTS, -17AA/+KTS, -17AA/-KTS) have been shown to be expressed in different cell types, tissues and contexts, and have different functions (Ito *et al.*, 2006; Jomgeow *et al.*, 2006). For example, the +17AA isoforms seem to be co-activated by Par4

which function together to regulate cell survival and proliferation (Richard, Royer-Pokora and Roberts, 2001). Interestingly, isoform-specific WT1 knock-outs also have varying phenotypic outcomes and survival, perhaps suggesting some isoforms are more essential than others. Selective loss of +KTS or –KTS isoforms, both result in neonatal death, though loss of –KTS isoforms induces a more severe and acute effect (Hammes *et al.*, 2001). Conversely, loss of the +17AA isoform (Natoli *et al.*, 2002), or other isoforms generated by alternative translational start sites (Miles *et al.*, 2003), produces essentially no phenotype. It is also worth noting that, from an evolutionary perspective, only the –KTS isoform is conserved across vertebrates (Davies, Bratt and Hastie, 2000).

The +KTS and –KTS isoforms are particularly important to the function of WT1. It was found that, while –KTS isoforms were found in transcription factor domains in the nucleus, +KTS isoforms were found to be associated primarily with splice factors. This prompted, the hypothesis that +KTS isoforms are involved in transcriptional regulation of DNA, while –KTS isoforms have a post-transcriptional role in RNA processing (Larsson *et al.*, 1995). It has subsequently been demonstrated that both isoforms can bind RNA and DNA, though +KTS has a higher affinity for RNA than –KTS, which more readily associates with DNA (Morrison, Viney and Ladomery, 2008). Concurrently, –KTS isoforms bind DNA much more readily than +KTS isoforms (Morrison *et al.* 2008). While both +KTS and –KTS bind to RNA *in vitro*, *in vivo* it appears that +KTS binds RNA much more efficiently and that the first zinc finger of WT1 is important for this RNA binding.

The ability of +KTS to bind RNA, is not unique to WT1 and has been observed in other zinc finger transcription factors (Burdach *et al.*, 2012). While all four zinc fingers bind to DNA, it appears that zinc finger 1 is essential for RNA-binding (Caricasole *et al.*, 1996) while fingers 2 and 3 contribute to the RNA binding affinity (Weiss and Romaniuk, 2009). To date, it has been found that +KTS WT1binds to a constitutive transport element (CTE) within the sequence of unspliced mRNA to facilitate transport from the nucleus (Bor *et al.*, 2006) and shuttles between the nucleus and cytoplasm (Niksic *et al.*, 2004). +KTS WT1 also binds alpha-actinin 1 (ACTN1) mRNA (Nurmemmedov *et al.*, 2010)

and a recent, comprehensive study confirmed that WT1 binds to the 3' untranslated regions (UTRs) of target mRNAs involved in developmental processes and regulates their turnover (Bharathavikru *et al.*, 2017). There is undoubtedly much more to be discovered in relation to +KTS WT1, its function and RNA binding targets.

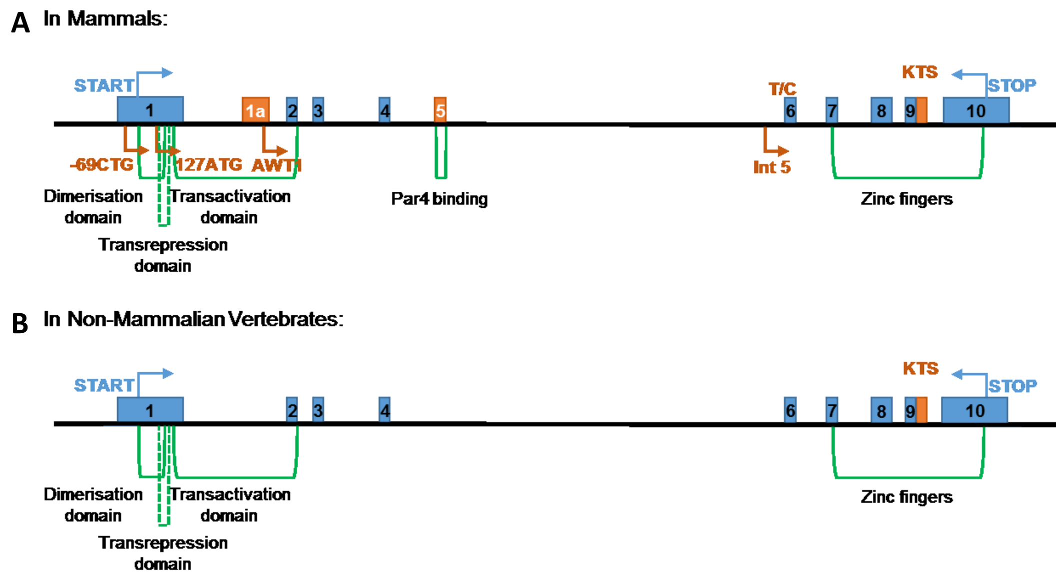

Figure 1.1. The *Wt1* gene. The structure of the WT1 gene is shown in **A)** mammals and **B)** non-mammalian vertebrates. Consistently transcribed exons are shown in blue, with alternative splices, RNA editing and alternative transcriptional and translational start sites in orange. Functional annotations are indicated in green. In mammals, this can give rise to 36 potential isoforms, in non-mammalian vertebrates, only +KTS and –KTS isoforms are currently documented. Adapted from Hastie 2017.

Hundreds of direct WT1 target genes, too many to discuss comprehensively here, have been discovered in mammals, with many more yet to be discovered no doubt (Toska and Roberts, 2014). There is strong *in vivo* evidence for many factors associated with WT1 and Chromatin immune-precipitation sequencing (ChIP-seq) has proved a powerful tool for validating these targets (Kim *et al.*, 2007, 2009; Sunny Hartwig *et al.*, 2010; Motamedi *et al.*, 2014; Kann *et al.*, 2015). Toska & Roberts (2014) review many of these validated direct targets of WT1 and others will be discussed later in this chapter in relation to the relevant roles of WT1. However, these genes are involved in a range of processes including: growth and development, cell differentiation (including

EMT/MET), cell adhesion and cytoskeletal organisation, cell proliferation, cell migration and apoptosis. Many of these direct targets of WT1 are, themselves transcription factors.

A number of high-affinity WT1-binding motifs have been discovered (Rauscher *et al.*, 1990; Wang *et al.*, 1993; Hamilton, Barilla and Romaniuk, 1995; Nakagama, Heinrich and Pelletier, 1995; Little *et al.*, 1996). As an added level of complexity, WT1 also binds to transcriptional co-factors including CREB-binding protein (CBP) (Wang *et al.*, 2001; Essafi *et al.*, 2011), Ewing's sarcoma gene (EWS) (Reynolds *et al.*, 2003), Brain acid soluble protein 1 (BASP1) (Green *et al.*, 2009) and Ten-eleven translocation protein 2 (TET2) (Wang *et al.*, 2015). These WT1 complexes may recognise different sequences, depending on the cofactors. WT1 also binds to a number of proteins involved in RNA turnover including, Wilms' tumour associating protein 1 (WTAP1) (Little, Hastie and Davies, 2000; Horiuchi *et al.*, 2013), U2AF65 (Davies *et al.*, 1998) and RBM4 (Markus *et al.*, 2006). Further, WT1 has regions capable of self-association (residues 1–45 and 157–253), which may act to inactivate the WT1 protein and antagonise its transcriptional regulation (Moffett *et al.*, 1995; Holmes *et al.*, 1997).

Considering the molecular genetics of the *Wt1* gene and WT1 protein; its multiple isoforms, role in transcriptional and post-transcriptional regulation, capacity to bind to DNA and RNA, multiple co-factors and target genes and its ability to self-regulate, it starts to become more evident why it is capable of so many functions and is intrinsic to so many processes. Some of these key roles of WT1 will be elaborated on in the following sections.

1.1.2 WT1 in Development

WT1 is an intrinsic factor in mammalian development with an essential role in normal development of a number of tissues and organs. WT1-null mice fail to develop complete gonads, spleen, kidneys, diaphragm and adrenal glands (Kreidberg *et al.*, 1993; Herzer *et al.*, 1999; Moore *et al.*, 1999; Hammes *et al.*, 2001). These mice die at an embryonic stage from heart failure characterised by malformation of the heart, lack of coronary vasculature and disrupted

epicardial development (Kreidberg *et al.*, 1993; Moore *et al.*, 1999). It is important to understand the role of WT1 in development, not only to understand its role in the adult, but also in disease and regeneration in which a recapitulation of developmental processes often occurs.

1.1.2.1 **Development of the Cardiovascular System**

In the foetal human and murine heart, WT1 is primarily expressed in the mesothelial lining of the heart (the epicardium) with temporal expression in endothelial cells of the cardiac vasculature, during development (Duim *et al.*, 2015, 2016). Cell fate mapping within the developing heart, revealed that a proportion of coronary vascular smooth muscle and endothelial cells derive from the $WT1^{+}$ cells of the proepicardium and septum transversum (Cano *et al.*, 2016). When WT1 is deleted only in the epicardium, the epicardium remains primarily intact, but mice die at E16.5-E18.5 with a dramatic depletion of both the coronary vasculature and epicardium-derived mesenchymal cardiovascular progenitor cells (Martínez-Estrada *et al.*, 2010).

It appears that much of the developmental role WT1 play in the heart is due to regulation of EMT. The result of epicardial WT1 KO (described above) was primarily attributed to disruption of EMT, with WT1 important for activating the major EMT regulator Snail and repressing E-Cadherin (Martínez-Estrada *et al.*, 2010). In a separate study, it was proposed that WT1 regulates epicardial EMT through the β-Catenin and retinoic acid (RA) pathways (von Gise *et al.*, 2011). Concurrently, WT1 regulates Raldh2 and Pdgfrα (components of RA pathways) (Bax and Oorschot, 2011; Guadix *et al.*, 2011) and cardiac malformations in Pdgfrα are associated with increased WT1 expression (Bax *et al.*, 2010). Epicardial WT1 Knock-down or mutation resulted in increased EMT and cell migration, attributed to WT1 directly upregulating Slug, another major regulator of EMT and member of the Snai family (Takeichi *et al.*, 2013). In reality, given the range of WT1 target genes, it is probable that all of these proposed mechanisms are valid. WT1 also activates the neurotrophin receptor TRKB (NTRK2), a key regulator of vascular development (Wagner et al. 2005). Epicardial WT1 also regulates the inhibitory chemokines Ccl5 and Cxcl10, both directly and indirectly through IRF7 (Velecela *et al.*, 2013). These

chemokines inhibit epicardial cell migration and cardiomyocyte proliferation, both important in cardiac development. Thus, WT1 is controlling development of the epicardium and cardiac muscle through suppression of these cytokines (Velecela *et al.*, 2013). In the foetal heart, WT1 is also expressed in endothelial cells of the developing vasculature, implicating it in vasculogenesis and developmental angiogenesis (Duim *et al.*, 2015, 2016)

1.1.2.2 Development of the Renal System

Wilms' tumour is a striking example of how perturbations of normal WT1 function *in utero* can adversely affect kidney development. Further, germline *Wt1* mutations play a role in development of Denys-Drash (Pelletier *et al.*, 1991) and Frasier syndromes (Barbaux *et al.*, 1997); one feature of which is glomerulosclerosis due to abnormalities in kidney podocytes. The kidney develops from two separate, interacting components derived from the intermediate mesoderm; the metanephric mesenchyme, which gives rise to nephrons and the ureteric bud, which gives rise to ureters. WT1 is expressed at varying levels throughout metanephric kidney development (Pritchard-Jones *et al.*, 1990; Armstrong *et al.*, 1993; Hastie, 2017), first at low levels in the intermediate mesoderm, with expression increasing as the metanephric mesenchyme condenses around the uretic bud and an MET event is initiated. WT1 expression remains widespread throughout nephrogenesis, eventually becoming restricted solely to the podocytes (Guo *et al.*, 2002). The development of the metanephric kidney is reviewed by Short & Smyth (2016), herein the role of WT1 will be discussed.

Inhibition at various stages of development has shown WT1 to be essential to all stages of kidney formation (Davies *et al.*, 2004; Berry *et al.*, 2015). WT1 appears to be essential for the regulation of metanephric mesenchyme survival (Motamedi *et al.*, 2014) and deletion of WT1 results in apoptosis of the metanephric blastemal and failed outgrowth of the ureteric bud from the Wolffian duct (Kreidberg *et al.*, 1993). Intriguingly, Wilms' tumours arising from *Wt1* mutations will often entail ectopic formation of muscle and occasionally bone, cartilage and fat within the kidney tissue (Schumacher *et al.*, 2003). Accordingly, WT1 deletion in the metanephric mesenchyme during kidney

development results in expression of myogenic markers and muscle formation (Berry *et al.*, 2015).

ChIP-seq experiments have been carried out to identify WT1 target genes in a renal context and have identified genes known to be essential to normal kidney development (Hartwig *et al.* 2010; Motamedi *et al.* 2014).These included *Sall1*, *Bmp4*, *Pax2* and *Bmp7*, a podocyte-derived protein essential for nephron development (Kazama *et al.*, 2008). Indeed, WT1 appears to be essential for normal development of podocytes; specialised glomerular cells of epithelial origin, but with many mesenchymal characteristics, that form a filtration barrier with endothelial cells. WT1 regulates the key podocyte gene Podocalyxin (*Pdxl*) at a transcriptional and post-transcriptional level (Palmer *et al.*, 2001; Bharathavikru *et al.*, 2017). In fact, WT1 binds to the regulatory regions of around half of the roughly 200 identified podocyte-specific genes and 18 of the 31 genes mutated in podocyte diseases (Dong, Pietsch and Englert, 2015; Kann *et al.*, 2015; Lefebvre *et al.*, 2015). As in the epicardium, WT1 also regulates *Wnt4* in the kidney (Sim *et al.*, 2002). The major MET event in early kidney development and essential for nephron formation is initiated by Wnt4 and therefore, interfering with WT1 at this stage disrupts MET, nephron formation and *Wnt4* expression (Kispert, Vainio and McMahon, 1998; Essafi *et al.*, 2011). Conversely, in the epicardium which undergoes the reverse process (EMT) WT1 represses *Wnt4* and this occurs through switching the chromatin from an active to a repressive state; a mechanism termed chromatin “flip-flop” (Essafi *et al.*, 2011).

1.1.2.3 **Gonadogenesis**

The well-established role of WT1 in sex determination and gonadogenesis in male and female tissues will be discussed in this section. Before WT1 was even discovered as the pivotal factor for Wilms’ tumour it was known that patients with Wilms’ tumour-aniridia (WAGR) syndrome often had gonadoblastoma and gonadal dysgenesis (Andersen *et al.* 1978). Despite their disparate localisation in the adult, the gonads and adrenal glands, derive embryonically from a common precursor, the adrenogonadal primordium (AGP). When WT1 is absent during development, mice fail to develop adrenal

glands and gonads (Kreidberg *et al.*, 1993). It is, therefore, intuitive that WT1 is affecting development of these two steroidogenic organs from an early stage. Indeed, WT1 transcriptionally activates the key AGP survival factor *Sf1* (Wilhelm and Englert, 2002), without which the AGP undergoes widespread apoptosis (Luo, Ikeda and Parker, 1994).

The gonad develops as a bipotential organ with the capacity to be directed down the male testicular development path or the female ovarian development path. In mammals, this is primarily determined by *Sry*, which activates *Sox9* in male sex determination and *R-Spondin 1* (*Rspo*), which activates *β-Catenin* in female sex determination (DeFalco and Capel, 2009). *Sry* and Müllerian inhibiting substance (Amh/Mis), a key sex determining hormone, are both directly regulated by WT1, particularly WT1 +KTS in association with GATA4 (Hossain and Saunders, 2001; Miyamoto *et al.*, 2008). This is in accordance with two observed instances of sex reversal, in Frasier syndrome (Barbaux *et al.*, 1997) and in the absence of +KTS WT1 which resulted in a reduction of *Sry* expression (Hammes *et al.*, 2001), establishing WT1 as an important factor in sex determination.

Following this event, WT1 has further roles in male and female gonadogenesis. In the testis, Sertoli cells form from *Sry/Sox9* expressing cells which orchestrate testis development, recruiting germ cells, vascular endothelial cells and myoid cells to form the seminiferous tubules and testicular cords. WT1 is widely expressed throughout (De Santa Barbara *et al.*, 2000), and is essential for testicular development (Wen *et al.*, 2014). Conditional *Wt1* deletion in Sertoli cells resulted in Sertoli cell death, disruption of developing seminiferous tubules and testicular cords, the latter due in part to WT1 regulation of basal lamina components *Col4a1* and *Col4a2* (Gao *et al.*, 2006; S.-R. Chen *et al.*, 2013). WT1 also inhibits *β-Catenin* in Sertoli cells (Chang *et al.*, 2008). Sertoli cells establish testis steroidogenesis and initiate differentiation of Leydig cells. While Sertoli cells continue to express *Wt1* into adulthood, Leydig cells stop expressing *Wt1* after forming from Sertoli cells (De Santa Barbara *et al.*, 2000).

Considerably less is known about WT1 in female gonadogenesis, however, a few studies have begun to dissect the relationship between WT1 and female fertility. The developing ovaries are formed from two cell populations: the granulosa cells of the coelomic epithelium and steroidogenic Theca cells. WT1 is expressed in the granulosa cells of follicles and the surface epithelium during development, with potential regulation of Inhibin-α (*IhbA*). This expression decreased over time. WT1 mutation in mice resulted in reduced ovary size and ova number, with a concurrent, dramatic reduction in fertility (Kreidberg *et al.*, 1999; F. Gao *et al.*, 2014; Nathan *et al.*, 2017). Among the factors controlling mammary development are Pax2 and Insulin like growth factor (IGF1) (Brown *et al.*, 2002; Silberstein, Dressler and Van Horn, 2002; Kleinberg *et al.*, 2009), both of which have been shown to be direct transcriptional targets of WT1 (Tajinda, Carroll and Roberts Jr., 1999; Discenza *et al.*, 2003).

1.1.2.4 Other Developmental Contexts

Other developmental contexts in which WT1 has been shown to have some influence are discussed below. Congenital diaphragmatic hernia (CDH), incomplete development of the diaphragm, can occur as a result of *Wt1* mutation in Denys-Drash, WAGR and Meacham syndromes (Scott *et al.*, 2005; Suri *et al.*, 2007; Antonius *et al.*, 2008). A recent study in which *Wt1* was deleted specifically from the septum transversum mesenchyme during diaphragmatic development showed a reduction in formation of a crucial mesenchymal cell population owing to impaired EMT and lower *Raldh2* expression, with retinoic acid (RA) also implicated (Carmona *et al.*, 2016). Therefore, this parallels the known role of WT1 in cardiac development, described previously (1.1.2.1).

During lung development, WT1 is discretely expressed in the embryonic mesothelium and this tissue gives rise to smooth muscle cells, endothelial cells, myocytes and cells of the bronchial and tracheal cartilage (Que *et al.*, 2008; Cano, Carmona and Munoz-Chapuli, 2013). *Wt1* deletion disrupts pulmonary development, with reduction in formation of these cell populations potentially attributable to impaired EMT (Cano, Carmona and Munoz-Chapuli,

2013). In liver development, $WT1^+$ septum transversum mesothelial cells generate hepatic stellate cells, perivascular mesenchymal cells, smooth muscle cells, portal fibroblasts and fibroblasts (Asahina *et al.*, 2011), with WT1 and RA shown to be important for stellate cell formation (Ijpenberg *et al.*, 2008). In the intestine, WT1 is expressed discretely in the coelomic epithelium and $WT1^+$ cells give rise to the visceral musculature, Cajal and Cajal-like interstitial cells and all cells of the intestinal vasculature (Carmona *et al.*, 2013).

One group has also examined the role of WT1 in the development of the retina and olfactory system. In the olfactory system, *Wt1* deletion resulted in thinner olfactory epithelia and fewer neuronal progenitor cells, with *Wt1* +KTS deletion having a more severe phenotype than *Wt1* –KTS deletion (Wagner *et al.* 2005). In retinal development, *Wt1* deletion resulted in thinner retinas, apoptosis of retinal ganglion cells and disruption of optic nerve growth (K.-D. Wagner *et al.*, 2002; N. Wagner *et al.*, 2002). WT1 has also been implicated in development of the sensory nervous system of the circumvallate taste buds (Y. Gao *et al.*, 2014). This begins to implicate WT1 in neuron development.

Immunohistological examination of human foetal tissues has also revealed extensive WT1 expression in skeletal muscle myofibres throughout development (Salvatorelli *et al.*, 2011; Magro *et al.*, 2015), though this requires further investigation. A relatively novel and exciting role for WT1 appears to be in adipose tissue formation, where it was shown that a proportion of visceral, but not subcutaneous adipose tissue develops from progenitors which arise from the adipose mesothelium and are $WT1^+$ (Chau *et al.*, 2014). This perhaps goes some way to explaining the otherwise idiosyncratic observation that Wilms' tumours occasionally form ectopic adipose tissue (Schumacher *et al.*, 2003).

WT1 is implicated in a broad range of developmental contexts, regulating development in numerous tissues in a temporal-specific manner. This suggests the actions of WT1 relate to regulation of the same cellular and molecular processes across contexts. Study to date suggests one of these processes is the regulation of EMT and MET (indeed the mammalian embryo

is generated by a number of cycles of EMT and MET) though there are undoubtedly other related or unrelated roles for WT1 in development.

1.1.3 **WT1 in Adult Tissue Homeostasis and Repair**

Despite its widespread expression and function in development, *Wt1* is expressed only in a few discrete cell populations in the healthy mammalian adult. Many of these cells are from tissues in which WT1 has an intrinsic developmental role. These include the podocytes of the kidney, a small proportion of the epicardial cells of the heart, the Sertoli cells of the testis, granulosa cells of the ovaries and a small proportion of cells in the bone marrow (Hastie, 2017). WT1 expression is also found in cells of the mammary gland, though this has not been examined in much detail (Artibani *et al.*, 2017). As previously shown, *Wt1* mutation or deletion in the developing embryo causes severe defects in numerous tissues. However, in a pioneering study, *Wt1* deletion in adult mice resulted in acute multiple organ failure (Chau *et al.*, 2011), specifically, oedema, glomerulosclerosis, deterioration of fat and bone, reduction in red blood cells, atrophy of the pancreas and spleen and a reduction in heart weight. This was attributed to effects on organ-specific progenitor and stem cell populations and a general reduction in some circulating serum proteins, including IGF-1. This demonstrates beyond doubt that WT1 is also essential for adult tissue homeostasis.

1.1.3.1 **Adult and Ischaemic Heart**

The main population of $WT1^{+}$ cells in the adult heart are found in the epicardial outer layer and vascular WT1 expression in only a small proportion of endothelial cells within the large blood vessels (Duim *et al.*, 2015). WT1 expression is increased in the epicardium in models of myocardial infarction (MI) in mice. Epicardial cells post-injury are activated, form epicardium-derived-cells (EPDCs), migrate inwards from the external epicardial layer and differentiate into cardiomyocytes and other cell types through EMT (van Wijk *et al.*, 2012). In a number of studies, it has been found that post-MI, EPDCs, cardiomyocytes and coronary vascular cells (vascular smooth muscle cells (VSMCs) and endothelial cells) proximal to the infarct area express WT1 (K.

Wagner *et al.*, 2002; Smart *et al.*, 2011; Duim *et al.*, 2015). Wt1 was also activated in the epicardium, EPDCs and cardiomyocytes and in areas of fibrosis in cardiac ischaemia reperfusion and in two models of hypertensive overload of the heart (constriction of the thoracic aorta and Angiotensin II (AngII) infusion) (Braitsch *et al.*, 2013). Furthermore, WT1 regulates adult epicardial cell EMT post-MI through direct transcriptional regulation of *Snail*, *VCAM-1* and *Pdgfrα* (Bax and Oorschot, 2011; von Gise *et al.*, 2011; Takeichi *et al.*, 2013). WT1 is therefore intrinsic to repair in the adult heart.

1.1.3.2 Kidney Homeostasis and Repair

The podocytes of the kidney are found within the glomerular capsule and align with endothelial cells of blood vessels, facilitating filtration. WT1 is expressed in adult podocytes throughout their life (Mundlos *et al.*, 1993). It is worth noting, given the known role of WT1 in EMT and MET, that while podocytes are effectively epithelial, they express high levels of a number of mesenchymal markers (Miller-Hodges & Hohenstein 2012). WT1 directly regulates the major podocyte gene *Nephrin* (Wagner *et al.*, 2004, 2006) and *Podxl* (Palmer *et al.*, 2001) and half the known podocyte-specific genes (Dong, Pietsch and Englert, 2015; Kann *et al.*, 2015; Lefebvre *et al.*, 2015). *Wt1* expression in normal and Denys-Drash podocytes inversely correlated with proliferation (Yang, Chen and Chen, 2004) and WT1 also appears to influence podocyte cytoskeletal architecture (Viney *et al.*, 2007).

As previously mentioned, (1.1.2.2) *Wt1* mutation results in formation of a number of developmental renal disorders, however, WT1 is also implicated in some acquired kidney diseases: for example in nephrotic syndrome not arising from *Wt1* mutation (Guan *et al.*, 2003). The mammalian kidney has limited regenerative capacity in adults. In contrast, in *Danio rerio*, which have the capacity to renew kidney nephrons throughout adulthood, adult nephron forming progenitors also overexpressed *Wt1a*; one of the two fish *Wt1* orthologues(Arora *et al.*, 2011). In mice, putative NCAM^{+} renal stem cells isolated from the kidney overexpressed *Wt1* (Harari-Steinberg, Pleniceanu and Dekel, 2011).

The adrenal gland, immediately proximal to the kidney has greater regenerative capacity than the kidney itself. In development of the adrenal gland, formation of mature adrenal cells requires downregulation of WT1, with continued expression of WT1 maintaining cells in a progenitor state (Bandiera *et al.*, 2013). Indeed, the adrenal gland has a proportion of WT1^{+} adrenal progenitor cells (APCs) in the outer mesenchymal capsule and zona glomerulosa, which migrate inwards and differentiate in regeneration of the adrenal (Wood and Hammer, 2011; Bandiera *et al.*, 2013). In this respect, these APCs and adrenal regeneration have striking similarities with regeneration from the epicardium (1.1.3.1).

1.1.3.3 **Liver Regeneration**

The adult mammalian liver has a relatively high regenerative capacity. This is primarily attributable to hepatic stellate cells (HSCs). Among other functions, HSCs are effectively a liver-resident mesenchymal stem cell (MSC) population, activated in damaged liver to initiate regeneration (Kordes *et al.*, 2014). As previously discussed, WT1 is essential to hepatic development and despite only being expressed in HSCs, *Wt1* KO livers lack a lobe, demonstrating the importance of WT1 in HSCs (Ijpenberg *et al.*, 2008). HSCs have similarities to other tissue-resident WT1-expressing cell populations. HSCs, podocytes and Sertoli cells have similar functions and roles, as all these cells are found in close association with endothelial cells and form a functional filtration barrier with blood; HSCs are found in the space of Disse, where they associate with vascular endothelial cells and hepatocytes. Like epicardial cells and EPDCs, HSCs are activated in injury, become proliferative and stop synthesising RA (Ohata *et al.*, 1997; Guadix *et al.*, 2011).

1.1.3.4 **Adult Reproductive Organs**

Sertoli cells of the testis and granulosa cells of the ovaries share a common *Wt1^{+}/Sox9^{+}* progenitor in the bipotential genital ridge prior to sex determination and these two distinct cell types then continue to express WT1 into adulthood (Albrecht and Eicher, 2001; Nakamura *et al.*, 2008). WT1 is essential for maintaining Sertoli cell identity and function throughout life. This is

demonstrated by *Wt1* KO in the testis, which results in transition of Sertoli cells into Leydig cells; conversely WT1 overexpression in Leydig cells causes them to express Sertoli cell markers (Zhang *et al.*, 2015). *Wt1* KO in adult Sertoli cells was also shown to induce Sertoli cell apoptosis and loss of adherence (Rao *et al.*, 2006). As previously mentioned, inhibition of WT1 in female reproductive tissues in development impairs fertility and similarly in this adult Sertoli cell *Wt1* KO, spermatogenesis was impaired and fertility reduced.

The female reproductive system is unique in mammals, in that it undergoes a constant cycle of tissue formation, degradation and regeneration due to menstruation. During menstruation there is constant expansion and turnover of the endometrial layer. It has been found that WT1 is expressed in the endometrial stroma cells throughout this process and is particularly upregulated during transition of stroma cells into decidual cells (Zhou, Rauscher and Bondy, 1993; Makrigiannakis *et al.*, 2001; Cousins *et al.*, 2014). There is a significant contribution of epithelial cell migration and MET to this process, with which WT1 was implicated (Cousins *et al.*, 2014). In endometriosis (ectopic formation of the endometrial lining of the uterus) WT1 protein and gene expression is reduced in the stromal cells of the endometrium (Matsuzaki *et al.*, 2005, 2006) and upregulated in the associated neurons (Coosemans *et al.*, 2009), but with no notable changes in microvascular WT1 expression.

WT1 has been studied extensively in mammary cancers, though to a much lesser extent in healthy mammary tissue. It has been noted, however, that WT1 is expressed in the breast and it is possible it could co-localise with a putative mammary gland progenitor cell niche (Villadsen *et al.*, 2007; Artibani *et al.*, 2017).

1.1.3.5 WT1 in Fat and Bone Marrow

As previously mentioned (1.1.3), *Wt1* KO in adult mice results in significant loss of fat and bone tissue (Chau *et al.*, 2011). WT1 expression was found in the adult mouse in all visceral fat depots: omental, retroperitoneal, perirenal, mesenteric, epididymal, and epicardial (Chau *et al.*, 2014). In the adult, WT1

is expressed in a very small proportion (~1%) of all CD34$^+$ haematopoietic precursor cells (Hosen *et al.*, 2002). However, this small population clearly has an important role, as these isolated cells, following adult *Wt1* KO were no longer capable of forming red blood cells (Chau *et al.*, 2011); an observation which was replicated *in vitro* (Cunningham *et al.*, 2013). This observation is explained by the fact that WT1 transcriptionally activated Erythropoeitin (EPO) which is required for red blood cell production (Dame *et al.*, 2006). After isolating healthy WT1$^+$ haematopoietic precursor cells it was found they could produce red blood cells, white blood cells and granulocytes and are therefore probably oligolineage-restricted haematopoietic progenitors (Chau *et al.*, 2011). In human haematopoietic cell lines, WT1 also appeared to control cell cycle progression and cell differentiation (L W Ellisen *et al.*, 2001).

Normal bone homeostasis relies upon bone synthesis by osteoblasts, which originate from MSCs, and bone reabsorption by osteoclasts, which originate from haematopoietic cells (Kusumbe, Ramasamy and Adams, 2014). A role for WT1 in these two cell types could explain the bone loss observed in adult *Wt1* KOs. In support of this, in these adult *Wt1* KO mice there was increased osteoclast formation, though *Wt1* KO mesenchymal cells had a reduced capacity to generate osteoblasts (Chau *et al.*, 2011).

In a number of the contexts explored above, it can be seen that WT1 is maintained in a small proportion of adult cells and that these cell populations often have a degree of pluripotency and a necessity for the regulation of EMT/MET. A proportion of these WT1-expressing cells (podocytes, HSCs and Sertoli cells) form a filtration barrier between their resident tissues and endothelial cells and capillaries. Disruption of WT1 in the populations of WT1-expressing adult cells has far reaching consequences, beyond effecting these WT1-expresing cells alone. Furthermore, these adult cells often have the capacity to recapitulate developmental processes to facilitate repair.

1.1.4 WT1 in Cancer

Despite its restricted expression in the adult, WT1 is widely expressed throughout a range of cancers, and as such, was ranked as the best

therapeutic cancer antigen by the National Cancer Institute (US) (Cheever *et al.*, 2009). Consequently, there are a number of ongoing Phase I, II and III clinical trials, though no commercially available WT1 immunotherapeutic agents have been produced as yet (Coosemans *et al.*, 2013; Koido *et al.*, 2014; Zauderer *et al.*, 2016; Bigalke *et al.*, 2017; Katsuda *et al.*, 2017). The relatively low expression of WT1 in adult tissues adds to the attractiveness of WT1 as a cancer therapeutic targets, as side effects could be limited. An established approach in multiple cancers is to sensitise the host immune system to a particular antigen and the majority of anti-WT1 trials have attempted this utilising either T-lymphocytes (Makita, Hiraki and Azuma, 2002; Oka *et al.*, 2004) or more recently dendritic cells (Coosemans *et al.*, 2013; Koido *et al.*, 2014; Takakura *et al.*, 2015; Bigalke *et al.*, 2017; Katsuda *et al.*, 2017) sensitised to WT1, with some positive outcomes on tumour regression. Incredibly, a proportion of $CD8^+$ T-lymphocytes were found to be spontaneously activated by the WT1 antigen in the absence of any intervention in acute myeloid leukaemia (Scheibenbogen *et al.*, 2002).

A full review of the role of WT1 in cancer is beyond the scope of this introduction. However, a recent meta-analysis of WT1 in human cancer patients found that WT1 was expressed in a variety of solid tumours and was significantly correlated with poorer prognostic outcome and reduced survival (Qi *et al.*, 2015). Intriguingly, WT1 can act as both a tumour suppressor, such as in Wilms' tumour (Call *et al.*, 1990), but as an oncogene in other cancers such as leukaemia, lung cancer and breast cancer (Miller-Hodges & Hohenstein 2012). As well as being expressed in cancers characteristic of all the tissues in which WT1 functions during adulthood and development, WT1 is also present in cancers of tissues in which WT1 is not normally present (reviewed by Sugiyama 2010) (Figure 1.2). First, an overview of the general function of WT1 in cancers, then a specific discussion of the role of WT1 in tumour angiogenesis will be provided.

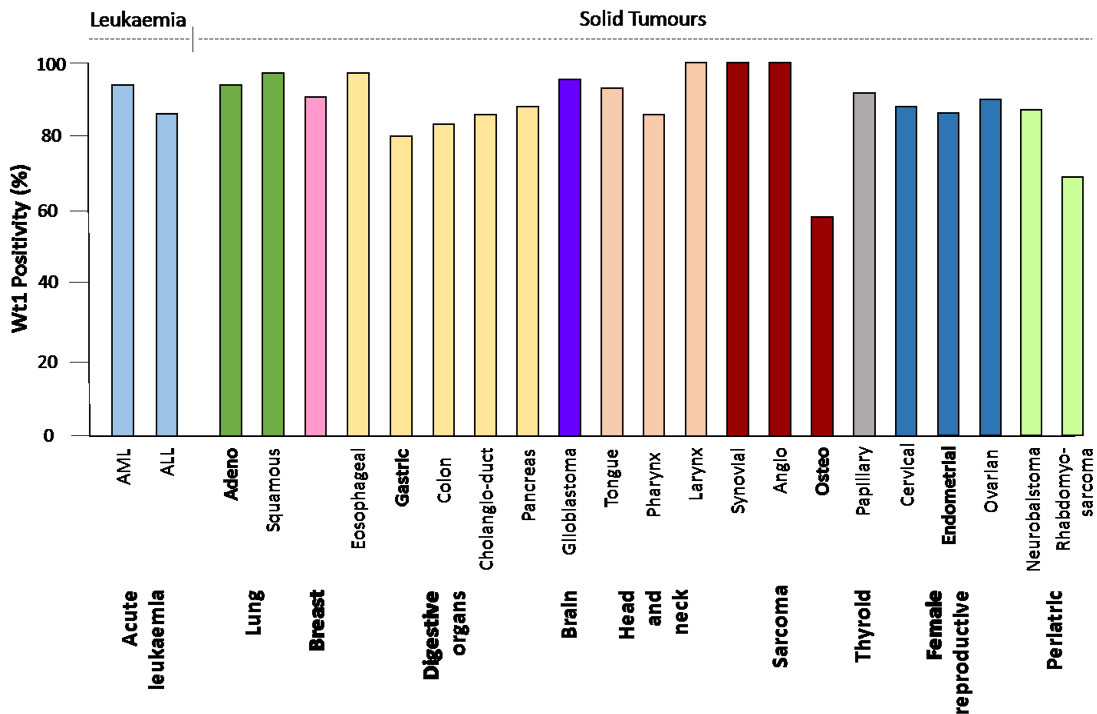

Figure 1.2. WT1 expression in a variety of cancers. Figure adapted from Sugiyama (2010). Overexpression was determined by WT1 immunohistochemistry and/or RT qPCR. Positivity (%) denotes the % of tumours with WT1 overexpression. AML = acute myeloid leukaemia, ALL = acute lymphoid leukaemia.

1.1.4.1 The Function of WT1 in Cancer

WT1 overexpression in cancers is evidently pathological, as WT1 expression in development, homeostasis and disease is tightly regulated in a spatiotemporal manner. This has proved sufficient to highlight WT1 as a therapeutic target, however, it is important to interrogate the role of WT1 in different cancers in order to better understand the effect of therapeutically targeting WT1, as well as provide a deeper understanding of the function of WT1 in general. Simplistically, research to date has shown WT1 in cancer to regulate four major processes: cell proliferation, apoptosis, cell migration and cell transition (specifically EMT and MET). Misregulation of these processes occurs in all cancers and drives growth and maintenance of the tumour microenvironment.

In a number of different cancer types, higher levels of WT1 expression within tumour tissue was associated with increased malignancy and worse patient outcomes (Miyoshi *et al.*, 2002; Cui *et al.*, 2014; Rauscher *et al.*, 2014). However, it should also be noted that in one study of serous ovarian carcinoma, high WT1 expression was a positive prognostic predictor of outcome (Taube *et al.*, 2016). This duplicity in WT1 action is often observed due to its bidirectional regulatory potential, though this could also demonstrate differences between different cancer types and how a process which is beneficial in one cancer can be detrimental in another.

Along with generally predicting a worse outcome, increased WT1 expression and decreased WT1-anti-sense (WT1-AS) RNA expression were associated with increased cell proliferation (Ashiba *et al.*, 2007; Xu *et al.*, 2013; Du *et al.*, 2015; Atik *et al.*, 2016; Barresi *et al.*, 2016). WT1 has also been shown to modulate apoptosis in tumour cells through regulation of p53 and bcl-2 (Maheswaran *et al.*, 1995; Mayo *et al.*, 1999; Tatsumi *et al.*, 2008) and cell migration (Barbolina *et al.*, 2008; Brett, Pandey and Fraizer, 2013a; Wu *et al.*, 2013; Lin *et al.*, 2016). In one *in vitro* study of prostate cancer cells, WT1 was shown to directly regulate *E-Cadherin*, consequently promoting EMT and cell migration (Brett, Pandey and Fraizer, 2013a). EMT in cancer is known to promote tumour growth and metastasis (Kudo-Saito *et al.*, 2009). WT1 target genes *Snail*, *Slug*, *Twist*, *VEGF* and β-*Catenin* are implicated in EMT in cancer (Mak *et al.*, 2010; Li and Zhou, 2011; Haslehurst *et al.*, 2012).

1.1.4.2 **WT1 in Tumour Angiogenesis**

WT1 is widely expressed in the vasculature of tumours and WT1 expression has been shown to correlate with increased tumour angiogenesis and therefore, greater micro-vessel density within tumours (Dohi *et al.*, 2010; Galfione *et al.*, 2014; Katuri *et al.*, 2014; De Palma *et al.*, 2016). WT1 directly regulates tumour angiogenesis in these cancer tissues through a number of mechanisms. WT1-expressing tumours have higher levels of pro-angiogenic factors such as VEGF, MMP9, Ang-1, and Tie-2 (Dohi *et al.*, 2010; Katuri *et al.*, 2014). Furthermore, Telomeric repeat binding factor protein 2 (TRF2), a direct target of WT1, transcriptionally regulates tumour angiogenesis by

activating *Pdgfrβ* (El Maï *et al.*, 2014, 2015). WT1 is also co-expressed with Proliferating cell nuclear antigen (PCNA), Nestin and ETS-1 in tumour vasculature (Wagner *et al.*, 2008). Angiogenesis is an essential process in establishment and progression of all cancers, as it provides tumour tissue with the oxygen and nutrients required. In metastasis, cancer cells use the tumour blood supply to disseminate throughout the body. Unsurprisingly then, increased WT1 expression in the tumour microvasculature is associated with an increased rate of tumour growth, higher recurrence rate and shorter survival (Barresi *et al.*, 2015, 2016). As a direct demonstration of the importance of WT1 in tumour angiogenesis, endothelial cell-selective *Wt1* KO induced dramatic regression of tumour vasculature which was sufficient to significantly reduce the size of tumours, reduce metastasis and consequently increase survival in mice (Wagner *et al.*, 2014).

1.2 Angiogenesis

Angiogenesis is the process of new blood vessel formation from existing vasculature. It can occur by two main mechanisms; sprouting angiogenesis and intussusception. Intussusceptive angiogenesis was first proposed in 1986, when electron-scanning microscopy revealed the formation of intraluminal pillars within the existing microvasculature with the capacity to induce vascular remodelling (Caduff, Fischer and Burri, 1986). Intussusception (reviewed by Makanya *et al.* 2009) is capable of inducing vessel branching, splitting or regression relatively rapidly. More recently discovered, a third possible process of angiogenesis, which may occur independently or concurrently with sprouting and intussusceptive angiogenesis, involves the recruitment of circulating endothelial progenitor cells (EPCs) into vessel walls (Asahara *et al.*, 1997).

In developmental contexts, vasculogenesis, *de novo* formation of vessels from precursor cells, and angiogenesis work in tandem to generate the vascular network. In the adult, angiogenesis is restricted to only a few specialised contexts and tissues, except in pathological circumstances, in which angiogenesis can be regenerative or degenerative. In the context of cardiovascular disease, arterial occlusion and the resultant downstream

hypoxia triggers angiogenesis which remodels the vasculature to resupply blood to the ischaemic tissue. This review will primarily cover sprouting angiogenesis, which has the greatest potential to reperfuse ischaemic tissues. Angiogenesis is initiated in and has the potential to form capillaries, arteries and veins (as well as lymph vessels in lymphangiogenesis). To avoid unnecessary repetition, description of sprouting angiogenesis will be covered from a capillary-centric standpoint, though the mechanisms in all forms of blood vessels are broadly the same. The sequential processes dictating angiogenesis will be outlined below, with some discussion of the key molecular signals required.

1.2.1 Cellular and Molecular Mechanisms of Angiogenesis

Sprouting angiogenesis is a complex and highly-regulated, multi-step process involving the coordinated action of a number of cell types, cellular processes and molecular players. In adult capillaries, quiescent endothelial cells line the luminal surface in a monolayer of phalanx shaped cells, interconnected by cell junctions and cell adhesion proteins. External to this layer, endothelial cells are in contact with pericytes, occasionally with other perivascular cells, fibroblasts and inflammatory immune cells, and in veins and arteries, a VSMCs. Quiescent endothelial cells and pericytes are attached to a common basement membrane. As the primary luminal surface of blood vessels and therefore the “tube” component through which blood flows, endothelial cells are effectively the functional unit of angiogenesis, though pericytes, smooth muscle cells, fibroblasts and immune cells all have an indispensable role. Herein is provided an overview of the main processes and players in angiogenesis, which should be sufficient to understand the work described in this thesis (Figure 1.3).

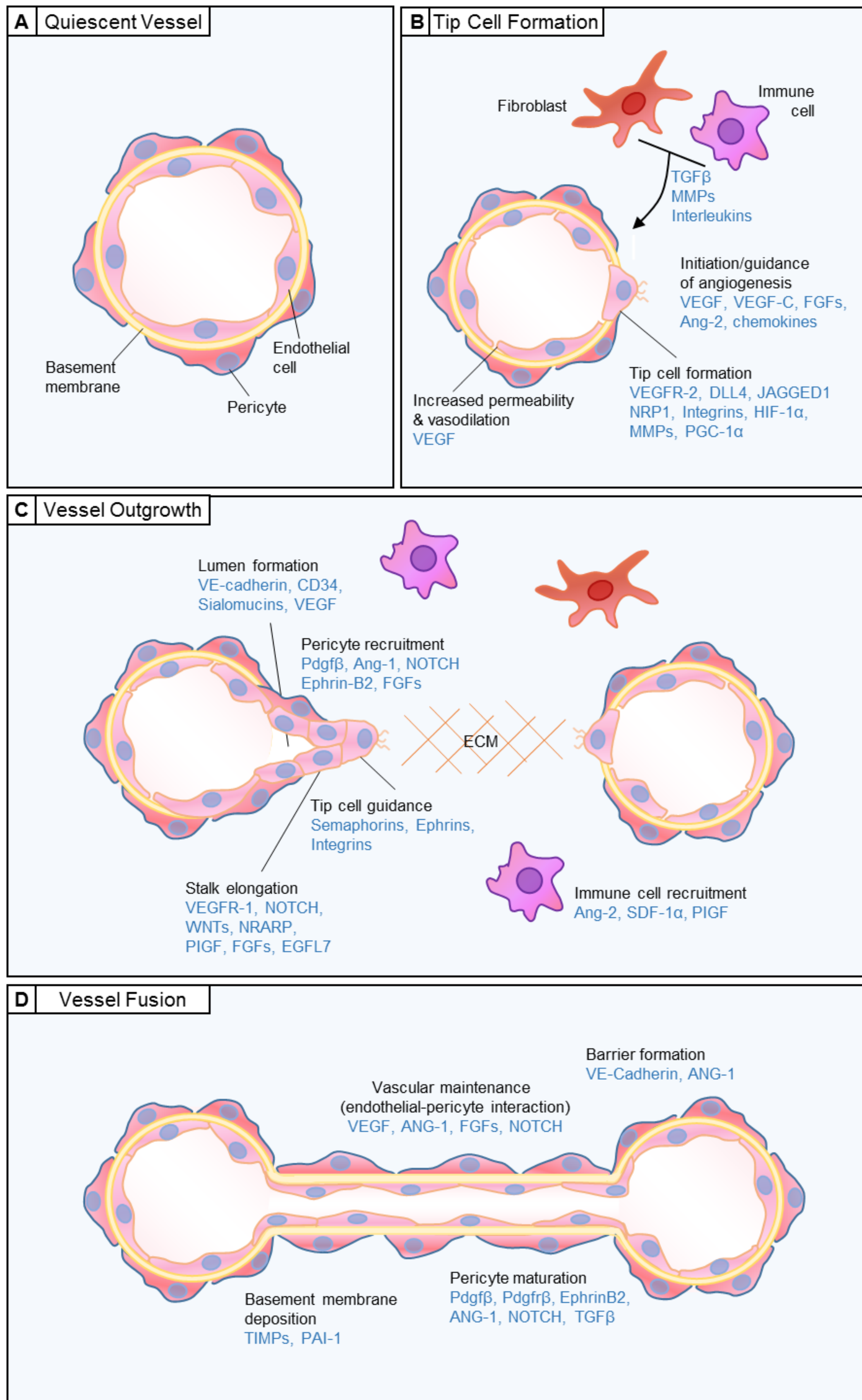

Figure 1.3. The cellular and molecular basis of sprouting Angiogenesis. Depicted is a cross-sectional representation of a capillary during new vessel formation by sprouting angiogenesis. **A)** A quiescent intact vessel is **B)** activated by angiogenic

factors, as a result of ischaemia, factors released from proximal vessels and signal from fibroblasts and inflammatory immune cells. This leads to a number of changes in vessel morphology, including prioritisation of an endothelial tip cell which will go on to guide the new vessel formation. **C)** Tip cell progresses, while the endothelial cells in the stalk proliferate and form a lumen. **D)** Eventually, the tip cells and stalk cells make contact with another vessel and fuse with it to form a competent vessel, which reverts back to a quiescent state. Cell types and components are labelled. Text in black denotes processes, while text in blue denotes proteins and genes associated with each process. Adapted from Carmeliet & Jain (2011).

1.2.1.1 Initiation of Sprouting Angiogenesis and Tip Cell Prioritisation

Quiescent vessels can be activated by detection of low oxygen levels (hypoxia), which brings about transcriptional changes, by external signals released by fibroblasts, inflammatory immune cells, or, in the case of tumour angiogenesis, by tumour cells. Endothelial cells are sensitive to local oxygen levels due to oxygen-sensing proteins such as proxyl hydroxylase domain (PHD) proteins. One of the targets of this family of proteins are HIFs, considered to be the key transcriptional activators of the response to hypoxia. In normoxia, PHDs hydroxylate HIF-1α and HIF-2α, but in hypoxia PHDs are inactive and HIFs become active, initiating transcription of a number of genes important to regulation of angiogenesis (Berra *et al.*, 2008; Fraisl *et al.*, 2009). HIF-1α and HIF-2α both activate transcription of VEGFs, essential proteins for the progression of angiogenesis (Nagy, Dvorak and Dvorak, 2007). Other key signals for initiation of sprouting angiogenesis include fibroblast growth factors (FGFs), Angiopoietins (ANGs) and chemokines (Carmeliet and Jain, 2011).

Commonly the first cells activated in angiogenesis, pericytes first detach in response to ANG-2 released from endothelial cells and proteolytically degrade the basement membrane through release of MMPs, including MMP9 (Augustin *et al.*, 2009). This occurs focally to allow liberation of endothelial cells from their monolayer without completely disrupting the morphology of the vessel. ANG-1 released by pericytes activates the Tie-2 receptor in endothelial cells (Augustin *et al.*, 2009). Mediated primarily by VEGF, endothelial cells loosen both their adherens cell junctions (involving catenins and cadherins such as VE-Cadherin) and their tight cell junctions (involving claudins and occludins)

(reviewed by Potente *et al.* 2011). Endothelial cells simultaneously induce vasodilation through release of nitric oxide (NO). VEGF and ANGs, also activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which regulates cell proliferation, adhesion, migration and invasion and, hence, has an important role in angiogenesis (Karar and Maity, 2011). The increased vascular permeability allows extravasation of plasma proteins, including fibrinogen and fibronectin which provide a provision matrix along which cells can migrate.

After these processes have effectively primed the vessel to undergo angiogenesis, a fascinating event characteristic of sprouting angiogenesis occurs, as an endothelial tip cell is established. In an elegant experiment, it was shown that endothelial cells dynamically compete for tip cell position, with relative expression levels of Vegfr1 and Vegfr2, in combination with delta-like 4 (Dll4)–Notch signalling, determining whether cells adopt a tip cell fate, or become supporting stalk cells (Jakobsson *et al.*, 2010). Tip cells have a distinctive morphology, with numerous extending filipodia and establish themselves at the tip of angiogenic outgrowths, with endothelial stalk cells comprising the main body of newly forming vessels. Formation of tip cell filipodia and associated stress fibres is regulated by the Rho family GTPases Cdc42, Rac1 and RhoA (Defilippi *et al.*, 1999; Pollard and Borisy, 2003; De Smet *et al.*, 2009). Tip cells have high migratory capacity and low proliferative capacity, whereas conversely, stalk cells have low migratory capacity and high proliferative capacity. Even with the same signal, VEGF-A binding to VEGFR2 in stalk cells results in increased endothelial proliferation, while in tip cells, a VEGF-A concentration gradient drives guided migration (Gerhardt and Betsholtz, 2003).

1.2.1.2 **Angiogenic Vessel Outgrowth and Lumen Formation**

In order for angiogenesis to be effective, angiogenic vessel outgrowths need to elongate, follow guidance signals, undergo controlled branching and be competent to support blood flow. Tight regulation of this process ensures that angiogenic vessels perfuse tissues most in need of an increased blood supply. Angiogenic vessel outgrowth progression, led by endothelial tip cell migration

and enabled by stalk cell proliferation and pericyte migration, is driven by a combination of attraction and repulsion guidance cues. This is demonstrated by the Neuropilin-1 receptor (NRP1), expressed in endothelial cells; binding of $VEGF_{165}$ to NRP1 promotes endothelial migration, whereas binding of Collapsin-1 or Semaphorin-3 (Sema3) inhibits migration (Miao *et al.*, 1999). As previously mentioned, endothelial tip cells migrate along an external VEGF concentration gradient; in addition other attraction guidance cues include SDF-1, CXCL12 and some repulsion cues include Netrin-1 and Robo4 (Potente *et al.* 2011). Semaphorins, Ephrins, Slits, Netrins and some other factors important in endothelial cell guidance in angiogenesis are also essential to axon guidance, while endothelial tip cells and axon growth cones have a number of morphological and functional similarities. This implies a degree of overlap in regulatory pathways between the two processes (Klagsbrun and Eichmann, 2005; Eilken and Adams, 2010).

Endothelial tip cells further mediate migration of angiogenic vessels through proteolytic degradation of the ECM to generate vascular guidance tunnels through which vessels can form. To mediate proteolytic degradation, tip cells produce MMP-14 and MMP-2, which are restricted to tip cells and not expressed in stalk cells during angiogenesis (Karagiannis and Popel, 2006; Yana *et al.*, 2007; Stratman *et al.*, 2009). Due to the dynamic competition for tip cell identity, stalk cells can adopt a tip cell fate, as previously described and migrate away from the existing angiogenic vessel outgrowth to form a new vessel branch. Branching structure is determined by a number of factors, including VEGF concentration, VEGF-A regulation of Flt-1/Flk-1signalling, Notch/Dll4 signalling, Heparin and ECM interactions (Ruhrberg *et al.*, 2002; Suchting *et al.*, 2007; Kappas *et al.*, 2008; Chappell *et al.*, 2016; Ubezio *et al.*, 2016).

Pericytes proximal to this forming endothelial vessel outgrowth proliferate and migrate onto the primitive vessel outgrowth, acting as structural support throughout sprouting angiogenesis. Endothelial cells release Pdgfβ which binds to the pericyte Pdgfrβ receptor, promoting pericyte proliferation and migration onto the forming endothelial outgrowth (Gaengel *et al.*, 2009). Sphingosine-1-phosphate (S1P) and S1P receptor (S1PR) signalling also

control endothelial cell and pericyte interactions, regulating cytoskeletal, cell adhesion, cell proliferation, migration and survival (Gaengel *et al.*, 2009; Lucke and Levkau, 2010).

In stalk cells, Notch-regulated ankyrin repeat protein (NRARP) and Sirtuin-1 (SIRT1) act to inactivate Notch, which promotes the increased stalk cell proliferation essential to elongation of the angiogenic vessel outgrowth (Phng *et al.*, 2009; Guarani *et al.*, 2011). As vessels grow and expand, stalk cells begin to form a vessel lumen in order to accommodate blood flow by adjusting their shape and rearranging their junctions. The contacts between stalk cells are mediated by VE-Cadherin junctions, which act with VEGF-A to recruit CD34, Sialomucins, Moesin, F-actin, and Myosin II to the endothelial cell surface and define the luminal surface (Strilic *et al.*, 2009). These negatively charged glycoproteins act to repel endothelial cells away from the junctions. Rho-associated protein kinase (ROCK) and Ras-interacting protein 1 (RASIP1) induce changes in cell adhesion and rearrange the cytoskeletal architecture to expand the endothelial lumen (Bryan *et al.*, 2010; Davis *et al.*, 2011; Xu *et al.*, 2011; Koo *et al.*, 2016).

1.2.1.3 **Fusion, Perfusion and Maturation**

Anastomosis is the process by which angiogenic vessel outgrowths fuse with other vessels and establish perfusion. Apposing vessels initiate tip cell contact and consolidate connections through VE-Cadherin junctions (De Smet *et al.*, 2009). Endothelial cells migrate outwards to form a functional vessel connection, extracellular matrix is deposited to generate a coherent basement membrane. Pericytes mature into a stable vascular lining through ANG-1/Tie-2 and TGFβ signalling (Augustin *et al.*, 2009; Pardali, Goumans and ten Dijke, 2010). Newly formed vessels are then capable of supporting blood flow and are shaped by haemodynamic forces. Shear stress of blood flow in the endothelial lumen activates the shear stress responsive transcription factor Krüppel-like factor 2 (KLF2), which in turn activates the PI3K and MAPK survival pathways (Nicoli *et al.*, 2010; Aksamitiene, Kiyatkin and Kholodenko, 2012; Boras *et al.*, 2014).

Evidently, effective tissue perfusion and nutrient delivery is dependent upon, not only competent individual vessels, but a well organised vascular network. The vascular network is structurally stabilised by pericytes, which deposit ECM and form cross-links between new vessel connections. Remodelling of the vascular network topology can occur by further sprouting angiogenesis and intussusceptive angiogenesis to fine-tune oxygen delivery. In contrast, vessels which are superfluous or do not support blood flow are pruned. Visualisation of vascular pruning demonstrates that this occurs by coordinated intussusception, endothelial cell apoptosis and endothelial cell migration (Lenard *et al.*, 2015). This can be mediated by low haemodynamic stress, low VEGF levels, FGD5 activation of Cdc42 and a number of Wnt ligands, including Wnt5a (Hlushchuk *et al.*, 2011; Cheng *et al.*, 2012; Korn *et al.*, 2014).

1.2.2 Non-Vascular Cells in Angiogenesis

Alongside endothelial cells and pericytes which can be thought of as the primary players in angiogenesis, it is becoming increasingly clear that a number of other cell types play an intrinsic role in regulation of angiogenesis, either by direct mediation or release of signals.

1.2.2.1 Monocytes, Macrophages and Dendritic Cells

Circulating chemoattractant angiogenic signals activate monocytes, macrophages and dendritic cells and recruit them to the site of angiogenesis from within the circulation and perivascular space. Among these signals are Granulocyte stimulating colony-stimulating factor (G-CSF) (Shojaei *et al.*, 2009; Schuett *et al.*, 2017), Monocyte chemoattractant protein 1 (MCP-1) (Ueno, Toi and Saji, 2000) and β-defensin (Conejo-Garcia *et al.*, 2004) released by endothelial cells.

Macrophages regulate and often initiate angiogenesis through paracrine effects and direct interaction with vascular cells. Macrophages secrete VEGF-A, MMPs, MCP-1, Interleukins, TGF-β and even microRNAs which elicit responses in endothelial cells, pericytes and VSMCs (Li *et al.*, 2013; Zajac *et al.*, 2013; Jaipersad *et al.*, 2014; Jetten *et al.*, 2014; Okizaki *et al.*, 2015). The

PI3K/Akt signalling pathway in macrophages controls their regulation of HIF-1α and HIF-2α in angiogenesis (Joshi *et al.*, 2014). HIF-1α is one of the factors controlling macrophage-endothelial cell-cell contact (Gerri *et al.*, 2017). Macrophages attach to endothelial cells by binding of their specific integrin receptor, Macrophage adhesion ligand 1 (Mac-1), to endothelial adhesion molecules and regulate processes, including ECM degradation and perhaps most importantly, anastomosis (reviewed by Jaipersad *et al.* 2014; Fantin *et al.* 2009; Outtz *et al.* 2011). There is a diversity of macrophage phenotypes and lineages and it has been suggested that anti-inflammatory M2 macrophages are more pro-angiogenic than pro-inflammatory M1 macrophages (Jetten *et al.*, 2014; Okizaki *et al.*, 2015). Indeed, endothelial cells seem to actively support differentiation of macrophages into an M2 state (He *et al.*, 2012).

Dendritic cells also produce a number of pro- and anti-angiogenic proteins, including VEGF-A and respond to angiogenic signals from other cells (Sozzani *et al.*, 2007). Immature, VEGFR2-expressing dendritic cells promoted angiogenesis in the endometrium, dramatically increasing endothelial cell migration (Fainaru *et al.*, 2007).

Intriguingly, monocytes, macrophages and dendritic cells appear to have some capacity to differentiate into endothelial-like cells. In damaged skeletal muscle tissue, macrophages were essential for vascular remodelling and regulated endothelial-to-mesenchymal transition (EndoMT), which occurred aberrantly in the absence of macrophages (Zordan *et al.*, 2014). In another study, macrophages treated with the angiogenic factors VEGF, FGFs and IGF-1 developed a more endothelial phenotype and were capable of forming tube-like structures (Schmeisser *et al.*, 2001). Accordingly, dendritic cells also differentiated into endothelial-like cells on treatment with VEGF-A and could form tube like structures *in vitro* (Pujol *et al.*, 2001).

1.2.2.2 **Neutrophils**

Neutrophils are essential to the inflammatory response, acutely accumulating in the early stages of tissue and vascular inflammation (reviewed by Phillipson

& Kubes 2011). Similarly, in angiogenesis neutrophils are primarily associated with angiogenic vasculature during initial onset of angiogenesis and have been implicated in initiating the angiogenic response in a number of contexts; the main neutrophil released factors in these contexts are VEGF-A, MMP9, macrophage inflammatory protein 1α (MIP-1α) and macrophage inflammatory protein 2 (MIP-2) (Nozawa, Chiu and Hanahan, 2006; Ardi *et al.*, 2007; Gong and Koh, 2010; Christoffersson *et al.*, 2013).

1.2.2.3 Mast Cells, Eosinophils and Basophils

The inflammatory myeloid cells, primarily responsible for allergic reactions, eosinophils, basophils and mast cells have a putative, but minimally investigated, role in angiogenesis. Through unknown mechanisms, eosinophils induce endothelial cell proliferation, upregulation of *VEGF* and induce angiogenesis *in vitro* and *in vivo* (Puxeddu, Alian, *et al.*, 2005). Mast cells influence angiogenesis to some extent and release angiogenic factors including VEGF, bFGF and IL-8 (Reed, Albino and McNutt, 1995; Boesiger *et al.*, 1998; Kim *et al.*, 2010). Basophils express the angiogenic factors NRP1, NRP2, VEGF-A, VEGF-B and VEGFR2 (de Paulis *et al.*, 2006). In allergic diseases such as asthma, and in tumour angiogenesis, eosinophils, basophils and mast cells seem to interact and overlap in their roles, resulting in increased angiogenesis (Puxeddu, Ribatti, *et al.*, 2005; Crivellato, Travan and Ribatti, 2010; Marone *et al.*, 2016).

1.2.2.4 T-Lymphocytes and B-Lymphocytes

The cells of the adaptive immune system, T- and B-lymphocytes, have been implicated in allergy-associated angiogenesis and tumour angiogenesis. It has been shown that lymphocyte express *IL-17F*, *STAT3* and *IL-25*, the latter of which induced the PI3K/Akt and Erk/MAPK pathways in endothelial cells (Starnes *et al.*, 2001; Corrigan *et al.*, 2011; Yang *et al.*, 2013). This is of relevance as these factors have all be implicated in angiogenesis (Starnes *et al.*, 2001; Wei *et al.*, 2003; Corrigan *et al.*, 2011; Karar and Maity, 2011). However, perhaps due to the normal localisation of lymphocytes in bone marrow and the lymph nodes and the necessity for their activation by antigen-

presenting cells, their role in angiogenesis appears tangential, with no evidence to date that disturbing lymphocyte function impairs angiogenesis.

1.2.2.5 **Fibroblasts**

Fibroblasts are the primary cell type responsible for extracellular matrix and collagen deposition and hence provide extracellular matrix in angiogenesis along which endothelial cells migrate during sprout formation. Following ablation of fibroblasts *in vitro*, angiogenic vessel outgrowths fail to form an endothelial lumen (Newman *et al.*, 2011). Fibroblasts also regulate angiogenesis through p53-mediated expression of Thrombospondin-1 (an anti-angiogenic factor) (Dameron *et al.*, 1994). EndoMT is capable of generating fibroblasts from endothelial cells. Fibroblasts appear to have a degree of native plasticity and also generate endothelial cells through the reverse process, mesenchymal-to-endothelial transition (Ubil *et al.*, 2014). This p53-regulated fibroblast-to-endothelial transition contributed to cardiac repair post-MI attributable to increased neovascularisation of the injured heart (Ubil *et al.*, 2014).

1.2.2.6 **Stem Cells and Progenitors**

Angiogenesis is effectively a recapitulation of developmental processes with an accompanying reversion towards a more pluripotent phenotype in angiogenic cells. It is therefore, intuitive that a number of stem and progenitor cell populations have been shown to participate in angiogenesis. As established, most immune cells have a degree of involvement in angiogenesis. In adults, there is a circulating niche of undifferentiated haematopoietic stem cells (HSCs); the progenitors to myeloid cells, lymphocytes, erythrocytes, megakaryocytes and platelets. HSCs also increasingly appear to be the origin of endothelial progenitor cells. This relationship is reciprocal, as bone marrow *Notch*$^{+}$/*Akt*$^{+}$ endothelial cells maintain HSC self-renewal through paracrine release of angiocrine signals (Butler *et al.*, 2010; Kobayashi *et al.*, 2010). EPCs have been found to secrete angiogenic growth factors including VEGF, Hepatocyte growth factor (HGF), G-CSF and GM-CSF (Rehman *et al.*, 2003).

Late, but not early EPCs were also capable of forming tubules and incorporating into existing endothelial cell tubules *in vitro* (Sieveking *et al.*, 2008). However, EPCs and HSCs synergistically facilitated angiogenesis to a greater extent than EPCs or HSCs in isolation (Yoon *et al.*, 2005).

Another stem cell population in adults is the mesenchymal stem cell (MSC) population, which is capable of generating adipose progenitor cells (APCs) and osteoblasts, among other cell types (Barclay *et al.*, 2012). Numerous studies with MSC implantation into the ischaemic myocardium post-MI have shown the angiogenic potential of MSCs to increase angiogenesis and repair through release of angiogenic signals and exosomes which influence endothelial cell function and transition of MSCs into endothelial-like cells (Silva *et al.*, 2005; Wu *et al.*, 2007; Hu *et al.*, 2008; Bian *et al.*, 2014; Anderson *et al.*, 2016; Gong *et al.*, 2017). APCs are capable of enhancing angiogenesis both *in vitro* and *in vivo* and like MSCs, release exosomes containing pro- and anti-angiogenic factors (Lopatina *et al.*, 2014; Bejar *et al.*, 2016; Cheng *et al.*, 2017). Of clinical relevance, in murine models of diabetes, the APC niche was depleted and this contributed to a proportion of the impaired angiogenesis observed in diabetes (Rennert *et al.*, 2014). Osteoblasts and the myeloid-derived cell type osteoclasts are primarily involved in bone formation, however, they are also capable of expressing VEGF-A and participating in angiogenesis (Tombran-Tink and Barnstable, 2004; Hoogendam *et al.*, 2014). Furthermore, osteoblasts enhance the migration of HSCs from the bone marrow and mediate the angiogenic activity of EPCs and HSCs (Kusumbe, Ramasamy and Adams, 2014; Chen *et al.*, 2017; Tsirkinidis *et al.*, 2017). As angiogenesis is essential for bone formation and regeneration, the involvement of osteoblasts and osteoclasts in angiogenesis is not counter-intuitive (Stegen, van Gastel and Carmeliet, 2015).(D'Alessio *et al.*, 2015)

1.2.3 Developmental Angiogenesis

Vasculogenesis is the primary mechanism of embryonic vascular network formation. In embryonic vessel formation, vasculogenesis generates a primitive vascular plexus by aggregation of angioblasts, which differentiate into endothelial cells and form new blood vessels. Angiogenesis occurs to remodel

this plexus into a competent vascular network. The mechanism of developmental sprouting angiogenesis is broadly similar to that described previously (1.2.1) and remodelling also occurs to some extent by intussusception and EPC recruitment. Angiogenesis is crucial for development of all organs to supply oxygen and nutrients to developing tissues and maintain this by remodelling the vasculature as these organs grow and develop.

1.2.4 Physiological Angiogenesis

Post-development, angiogenesis is arrested in most organs, except in a few discrete contexts. The main site of angiogenesis in the healthy adult organism is the female reproductive system, specifically the mammary gland and uterine endometrium. Mammalian vertebrates have a regular menstrual cycle, during which the endometrial lining of the uterine tract undergoes a formidable cycle of tissue expansion, degradation and shedding. This is accompanied by tissue neovascularisation by angiogenesis followed by vessel breakdown (Cousins *et al.*, 2014). This degree of tissue turnover in any other mammalian tissue would cause extensive, irreversible damage, though due to coordinated tissue formation and angiogenesis the uterus is capable of undergoing up to 400 cycles during a woman's life (Cousins *et al.*, 2014). Vessels in the endometrium have an unusual tortuous, spiral morphology (Pijnenborg, Vercruysse and Hanssens, 2006). If an ovum embeds in the uterine lining, further angiogenesis occurs to support the continued expansion of the decidualised endometrium and progesterone secreting corpus luteum during establishment of pregnancy and to establish the placenta.

Unlike in other tissues, progesterone and oestrogen play a crucial role in initiating and regulating angiogenesis in the endometrium. These two hormones activate angiogenic signals, including VEGF-A, in normal uterine physiology and in pregnancy (Greb *et al.*, 1997; Walter, Rogers and Girling, 2005; Kim *et al.*, 2013; Zhang *et al.*, 2017). The MAPK pathway and Leptin-activated STAT3 have also been implicated in endometrial angiogenesis (Hu and Zhang, 2017; Vargas *et al.*, 2017). Leukocytes play a regulatory role by releasing angiogenic factors in the uterus, including Placental growth factor

(PGF), essential to angiogenesis in pregnancy and the placenta (Li *et al.*, 2001; Lima *et al.*, 2014; Rätsep *et al.*, 2014).

Similarly, the adult mammary gland goes through a physiological, cyclic expansion, differentiation and degradation in response to the menstrual cycle and more broadly, the oestrus cycle (Andres and Strange, 1999). Therefore, the mammary gland also undergoes regular cycles of angiogenesis (Durando *et al.*, 2011; Shao and Zhao, 2014).

1.2.5 **Tumour Angiogenesis**

Tumours require a vascular network to grow; angiogenesis is a hallmark of all cancers and is even enhanced in leukaemia (Schmidt and Carmeliet, 2011). While the majority of pathways and mechanisms of tumour angiogenesis overlap with angiogenesis in other contexts, tumour angiogenesis, like tumorigenesis in general is a mis-regulated pathological process and therefore, results in an abnormal vascular morphology. Tumour angiogenesis is generally initiated by VEGF-A overexpression as a result of upregulation of *HIF-1α* and *STAT3* in tumour cells (Zhong *et al.*, 1999; Wei *et al.*, 2003; Olsson *et al.*, 2006; Nagy, Dvorak and Dvorak, 2007). Consequently, a lack of organised angiogenesis results in torturous, serpentine blood vessels, which are permeable, prone to bleeding, frequently lack pericytes and may carry blood in one direction one moment, only to be perfused in the opposite direction the next (Nagy *et al.*, 2009). These vessels are even sometimes completely unperfused (Nagy *et al.*, 2009). While tumour angiogenesis is essential to provide nutrients and oxygen to the developing tumour, poorly-regulated angiogenesis is also responsible for areas of tumours becoming ischaemic and necrotic as they expand.

Recognising the importance of angiogenesis in cancer progression, there are now a number of anti-angiogenic drugs deployed in combination with chemotherapy (reviewed by Vasudev & Reynolds 2014). These include inhibitors of VEGF signalling such as Bevacizumab, Sunitinib and Aflibercept which have proved effective in slowing progression of a range of solid tumour cancers.

1.2.6 Pathological Angiogenesis

The main disease associated with pathological angiogenesis is retinopathy, damage to the ocular retina by vessel remodelling and expansion. There are broadly two types of retinopathy; non-proliferative retinopathy, as a result of hypertension (hypertensive retinopathy) or premature birth (diabetes of prematurity) pathologically remodelling or damaging the retinal blood vessels and proliferative retinopathy as a result of diabetes (diabetic retinopathy) bringing about aberrant angiogenic overgrowth of the vascular network. This can ultimately result in blindness. The angiogenesis which occurs in proliferative retinopathy is closely linked to inflammatory processes and consequently, numerous angiogenic factors and inflammatory cytokines are implemented in the progression of retinopathy (Gologorsky, Thanos and Vavvas, 2012; Zhou, Wang and Xia, 2012; Simó, Sundstrom and Antonetti, 2014; Babapoor-Farrokhran *et al.*, 2015).

Endometriosis is a common gynaecological disease in which endometrial tissue arises outside the uterine tract. As with normal endometrial tissue, these ectopic endometrial lesions require a dense vascular network to survive and proliferate. Consequently, a combination of vasculogenesis and angiogenesis occurs to establish and maintain endometrial lesions in angiogenesis. Alongside VEGF from epithelial and stromal endometriotic cells, angiogenic and inflammatory signalling by immune cells drives pathological angiogenesis in endometriosis, particularly VEGF and Interleukin release by peritoneal macrophages, neutrophils and lymphocytes (reviewed by Gazvani & Templeton 2002; Groothuis 2012). Anti-angiogenic therapy is not routinely deployed to treat endometriosis, though experimentally inhibiting angiogenesis has been effective in reducing lesion size and impairing growth. This suggests that anti-angiogenic drugs could be more routinely deployed in endometriosis in the future (Laschke *et al.*, 2006; Laschke and Menger, 2012; Edwards *et al.*, 2013).

1.2.7 Regenerative Angiogenesis

While angiogenesis in the adult is restricted to a few discrete situations and pathological processes, angiogenesis has huge regenerative capacity across

a range of diseases, supporting tissue remodelling and formation of new tissue. Some of the myriad contexts in which angiogenis is beneficial include, wound healing, bone regeneration, liver repair and ischaemic reperfusion in renal and cardiovascular disease. Given the cardiovascular focus of this work, the regenerative potential of angiogenesis in cardiovascular disease is of particular interest.

Ischaemic cardiovascular diseases include myocardial infarction, peripheral vascular disease and stroke. The primary cause of these is arterial occlusion, ordinarily the result of atherosclerosis and eventual plaque rupture, which restricts downstream blood flow, thereby starving tissues of oxygen. The resulting ischaemia causes devastating tissue damage and can lead to irreversible degradation and death if blood flow is not re-established. Angiogenesis, in tandem with arteriogenesis (redirection of blood flow within existing vasculature), is capable of restoring oxygen to ischaemic tissues; reducing damage and inducing tissue regeneration. In the heart, following myocardial infarction, angiogenesis acts to reperfuse ischaemic myocardium and thereby reduces the damage sustained. However, this rarely occurs without extensive scarring and damage to the heart. This scarring occurs when angiogenesis is unable to reperfuse the infarct area rapidly or extensively enough to prevent irreversible cardiomyocyte death. In principle, this also applies to the neurons of the brain in stroke and skeletal muscle in peripheral vascular disease. As angiogenesis does not consistently occur to a sufficient extent to induce complete tissue reperfusion, therapeutically targeting ischaemic angiogenesis is of considerable interest to improving patient outcomes in cardiovascular disease.

1.3 WT1 in Angiogenesis

In contrast to the number of anti-angiogenic drugs currently licenced for therapeutic targeting of tumour and pathological angiogenesis, only one pro-angiogenic drug has, to date, reached the clinic, Beclapermin (Regranex®). Beclapermin, approved by the FDA in 1997, is a PDGF-based gel applied for the treatment of lower extremity chronic diabetic neuropathic ulcers (Fang and Galiano, 2008). While this treatment is effective for increasing angiogenesis

into the affected area, it has a localised effect and limited depth of penetration. Systemic pro-angiogenic therapeutics, primarily based around targeting VEGF or FGF signalling have all failed to pass clinical trials due to limited or no beneficial outcome (Simons and Ware, 2003), a case repeated in a recent phase III G-CSF and Sitagliptin clinical trial, SITAGRAMI (Brenner *et al.*, 2016). This demonstrates the unmet clinical need for a targeted pro-angiogenic therapeutic, not based on generic angiogenic growth factors. Indeed, while upregulating angiogenesis is evidently essential for facilitating regenerative angiogenesis, increasing new neovascularisation in non-specific manner may have no meaningful impact on targeted tissue reperfusion. WT1 has recently been implicated in angiogenesis and as a transcriptional regulator with discrete temporal and spatial expression in angiogenesis and other tissues, could be a more effective therapeutic target to enhance regenerative angiogenesis.

WT1 is largely absent from non-regenerating adult vasculature, except potentially in the vasa vasori (microvessels in the vessel wall of large vessels) which undergo a degree of physiological remodelling and angiogenesis (Ritman and Lerman, 2007; Vasuri *et al.*, 2012). However, WT1 is expressed in endothelial cells, fibroblasts, pericytes and inflammatory cells in vasculature undergoing angiogenesis (McGregor et al. 2014, Katuri *et al.* 2014; Dohi *et al.* 2010). Given the widely accepted role for WT1 as an oncogenic target, it was first recognised that WT1 could be a potential transcriptional regulator of angiogenesis in cancers, where it is found in vascular cancers and in the tumour vasculature of solid cancers (Timár *et al.*, 2005; Wagner *et al.*, 2008, 2014; Dohi *et al.*, 2010; McCarty, Awad and Loeb, 2011; Trindade *et al.*, 2011; Iranparast *et al.*, 2014; Katuri *et al.*, 2014; Barresi *et al.*, 2015, 2016). In endometrial cancer, WT1 expression was found to correlate with angiogenesis (Dohi *et al.*, 2010). In acute leukaemia, WT1 expression correlated with *VEGF* expression which was pro-angiogenic (Iranparast *et al.*, 2014) whilst in Ewing sarcoma not only was WT1 correlated with *VEGF* expression, but it directly regulated its transcription, along with *MMP9* (McCarty, Awad and Loeb, 2011; Katuri *et al.*, 2014). It was discovered that WT1 regulates angiogenesis in a number of tumours, working by transcriptionally activating the ETS-1

transcription factor to control endothelial cell migration and proliferation (Wagner *et al.*, 2008). Furthermore, induction of *Wt1* KO specifically in the vascular endothelium of tumours dramatically impaired angiogenesis and resulted in reduced tumour growth and increased tumour regression (Wagner *et al.*, 2014).

The role of WT1 in angiogenesis does not, however, appear to be a peculiarity of tumour angiogenesis. Following induction of MI in rats, WT1 was found to be expressed in endothelial cells and supporting vascular cells of the coronary vasculature proximal to the site of infarction and this expression was colocalised with PCNA and VEGF (K. Wagner *et al.*, 2002). These observations were later replicated in a mouse model of MI (Duim *et al.*, 2015). Intriguingly, at least a proportion of these WT1$^+$ endothelial cells post-MI are generated from the epicardium and EPDCs (S. Chen *et al.*, 2013). Given the known role and widespread expression of WT1 in the developing heart, it is likely this represents a reactivation of angiogenesis as seen in development (Martínez-estrada *et al.*, 2010; Zhou and Pu, 2012).

Work *in vivo* and *in vitro* has since provided strong evidence that WT1 is transcriptionally regulated by HIF-1 binding in hypoxic conditions (Wagner *et al.*, 2003) and in turn transcriptionally regulates *VEGF* (Graham *et al.* 2006; Hosen *et al.* 2007; Amin *et al.* 2011; McCarty *et al.* 2011; Iranparast *et al.* 2014). WT1 also appears to directly regulate the pro-angiogenic Interleukin IL-10 (Sciesielski *et al.*, 2010). *In vitro*, hypoxia was shown to induce a ~3 fold increase in WT1 expression in HUVECs (Duim *et al.*, 2015). In HUVECs and C166 mouse endothelial cells, it was shown that WT1 inhibition impaired cell migration and cell proliferation *in vitro* (Wagner *et al.*, 2008, 2014; Duim *et al.*, 2015). WT1 knock-down also impaired angiogenic vessel formation in endothelial cells in an *in vitro* 2D tube formation assay (Wagner *et al.*, 2008, 2014; Duim *et al.*, 2015). In a more complex *ex vivo* aortic ring assay of angiogenesis *Tie2Cre*ER^{T2} *Wt1* KO almost completely abolished new vessel formation (Wagner *et al.*, 2014). All of this demonstrates that WT1 has an important role to play in regenerative cardiovascular angiogenesis, in endothelial cells in particular and raises the exciting possibility that WT1 could be therapeutically harnessed as a pro-angiogenic target in angiogenesis;

moreover, a therapeutic capable of regulating angiogenesis in a controlled, coordinated manner as necessary to enhance regeneration.

1.4 Hypothesis

Given the developing consensus that WT1 is an important regulator of angiogenesis, the main goal of the work described in this thesis is to determine the role of WT1 in cardiovascular diseases, specifically in endothelial cells in ischaemic angiogenesis. This will be investigated using *in vivo* and *ex vivo* models of angiogenesis, alongside a mouse model of inducible vascular endothelial cell-specific WT1 knockout (KO). Thus, the hypothesis addressed in this thesis is that:

> *"The expression of Wt1 in vascular endothelial cells is central to the angiogenic formation of new vasculature"*

1.5 Aims

To address this hypothesis, investigations have been designed to:

1. Determine the expression pattern of WT1 in *in vivo* and *ex vivo* models of angiogenesis and ischaemia.
2. Determine whether selective deletion of WT1 from the vascular endothelium inhibits angiogenesis in *in vivo* and *in vitro* models.
3. Determine the function of WT1 in the endothelium during angiogenesis.
4. Gain further insight into the mechanistic role of WT1 in tissue repair and regeneration.

CHAPTER 2:

Materials and Methods

2.1 Experimental Animals

2.1.1 Vascular Endothelial WT1 KO Mouse (VE-Wt1 KO)

All mice were bred and caged in line with Home Office conditions at the Little France Animal Facility (University of Edinburgh). Licenced procedures were carried out in strict accordance with the Home Office Animals (Scientific Procedures) Act 1986, under project Licence 60/4523 and personal licence I93FA603D.

Mice expressing tamoxifen-inducible *Cre*-recombinase under the control of the VE-cadherin promoter (VE-Cadherin-*Cre*ER^{T2}) (provided by Melissa Cudmore (University of Edinburgh)) (Monvoisin *et al.*, 2006) and mice with *LoxP* flanked WT1 gene (WT1 co/co) (provided by Nick Hastie (University of Edinburgh)) were crossed to generate vascular endothelial *Wt1* conditional knock-out mice (VE-Wt1 KO) (Figure 2.1) in which knock-out (KO) of WT1 can be induced specifically in VE-Cadherin expressing cells by tamoxifen injection.

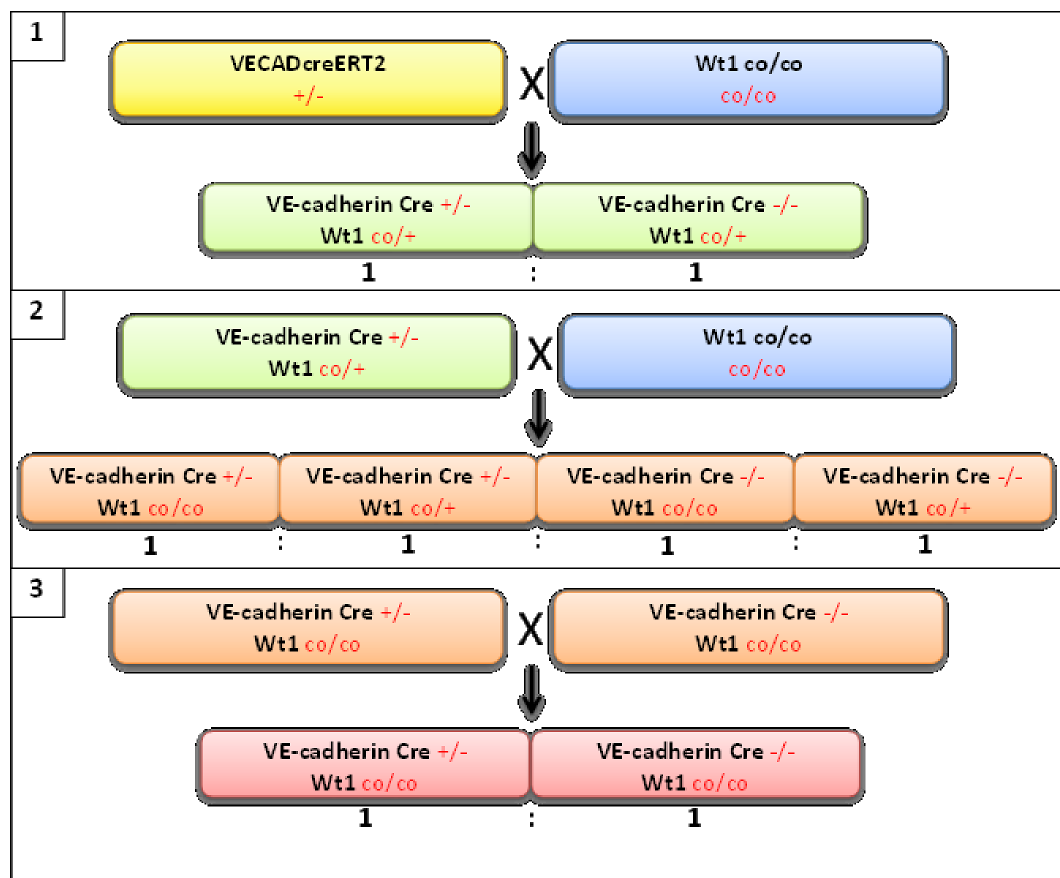

Figure 2.1: Three stage breeding strategy for generating a new mouse strain from initial VECADcreER^{T2} and WT1 co/co mouse lines. Genotypes are shown in red and Mendelian ratios shown beneath offspring. For tamoxifen-inducible VE-cadherin Cre, '+' indicates the presence of the VE-cadherin Cre, while '-' indicates its

absence. For WT1 'co' indicates the presence of a floxed WT1 gene, while '+'indicates a WT1 gene which cannot be conditionally knocked-out.

2.1.2 Tamoxifen injection regime

VE-Wt1 KO was induced by once daily intraperitoneal injection of tamoxifen (33mg/kg in glyceryl tridodecanoate) for 5 days. *Cre* control (CC) mice also received these injections, while vehicle control (VC) mice received the same regime of vehicle (glyceryl tridodecanoate) solution injection (Table 2.1) (Figure 2.2).

Experimental Mouse Line	Genotype		Injection
	VE-Cadherin *Cre*ER^{T2}	*Wt1*	
VE-Wt1 KO	Present	*Wt1*$^{Co/Co}$	Tamoxifen
CC	Present	*Wt1*$^{+/+}$	Tamoxifen
VC	Present	*Wt1*$^{Co/Co}$	Vehicle

Table 2.1: Experimental mice. The genotype and type of injection received by the different experimental mice are shown. All mice have VE-Cadherin *Cre*ER^{T2} present, while only vascular endothelial *Wt1* KO (VE-Wt1 KO) and vehicle control (VC) mice have the homozygous *LoxP* flanked *Wt1* allele (*Wt1*$^{Co/Co}$) present (*Cre* control (CC) mice have the homozygous wild-type *Wt1* allele (*Wt1*$^{+/+}$). VE-WT1 KO and CC mice receive tamoxifen injections, while VC mice receive vehicle injections.

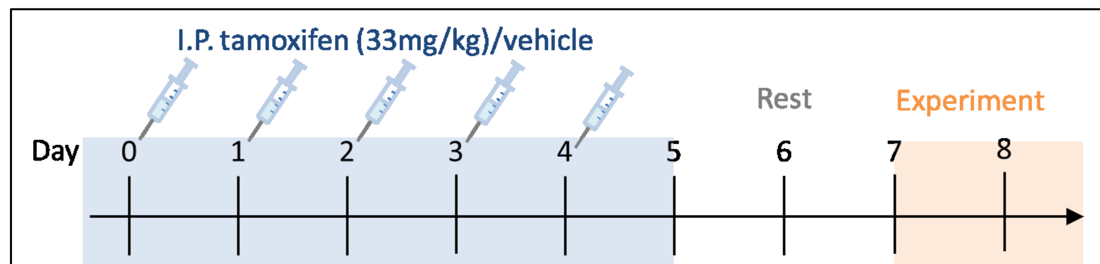

Figure 2.2: Tamoxifen/vehicle injection regime. Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) and *Cre* control (CC) mice received once daily intraperitoneal injection of tamoxifen (33mg/kg in glyceryl tridodecanoate) for 5 days. Vehicle control (VC) mice received the same regime of vehicle (glyceryl tridodecanoate) solution injection. Mice were rested for 2 days post-injection before commencing experimental procedures.

2.1.3 WT1-GFP

A WT1 reporter mouse with a knock-in green fluorescent protein under the control of the WT1 promoter (WT1-GFP), generated by Hosen *et al.* 2007, was

used. In this mouse, GFP is expressed in the nucleus of mice upon activation of the WT1 promoter and transcription of the WT1 gene, allowing detection of WT1 by GFP expression.

2.1.4 **C57Bl/6**

Where mentioned, C57Bl/6 mice used were C57Bl/6J mice. This inbred strain is considered to be wild-type (WT).

2.2 **Genotyping**

2.2.1 **DNA extraction**

DNA for genotyping was extracted from mouse ear clips. Ear clips were digested in 75µl digestion solution (25mM NaOH, 0.2mM EDTA, pH=12) per sample at 95°C for 45mins to create a crude lysate. Solution was allowed to cool, then neutralised with 75µl digestion solution (40mM Tris-HCl, pH=5) per sample.

2.2.2 **VE-cadherin-*Cre* PCR**

For each sample, 4µl of extracted DNA lysate were added to 7.5µl master mix (GoTaq G2 Hot Start Green Master Mix 100rxn, C-M7422-X), 3µl distilled water (dH_2O), 0.75µl forward primer and 0.75µl reverse primer.

Primer sequences:
5'- TCGATGCAACGAGTGATGAG -3'
5'- AGTGCGTTCGAACGCTAGAG -3'

PCR was run under the following conditions:

- 94°C 5mins
- 94°C 30s
- 55°C 30s
- 72°C 10mins
- Samples held at 4°C

(94°C 30s – 72°C 10mins: 30 cycles)

2.2.3 **WT1 co/co PCR**

For each sample, 5µl of extracted DNA lysate were added to 12.55µl dH_2O, 2.5µl 10x buffer, 1.25µl $MgCl_2$, 0.2µl *Taq* DNA polymerase (all Invitrogen,

10342-020), 0.5µl forward primer, 0.5µl reverse primer and 2.5µl dNTPs (Invitrogen, 10297-018).

Primer sequences:

5'- TGGGTTCCAACCGTACCAAAGA -3'

5'- GGGTTATCTCCTCCCATGT -3'

PCR was run underthe following conditions:

- 94°C 2mins
- 94°C 15s
- 58°C 30s
- 72°C 1min
- 72°C 5mins
- Samples held at 4°C

35 cycles

2.2.4 Agarose Gel Electrophoresis

Agarose gels were made up at 1g/100ml agarose in 1x Tris-acetate-EDTA (TAE) buffer (40mM Tris acetate, 1mM EDTA) for *Cre* and WT1 co/co PCR. Gels were run at 100V for ~45mins until bands were sufficiently separated for visualisation. Gels were visualised under UV light. For VE-cadherin *Cre* genotyping, a band at 375bp indicated the presence of *Cre* (Figure 2.2). For WT1 co/co genotyping bands were at 196bp and 230bp (Figure 2.3).

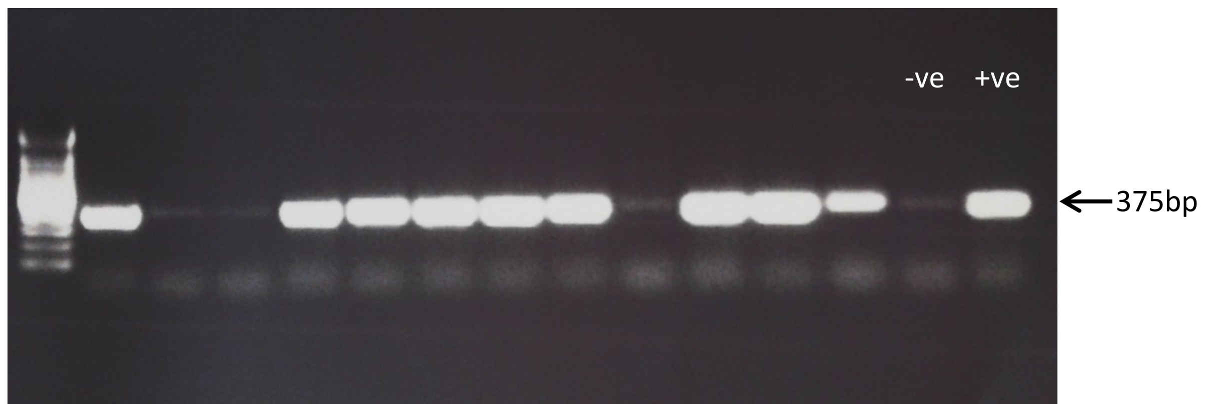

Figure 2.3: A representative agarose gel electrophoresis gel for *Cre* genotyping. A band at 375bp indicates the presence of a *Cre* allele. 100 bp DNA ladder is represented far left of the image. The two rightmost lanes are negative (-ve) and positive (+ve) controls respectively.

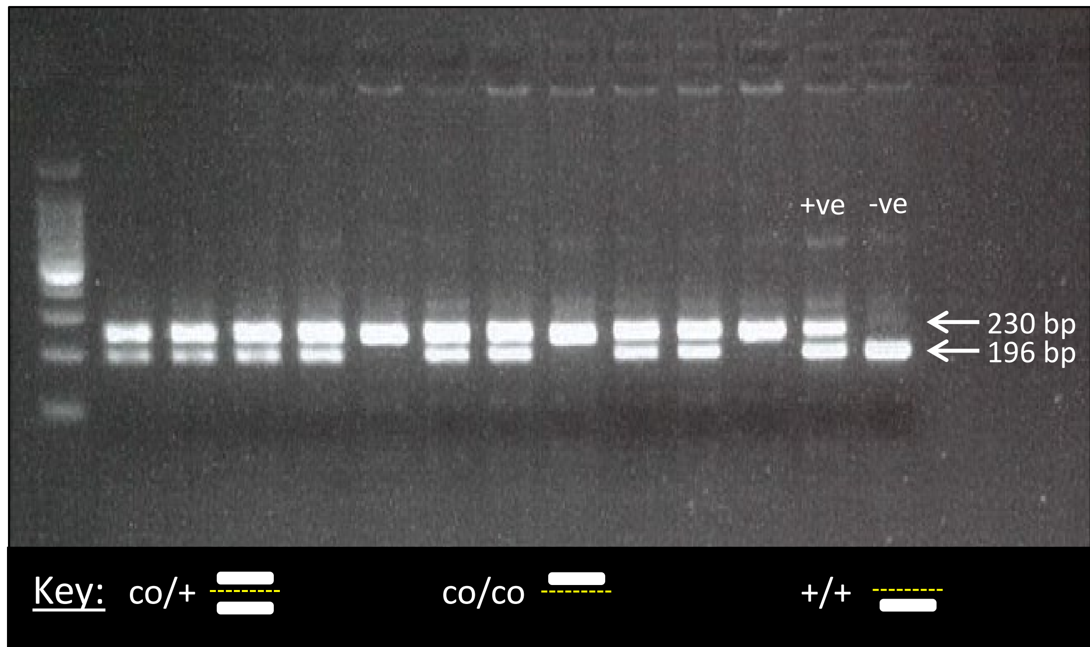

Figure 2.4: A representative agarose gel electrophoresis gel for WT1 co/co genotyping. The wild type (+) *WT1* allele forms a band at 196 base pairs (bp), while the conditional WT1 KO allele (co) forms a band at 230 bp. A key for interpreting the gels for different genotypes is shown. 100 bp DNA ladder is represented on the far left of the image. The two rightmost lanes are positive (+ve) and negative (–ve) controls, respectively.

2.3 Isolation of Mouse Aorta

Mice were killed by asphyxiation in CO_2. The ribcage was exposed by subcutaneous excision along the ventral midsagittal plane with surgical scissors. The diaphragm was pierced and thoracic cavity exposed by cutting through the sternum, ribs and diaphragm, as necessary to allow appropriate visibility and accessibility. Lung and oesophageal tissue were excised and the thoracic aorta was bisected immediately anterior to the diaphragm, using a cotton bud to absorb any resulting blood. Fat and adventitia were then cleaned from the aorta *in situ* using fine surgical scissors, from the site of bisection to the start of the aortic arch. The aorta is bisected at the aortic arch and the section of aorta is removed.

2.4 Aortic Ring Assay

Thoracic aortae were isolated as described (Section *2.3*). Aortae were cut into rings (~0.5mm in length) under a dissecting microscope (Olympus CK40, Olympus) using fine surgical spring scissors (Fine Science Tools). In a 96-well plate, one aortic ring per well was embedded in 50µl of collagen type 1 gel

(Millipore 08-115), made up to 1 mg/ml in OptiMEM (Gibco, 51985-026) with pH equalised by addition of NaOH (~10µl/ml 1M NaOH). Collagen gel was then left to set for 15mins at room temperature and 1hr at 37°c. 150 µl of one of four media preparations were then added to each well; a) OptiMEM only, b) OptiMEM + 5ng/ml VEGF (Peprotech, 450-32), c) OptiMEM + 2% FBS, d) OptiMEM + 2% FBS for the first three days and + 5ng/ml VEGF thereafter. Vessels were cultured in triplicate at 37°C in a humidified atmosphere at 5% CO_2.

Media were changed every 2-3 days and vessel growth was measured at intervals up to 12 days post-embedding. Vessel number was counted, blinded to treatment and the maximum vessel length was measured.

2.5 Myography

Aortae were isolated as described in Section *2.3*. Myography was performed using(del Campo and Ferrer, 2015) a Multi Myograph Model 610M wire myograph and Myo-Interface Model 310A (both Danish Myo Technology) with Labchart 8 software (ADInstruments). Prior to mounting vessels, physiological saline solution (PSS) (per 1L: 7.2g NaCl, 0.37g KCl, 0.17g $CaCl_2$, 1.8g Na_2HPO_4, pH 7.3) and PSS with 12mM KCl by equimolar substitution with NaCl (KPSS) were aerated with carbogen (95% O_2, 5% CO_2) and warmed to 37°C in a water bath. Myograph wells were filled with PSS and a section of aorta (~2mm in length) was mounted between the jaws of the myograph on 40µm steel wire wire using a dissecting microscope. If necessary, the luminal surface of the vessel was rubbed with a wire to remove the endothelium. Vessels were stretched to their optimal resting force; 7.34mN for aortae and 3.2mN for mesenteric arteries. Vessels were left to equilibrate, adjusting until force remained constant.

KPSS (6ml) was added to each well and maximum contraction response was recorded. Wells were thoroughly rinsed with PSS and then 6ml PSS was added to each well. Response to noradrenaline (NA), acetylcholine (ACh), serotonin (5-HT) and sodium nitroprusside (SNP) was then assessed by cumulative addition of each drug so the final concentration in the well incrementally increased (Table 2.2). On the day, drugs were diluted in dH_2O

to make stock solutions of $1x10^{-6}$M, $1x10^{-5}$M, $1x10^{-4}$M and $1x10^{-3}$M. For aortae, NA was only added up to a concentration of $3x10^{-6}$. Prior to assessment of relaxant responses to ACh and SNP, vessels were pre-constricted with NA at a concentration that achieved 50% of their maximum contractile response. Wells were rinsed with PSS between each different drug treatment and vessel left to equilibrate until they returned to basal tone.

Volume & Concentration Added	Final Concentration
6µl $1x10^{-6}$	$1x10^{-9}$M
12µl $1x10^{-6}$	$3x10^{-9}$M
42µl $1x10^{-6}$	$1x10^{-8}$M
12µl $1x10^{-5}$	$3x10^{-8}$M
42µl $1x10^{-5}$	$1x10^{-7}$M
12µl $1x10^{-4}$	$3x10^{-7}$M
42µl $1x10^{-4}$	$1x10^{-6}$M
12µl $1x10^{-3}$	$3x10^{-6}$M
42µl $1x10^{-3}$	$1x10^{-5}$M
120µl $1x10^{-3}$	$3x10^{-5}$M

Table 2.2: Myography drug treatments. The volume of NA, ACh, 5-HT and SNP added to each well, along with the final concentration achieved in the well is shown.

2.6 Anaesthesia and Analgesia

Pre-surgery, mice were anaesthetised with inhaled isoflurane (5%) in O_2 and anaesthesia was maintained thereafter with 3-4% isoflurane in O_2. At the site of incision, hair was removed exposed with clippers and sterilised with Povidone Iodine solution (Videne, 2122828). Vetergesic (buprenorphine) (Ceva) was diluted 1:10 in sterile H_2O and injected subcutaneously at dose of 0.05mg/kg body weight. During surgery, 1% lidocaine HCl (Hospira, RL-1045) was applied topically to lubricate exposed tissue if necessary. Post-surgery, EMLA cream (Astra Zeneca, 00651) was applied topically. All surgery was carried out on a heated mat to regulate body temperature under anaesthesia.

2.7 Subcutaneous Sponge Implantation Model

A roughly 1.5cm horizontal incision between the scapulae was made and, using blunt dissection, this incision was extended subcutaneously to create

two tunnels extending caudally over the left and right hindquarters. One sterile polyurethane sponge (0.5cm x 0.5cm x 1cm) was inserted into each side and the wound was closed using closure clips (1.75mm x 1.75mm; Fine Science Tools, 12040-01). Mice were allowed to recover and clips were removed after 7-10 days.

Mice were culled by CO_2 21 days post-implantation. Sponges were then excised, cut in half across the shortest axis and either fixed in 4% zinc formalin for 24hrs or snap frozen on dry ice and stored at -80°C prior to analysis.

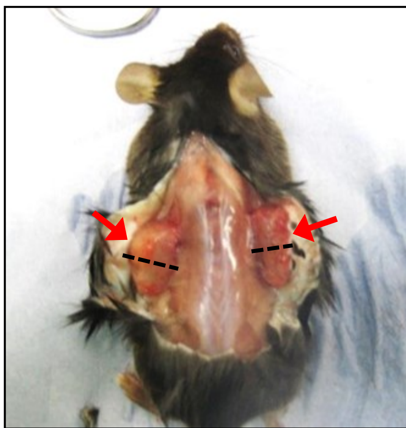

Figure 2.5: Exposed subcutaneous sponges *in situ* 21 days after implantation. Sponges are indicated by red arrows and the dotted line represents the axis along which sponges were cut for processing.

2.7 Hindlimb Ischaemia

2.7.1 Femoral Artery Ligation

Protocol was carried out in line with that previously described for the hindlimb ischaemia model (Limbourg *et al.*, 2009; Niiyama *et al.*, 2009). Fur was removed from the ventral surface of both hindlimbs. Using a laser Doppler scanner (Moor Instruments) and Moor LDI software (5.3) (Moor Instruments) a pre-operative laser Doppler scan was taken. Mice were placed under the scanner and using a laser blood flow in the murine hindlimbs were assessed.

Then, a 1cm incision was made in the medial thigh, starting at the mid-point of the inguinal ligament. The femoral vessels were revealed using blunt dissection and the femoral artery was separated from the femoral vein. Two double-knotted ligatures were applied to the femoral artery with 5/0 suture silk

(Fine Science Tools, 18020-50), proximal to the inguinal ligament and another two ~20mm distally. The femoral artery was then bisected along its length *in situ*. The skin was closed with non-absorbable 5/0 monofilament sutures (Ethilon, W1618T). Another laser Doppler was performed post-operatively to confirm ablation of blood flow and further laser Doppler scans were carried out at 3, 7, 14, 21, 28 days. Colour quantification of laser Doppler images was analysed by MoorLDI software (5.3) (Moor Instruments) to quantify the extent of blood flow, represented by colour spectrum.

2.7.2 Muscle Isolation

After 28 days and completion of all laser Doppler scans, mice were killed by asphyxiation in CO_2. The adductor and gastrocnemius muscles were isolated from both the injured and uninjured hindlimbs. Muscles were then cut in half across the shortest axis and either fixed in 4% zinc formalin for 24hrs or snap frozen on dry ice and stored at -80°C prior to analysis.

2.8 Murine Models of Arterial Remodelling

2.8.1 Wire Injury

Intra-luminal injury was surgically induced in the left femoral artery using a wire injury model (Sata *et al.*, 2000). A 1cm incision was made in the medial thigh, starting at the mid-point of the inguinal ligament. The femoral vessels were revealed using blunt dissection and a small, homemade retractor. The branch of the femoral artery was ligated distal to the site of incision and a small incision was made to the profunda femoralis at the branch of the femoral artery and the profunda femoralis. An angioplasty wire was inserted, in a retrograde direction, ~5mm proximally into the common femoral artery. The wire was left in place for 30s before removal. Permanent ligatures (Fine Science Tools, 18020-50) were applied to the profunda femoralis proximal to the site of incision. Temporary ligatures were removed and the skin was closed with non-absorbable 5/0 monofilament sutures (Ethilon, W1618T). Mice were allowed to recover.

2.8.2 Optical Projection Tomography

Following wire injury, vessel were isolated and embedded in 1.5% agarose (Invitrogen, 16520050), dehydrated in 100% methanol for 48hrs and then suspended in a solution of 1:2 (vol/vol) benzyl alcohol:benzyl benzoate for 48hrs. Optical projection tomography (OPT) was performed on a Bioptonics 3001 OPT tomograph. Exposure time was adjusted for each sample to provide high resolution images. 3D files were generated from raw images using NRecon software (Skyscan). Images were then viewed using CTan software (Skyscan). (Kirkby *et al.*, 2011; Zhao *et al.*, 2015).

2.9 Murine Menstruation Model

Uterine samples from C57Bl/6 female mice were kindly provided by Philippa Saunders and Douglas Gibson (QMRI, University of Edinburgh). This group has developed a mouse model of menstruation (Brasted *et al.*, 2003) modified to enable non- surgical induction of decidualisation and a longer decidualisation period. This is carried out using β-oestradiol, progesterone and an intra-uterine injection of sesame oil to induce decidualisation; described in full by Cousins *et al.* (2014). In this model, one uterine horn was decidualised, while the other was used as a non-decidualised control.

2.10 Quantitative PCR

2.10.1: RNA Extraction

Frozen sponge tissue was kept on dry ice prior to RNA extraction. 700µl of Qiazol (Qiagen) was added to each half sponge which was homogenised using an ultra turrax homogeniser (IKA T10 Basic) for ~3mins until smooth. Samples were then halved and a further 350µl of Qiazol was added to each half, prior to further homogenisation; the sampes were then left to rest for 5mins on wet ice. 200µl of chloroform was added and samples were mixed and left for 2mins at R.T. Samples were then centrifuged at 12,000 G at 4°C for 15mins. The aqueous phase was removed and combined from the two halves of the same sample. Further RNA purification was carried out using an RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. RNA quality and concentration were determined using a Nanodrop ND-1000 (Thermo

Scientific) spectrophotometer. RNA integrity was determined by agarose gel electrophoresis on a 1% agarose gel made up with TAE and stained with Gel Red (Figure 2.5).

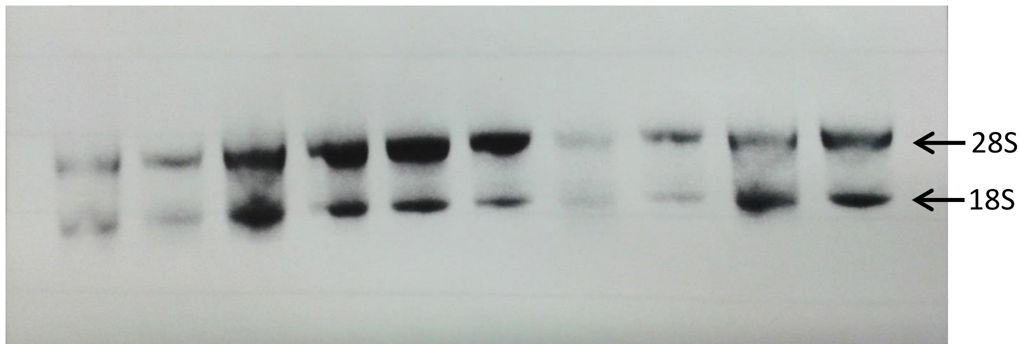

Figure 2.7: Representative RNA Integrity analysis. Good RNA integrity is shown by clear bands and a roughly 2:1 ratio between 28S and 18S bands, respectively. Weaker bands are representative of a lower RNA concentration.

2.10.2 cDNA Synthesis

cDNA synthesis was carried out using a QuantiTect Reverse Transcription Kit (Qiagen) on ice. Using RNA concentration values from the Nanodrop spectrophotometer, 500ng of RNA was diluted to a final concentration of 12µl in nuclease-free H_2O per sample. To this, 2µl of gDNA Wipeout Buffer was added and samples were incubated at 42°C for 2mins and then replaced on ice. To each sample, 4µl Quantiscript RT Buffer, 1µl RT Primer Mix and 1µl Quantiscript Reverse Transcriptase were added. Samples were then incubated at 42°C for 15mins, then 95°C for 3mins. As negative controls, one sample was prepared with 12µl H_2O instead of RNA and one sample per experimental group with 1µl H_2O instead of Reverse Transcriptase. cDNA was stored at -20°C.

2.10.3 Quantitative Real Time PCR

Concentrated cDNA was diluted 1/10 or 1/20 in nuclease-free H_2O. Standards were made up by pooling cDNA from all samples (except negative controls) and serially diluting 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 in nuclease-free H_2O. All procedures were carried out on wet ice unless otherwise stated. Mastermix was prepared as shown (Table 2.2) and was distributed on a 96-well plate at 8µl per well with 2µl cDNA. Samples and standards were loaded in triplicate. Wells were covered and centrifuged at

1500rpm for 2mins. 96-well plates were loaded onto a LightCycler 480 Instrument (Roche) and run on LightCycler 480 software (Roche). All primers (Table 2.3), probes and mastermix were from Roche.

Reagent	Volume (µl/well)
Nuclease-free water	2.7
Probe Master	5
Primer (forward)	0.1
Primer (reverse)	0.1
Probe	0.1
Total	8

Table 2.3: qRT-PCR Mastermix. The volume of each reagent added to create 8µl mastermix for addition to each 2µl cDNA sample for qRT-PCR.

Gene	Primer Sequences	Probe Used
WT1	5'- GCACTAGACAAAGTTCACCTGAGA -3'	#95
	5'- CGCTATCCACATCAAAGCAA -3'	
HIF-1α	5'- GCACTAGACAAAGTTCACCTGAGA -3'	#95
	5'- CGCTATCCACATCAAAGCAA -3'	
VEGF-A	5'- AAAAACGAAAGCGCAAGAAA -3'	#1
	5'- TTTCTCCGCTCTGAACAAGG -3'	
CD31	5'- CGGTGTTCAGCGAGATCC -3'	#45
	5'- ACTCGACAGGATGGAAATCAC -3'	
Snail	5'- CTTGTGTCTGCACGACCTGT -3'	#71
	5'- CAGGAGAATGGCTTCTCACC -3'	
Slug	5'- CATTGCCTTGTGTCTGCAAG -3'	#71
	5'- AGAAAGGCTTTTCCCCAGTG -3'	
PDGFR-β	5'- TGATGAAGGTCTCCCAGAGG -3'	#1
	5'- CTGCTTGCTGTGGCTCTTCT -3'	

Table 2.4: Genes and primer sequences used in RT qPCR along with the probe number used for each. Sequences of qPCR probes for each gene and the corresponding probes to use were identified using the Roche UPL probe identifier tool.

2.11 Histological Techniques

2.11.1 Preparing Slides

Samples were fixed overnight in 4% zinc formalin and then stored in phosphate-buffered saline (PBS) prior to embedding in wax blocks. Wax blocks were sectioned at 5µm on a Leica RM2235 Rotary Microtome (Leica Biosystem, UK), mounted on slides and dried at 37°C overnight. Slides were dewaxed in xylene for 10mins then rehydrated in 100%, 95%, 80% and 70% ethanol for 20secs each before washing in H_2O. Staining was then carried out as per individual protocols.

2.11.2 Haematoxylin and Eosin

Slides were submerged in Mayer's Hematoxylin (Dako, UK) for 5mins, then Acid ethanol (1% HCl in 70% EtOH), Scott's tap water for 20-30secs and Eosin for 5-10 secs (Dako). A wash in tap water was carried out between each of the above steps. Sections were then dehydrated in 70%, 80% 90% and 100% ethanol for 20s each and xylene for 10mins prior to mounting coverslips with mounting medium (Pertex, SEA-0104-00A).

2.11.3 Picrosirius Red

Picrosirius red staining was carried out by the Histology department (Shared University Research Facilities (SURF), University of Edinburgh) at The Queen's Medical Research Insitute, University of Edinburgh.

2.11.4 Immunoprecipitation Staining

Antigen retrieval was carried out by submerging slides in 10mM sodium citrate buffer and boiling for 5mins in a pressure cooker. Slides were then submerged in 0.3% H_2O_2 solution in dH_2O for 30mins and 50mM NH_4Cl in PBS (pH7.4) for 30mins, rinsing thoroughly in between and after in dH_2O. Samples were blocked with 10% serum (in PBS) from the animal in which the primary antibody was raised, for 1hr at R.T. Samples were incubated with the primary antibody overnight at 4°C in a humidified chamber.

Slides were then thoroughly washed in PBS and incubated with the secondary peroxidase-conjugated antibody for 1hr at R.T., rinsing thoroughly after with

PBS. DAB staining was then carried out for 10mins using ImmPACT DAB Substrate (Vector, UK) as per manufacturer's instructions. Slides were counterstained with Haematoxylin and Scott's tap water, dehydrated and mounted as described in H&E Section.

2.12 **Immunofluorescent Staining**

2.12.1: **Paraffin-embedded, Formalin-fixed Tissue**

Slides were prepared as in section *2.9.1*. Antigen retrieval was carried out by submerging slides in 10mM sodium citrate buffer and boiling for 5mins in a pressure cooker. Slides were then submerged in 0.3% H_2O_2 solution in dH_2O for 30mins. Samples were blocked with 10% serum in PBS from the animal in which the primary antibody was raised for 1hr at R.T. Samples were then incubated with primary antibodies overnight at 4^oC in a humidified chamber.

Slides were then thoroughly washed in PBS and incubated with the appropriate secondary antibody for 1hr at R.T., rinsing thoroughly after with PBS. Counterstaining with DAPI (1/1000 in PBS) was carried out for 10mins. Slides were thoroughly rinsed in PBS and mounted with coverslips using PermaFluor (Thermo Scientific).

2.12.2 **Aortic Rings**

Aortic rings were stained in 96-well plates with 50µl /well of all reagents and at R.T. unless otherwise stated. Culture medium was removed and wells were washed with PBS. Aortic rings were fixed with 4% zinc formalin per well for 30mins, then thoroughly washed in PBS. 0.25% Triton X-100 in PBS was added for 30mins, followed by thorough washing with PBS. Aortic rings were blocked with 10% serum in PBS from the animal in which the primary antibody was raised for 1hr. Samples were then incubated with primary antibodies overnight at 4^oC.

Wells were thoroughly rinsed with PBS + 0.1% (vol/vol) Triton X-100 before incubation with secondary antibodies for 1hr. DAPI was then added at 1/1000 for 10mins, washing with PBS before and after.

Each well was refilled with dH_2O and collagen gels containing the aortic tissue were detached by running a needle around the edge of the well. Gels were removed from the bottom of the well using fine forceps and manipulated up the side of the well onto a slide. Up to six gels were mounted per slide and mounted with coverslips using PermaFluor (Thermo Scientific).

2.12.3 Tyramide Signal Amplification

For WT1 immunofluorescence, tyramide signal amplification was carried out using TSA™ Plus System (PerkinElmer, USA) conjugated to Fluorescein, Cyanide 3 or Cyanide 5 fluorescent proteins. As per the protocol in section 2.11.14, a peroxidase-conjugated anti-rabbit antibody was added at the secondary antibody step for 1hr at R.T. Then, following a thorough rinse with PBS, TSA™ Plus tyramide was added at 1/50 in the provided reaction diluent for 10mins at R.T. Slides were rinsed again in PBS and the rest of the protocol was carried out as for Paraffin-embedded Sections.

2.13 Transcriptomics

2.13.1 WT1-GFP FACs Sorting

FACs was carried out by Richard McGregor with the help of the QMRI FACs facility. Subcutaneous sponges were implanted in WT1-GFP reporter mice as described in section *2.7*. Sponges were removed at day 7 or day 21. Cells were extracted from sponges and sorted by GFP expression into GFP^+ and GFP^- cells.

2.13.2 Whole Genome RNA Expression Profiling

Sorted cells from section *2.13.1* were submitted to the Illumina Whole Genome Expression Profiling Service (Qiagen). In brief, extracted whole genome total RNA was screened for RNA expression on a MouseWG-6 or MouseRef-8 v2 Expression BeadChip with Illumina iScan. This provided expression levels of RNAs expressed in GFP^+ and GFP^- cells at Day 7 and Day 21.

2.13.3 **Transcriptomic Data Analysis**

Data from section *2.13.2* was processed with the help of Graeme Grimes and the IGMM Bioinformatics Core. Microarray data was read in R using the bioconductor package 'limma' and raw expression profiles were normalised so that the intensities or log-ratios had similar distribution across a set of arrays. The raw data was checked using expression distribution and found to be of good quality. Probes not expressed in any samples were removed using a detection filter of $p< 0.01$. Differential expression analysis was conducted using limma. Genes which had a significantly different levels of gene expression between GFP^+ and GFP^- groups were identified using an FDR cut off of $q<0.05$.

Processed data were analysed using the online programs GOrilla (Eden *et al.*, 2007, 2009), ReviGO (Supek *et al.*, 2011) and DAVID (Huang, Sherman and Lempicki, 2008, 2009).

2.13.4 **Identification of Potential WT1 Target Genes**

In order to determine potential genes with which WT1 may interact, the location of known motifs to which WT1 binds (identified from previous experiments; Table 2.4), was determined within the mouse genome. This was performed using two online programs; BLASTN from Ensembl [release 88] (Aken *et al.*, 2016) and RSAT Metazoa (Medina-Rivera *et al.*, 2015).

	WT1-binding sequence	Identified by
1.	5'- GCGGGGGCG -3'	Rauscher *et al.* 1990
2.	5'- CGCCCCCGC -3'	Rauscher *et al.* 1990
3.	5'- TCCTCCTCCTCCTCTCC -3'	Wang *et al.* 1993
4.	5'- GCGTGGGAGT -3'	Nakagama *et al.* 1995
5.	5'- GCGTGGGCG(T/G)(G/A/T)(T/G) -3'	Hamilton *et al.* 1995
6.	5'- GGAGAGGGAGGATC -3'	Little *et al.* 1996

Table 2.6: WT1-binding sequences identified from previous literature. Sequences of identified WT1-binding motifs used to identify potential direct WT1-target genes, along with the paper in which each sequence was originally identified.

BLASTN was used to screen the entire C57Bl/6J mouse genome for the location of WT1-binding sequences. Search parameters were confined as follows:

Search Sensitivity:	Short sequences
Max E-value:	1000
Word size for seeding assignments:	7
Filters:	None

RSAT was used to extract promoter sequences of genes whose expression were significantly changed between GFP^{+} and GFP^{-} groups at both Day 7 and Day 21 in data from section *2.13.2*. Extracted promoter sequences were then screened for the presence and location of WT1-binding sequences in both the reverse and forward strands using the RSAT tool "DNA-pattern", allowing for no substitutions in target sequence to reduce the probability of false positive hits. Mouse genome release for reference was "Mus Musculus GRCm38".

To control for random hits in BLASTN and RSAT, analysis was repeated for non-sense control sequences. These randomly generated sequences were 9bp long with a GC content of 0.5.

Lists of genes with potential WT1-binding sites were analysed using GOrilla, Revigo and DAVID as before.

2.14 **Statistics and Analysis**

Statistical analysis was carried out using Minitab 16 software (Minitab) and Prism 5 software (GraphPad). Prism 5 was used for all graphical analysis. Image analysis, including DAB and PSR staining analysis and nuclei counting was carried out using Image J. Data was assessed for a normal Gaussian distribution and parametric or non-parametric analysis was carried out accordingly. Student's t-test, or Mann-Whitney u-test were carried out for data with two groups. For data with more than two groups, one-way ANOVA was carried out with post-hoc Bonferroni tests, or a Kruskal-Wallis one-way ANOVA. For data with more than two groups and two dependent variables, two-way ANOVA was carried out. To determine correlation, linear regression was applied. All data was shown as mean +/- s.e. mean. Groups were considered to be significantly different if $p<0.05$.

CHAPTER 3:

WT1 in Angiogenesis and Vascular Function *Ex Vivo*

3.1 Introduction

WT1 has been investigated thoroughly in a number of contexts, including development, developmental kidney disorders and cancer. However, the role of WT1 in the vasculature, specifically in the context of cardiovascular disease is much less well-characterised. In order to gain an initial understanding of the role of WT1 in the vasculature and endothelial cells, *in vitro* and *ex vivo* model have often been used. These have a number of advantages over *in vivo* approaches in that, while they are sometimes less representative of physiological processes, they are simple, reproducible, easily manipulated, readily observable and variables can be more effectively controlled. For these reasons, *in vitro* and *ex vivo* experimental approaches have often been employed to examine the functional role of WT1, either independently or in combination with *in vivo* experiments. This approach was also employed for the experiments in this chapter. For context, the work to date on WT1 *in vitro* and *ex vivo* of relevance to vascular function is outlined below.

As previously discussed (1.3), WT1 is expressed *in vivo* in developing vasculature and in vasculature undergoing angiogenesis (reviewed by Scholz *et al.* 2009). Work performed *in vitro* has attempted to elucidate the role of WT1 in the vasculature in angiogenesis. The coordinated action of WT1 and two major regulators of angiogenesis, HIF-1α and VEGF has been demonstrated *in vitro* and *in vivo*, in non-vascular cell lines; WT1 directly regulates *VEGF* and is itself transcriptionally regulated by HIF-1α (Wagner *et al.*, 2003; Cash *et al.*, 2007; Hanson *et al.*, 2007; McCarty, Awad and Loeb, 2011). Whilst these relationships have not been proven in endothelial cells, it would be surprising if the same transcriptional mechanisms did not occur since HIF-1α is widely expressed in endothelial cells in angiogenic vasculature (K. Wagner *et al.*, 2002). HIF-1α and other hypoxia-inducible factors are the main regulators of the vascular response to hypoxia and concordantly, hypoxia induces an increase in WT1 expression (~3 fold) in HUVECs (Duim *et al.*, 2015). This is of relevance as endothelial cells, VSMCs and pericytes are all sensitive to oxygen levels and hypoxia is the main inducer of pathological and regenerative angiogenesis (Moeller *et al.*, 2004; Semenza, 2014).

On a cellular level, WT1 has previously been demonstrated to play a role in cell proliferation, growth, migration, transition, adhesion, survival and apoptosis (reviewed by Hastie 2017). In endothelial cells, many of the same processes seem to be of relevance. In HUVECs, it was found that $WT1^+$ cells had much higher levels of PCNA expression (Duim *et al.*, 2015) and reducing WT1 protein levels by siRNA resulted in reduced proliferative capacity, alongside reduced PCNA and BrdU expression, with no significant effect on apoptosis (Wagner *et al.*, 2008). Reducing WT1 expression by shRNA in a scratch assay significantly reduced cell migration, though interestingly, overexpressing C-kit, a direct target of WT1, somewhat restored the migratory response (Wagner *et al.*, 2014).

Perhaps the most commonly deployed *in vitro* angiogenic assay is the 2D-tube formation assay. Here, endothelial cells are plated onto a basement membrane and spontaneously form luminal endothelial vessels. This is a quantifiable, reproducible assay involving endothelial cell, migration, adhesion, ECM degradation and tubule formation (Arnaoutova and Kleinman, 2010). The role of endothelial WT1 in this 2D-tube formation assay is clear. WT1 expression increased vessel formation in this assay for HUVECs cultured with Ewing's sarcoma cell lines (McCarty, Awad and Loeb, 2011), in HUVECs silenced with *Wt1* siRNA (Wagner *et al.*, 2008) and C166 mouse endothelial cells with *Wt1* shRNA (Wagner *et al.*, 2014). A more complex, *ex vivo* angiogenic assay involving a number of vascular cell types is the aortic ring assay, in which the thoracic aorta is isolated from mice and embedded in collagen or Matrigel. Recent results from this assay used a *Tie2Cre*ER^{T2} *Wt1* KO (Wagner *et al.*, 2014). *Tie2* is a gene expressed in endothelial cells and a proportion of cells of a haematopoietic lineage including macrophages, B- and T-lymphocytes, natural killer cells and dendritic cells, so the inducible *Tie2CreER*^{T2} *Wt1* KO will reduce *Wt1* expression in all these cells (Constien *et al.*, 2001; Kisanuki *et al.*, 2001). In the aortic ring assay, *Tie2CreER*^{T2} *Wt1* KO almost entirely abolished angiogenic vessel formation (Wagner *et al.*, 2014). All of this strongly implicates WT1 in endothelial angiogenesis.

As WT1 is not expressed in adult quiescent vasculature (McGregor et al.), it would not be expected to play a physiological role in most tissues. Therefore,

the role of WT1 in regulation of vascular tone has not been directly investigated, despite long having been known that WT1 mutation in Denys-Drash syndrome and other WT1-associated glomerulopathies can manifest with hypertension (Little *et al.*, 1993; Wagner *et al.*, 2008; Lipska *et al.*, 2014). In congenital obstructive nephropathy, *Wt1* is downregulated resulting in apoptosis, however nitric oxide (NO)/Hsp70 expression reduces this apoptosis and in kidney cells NO regulates *Wt1* mRNA expression (Mazzei *et al.*, 2010). NO-mediated regulation of *Wt1* transcription was also observed in lung epithelial cells and lymphocytes (Marcet-Palacios, Davoine, *et al.*, 2007; Marcet-Palacios, Ulanova, *et al.*, 2007). This is of particular interest because NO, as a potent vasodilator, is essential for maintenance of endothelial cell function and vascular tone. Intriguingly, as well as NO influencing *Wt1* transcription, WT1 is capable of directly regulating inducible nitric oxide synthase (iNOS), which in turn synthesises NO (Johannesen *et al.*, 2003; Mazzei *et al.*, 2010). The inextricable link between vasodilation and angiogenesis is a paradigm well accepted in the case of VEGFs and NO also has a dual role in these entwined processes, as a target of VEGF (Coletta *et al.*, 2012).

Therefore, while some interesting insights into the role of WT1 in vascular function have arisen from *in vitro* and *ex vivo* experiments, there remains much that has been either completely neglected or remains partially investigated. It is clear there is a role for WT1 in regulating angiogenic function of the endothelium in the 2D-tube formation assay, but it is unclear through what mechanism. For example, while a number of groups have suggested WT1 is important in *in vitro*/*ex vivo* angiogenesis, none have examined its expression during this process. The work herein will attempt to provide more clarity to this situation by further investigating the role of WT1 in endothelial cell function and angiogenesis.

3.1.2 Hypothesis

Given the previous implication of WT1 in angiogenesis *in vitro*, the work described in this chapter addressed the hypothesis that:

"As Wt1 is intrinsically involved in angiogenesis in endothelial cells, Wt1 KO in the vascular endothelium will impair ex vivo *angiogenesis."*

3.1.3 **Aims**

1. Introduce the VE-WT1 KO model of inducible endothelial-selective WT1 deletion to Edinburgh.
2. Determine whether selective deletion of WT1 from endothelial cells (VE-WT1 KO) alters endothelium-dependent relaxation.
3. Characterise and optimise the *ex vivo* aortic ring angiogenesis assay.
4. Determine whether selective deletion of WT1 from endothelial cells (VE-WT1 KO) inhibits *ex vivo* angiogenesis.

This VE-Wt1 KO mouse under the control of *VE-Cadherin Cre* is advantageous in comparison to the *Tie2 Cre* mouse for investigating endothelial WT1, as in contrast to *Tie2 Cre*, it only knocks-out *Wt1* in adult endothelial cells and not haematopoietic cells (Monvoisin *et al.*, 2006).

3.2 Materials and Methods

For full materials and methods refer to Chapter 2.

3.2.2 VE-Wt1 KO Mouse

In order to confirm the KO of *Wt1*, female VE-Wt1 KO mice, 16-18 weeks were used. *Cre* control (CC) and VE-Wt1 KO were used in this experiment (n=5). I.P. tamoxifen injections were carried out as described in 2.1.1. Uterine and mammary tissue were harvested 1 week after finishing injections, fixed in 10% formalin and wax embedded prior to staining as described (2.12.2).

Immunohistochemistry using antibodies against CD31/αSMA/WT1/DAPI was carried out on uterine tissue. Rabbit v mouse anti-CD31 was used at 1/500 (ab28364, Abcam), with secondary donkey v rabbit Alexa 488 (1/200) (Invitrogen, A21206); Rabbit v mouse anti-WT1 (1/500) (Abcam, ab89901) with secondary peroxidase IgG (1/200) (Vector, PI-1000) and TSA Tyramide Cy3 (PerkinElmer, SAT705A001EA); Rabbit v mouse anti-αSMA (1/500) (C6198, Sigma); DAPI (1/1000) (D9542, Sigma).

This staining was quantified in two ways: as the proportion of $CD31^+$ vessels which have any $WT1^+$ endothelial cells compared with those vessels with no $WT1^+$ endothelial cells and as the proportion of $CD31^+$ endothelial cells (identified by $DAPI^+$ nuclei) which were $WT1^+$.

Male VE-Wt1 KO mice, 11-19 weeks old were culled 4 weeks after the last injection of tamoxifen and heart, liver, spleen, kidney, kidney and testes were harvested fixed in 10% formalin and wax embedded as described (2.12.2). All these tissues were H&E stained. Testes, epididymal fat and seminal vesicles were harvested and weighed.

3.2.1 Myography

Myography was initially carried out on aortae from C57Bl/6 male mice, 12-16 weeks of age (n=3-5), in order to optimise the protocol and characterise the normal response of vasculature to NA, 5-HT, ACh and SNP.

To assess the effect of VE-Wt1 KO on vascular function, aortae from VE-Wt1 KO male mice, 11-16 weeks of age (n=7-8) were subjected to myography (see section 2.4, for full protocol).

3.2.2 **Aortic Ring Assay**

The murinethoracic aorta was isolated from mice and embedded in 1mg/ml collagen type 1 gel (Millipore 08-115). See section 2.5 for full details. Characterisation and optimisation of the aortic ring model was initially carried out in C57Bl/6 male mice, 12-20 weeks of age (n=5-6). Vessel outgrowths were quantified as shown in Figure 3.2.1.

For quantification of the effect of endothelial cell-selective *Wt1* KO on angiogenesis in the aortic ring assay, VE-Wt1 KO male mice 12-19 weeks of age were used (n=8). VE-Wt1 KO was induced by I.P. tamoxifen injection over 5 days as described in section 2.1.1. Aortic tissue was harvest 1-4 weeks after finishing the tamoxifen injection regimen.

For imaging WT1 in the angiogenic vessel outgrowths of the aortic ring assay, aortic section from a WT1-GFP (see section 2.1.2) male mouse 10 weeks of age was used (n=1). GFP was induced by including 4-OH Tamoxifen in the aortic ring media at a concentration of 1µM for the duration of the experiment.

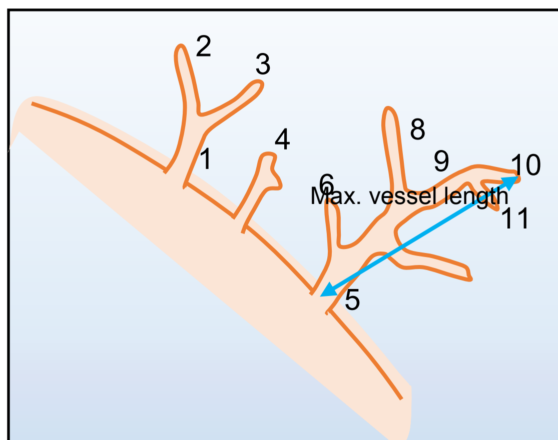

Figure 3.2.1 Quantification of angiogenic outgrowths from aortic rings. Outgrowths were counted at each new branch point, as shown, with the maximum vessel length quantified as the maximum distance of vascularisation.

Histological staining of aortic rings was carried out as described in section 2.12.2. The antibodies used for individual staining and the concentrations they were used at are shown in Table 3.2.1.

1º Antibody	2º Antibody
Rabbit v mouse anti-CD31 (1/500) (Abcam, ab28364)	**Goat v rabbit Alexa 488** (1/200) (Molecular Probes, GAR-A488)
Isolectin-B4 (1/500) (Invitrogen, 1110271)	**Streptavidin - Alexa 488** (1/200) (Invitrogen, A-32354)
Rabbit v mouse anti-αSMA (1/500) (C6198, Sigma)	Directly conjugated to Cy3
Rabbit anti-GFP (1/200) (Abcam, ab6556)	**Goat v rabbit peroxidase IgG** (1/100) (Vector, PI-1000) **TSA Tyramide Cy5** (1/50) (PerkinElmer, SAT705A001EA)
DAPI (1/1000) (Sigma, D9542)	N/A

Table 3.2.1: Antibodies used for aortic ring immunohistochemistry. Primary antibodies are provided with the relevant secondary antibodies used in the adjacent column. Antibody description/name, concentration used, provider and catalogue number are all provided.

3.3 Results

3.3.1 The VE-Wt1 KO Mouse Model

3.3.1.1 Confirmation of WT1 KO

Uterine tissue from VE-Wt1 KO mice was assessed and quantified from WT1/CD31/DAPI IHC. In CC (Figure 3.3.1 A-C) and VE-Wt1 KO (Figure 3.3.1 D-F) a proportion of vascular endothelial cells in some vessels were $WT1^+$, though the strength of staining for WT1 was lower than in the surrounding stromal cells, which were highly $WT1^+$. In VE-Wt1 KO tissue, a smaller proportion vessels had $WT1^+$ endothelial cells than in CC vessels ($p<0.05$, unpaired t-test) (Figure 3.3.1G). The total number of $WT1^+$ vascular endothelial cells was also quantified and had a trend towards being lower in VE-Wt1 KO in comparison to CC ($p=0.051$) (Figure 3.3.1H).

A number of organs isolated from VE-Wt1 KO male mice were H&E stained. General morphology of these tissues was assessed by a pathologist (Tim Aitman, University of Edinburgh) to determine whether there was any overt phenotypic different between VE-Wt1 KO, CC and VC mice. It was determined that there was no evident difference in morphology of the heart, liver, spleen or kidney between groups (Figure 3.3.2).

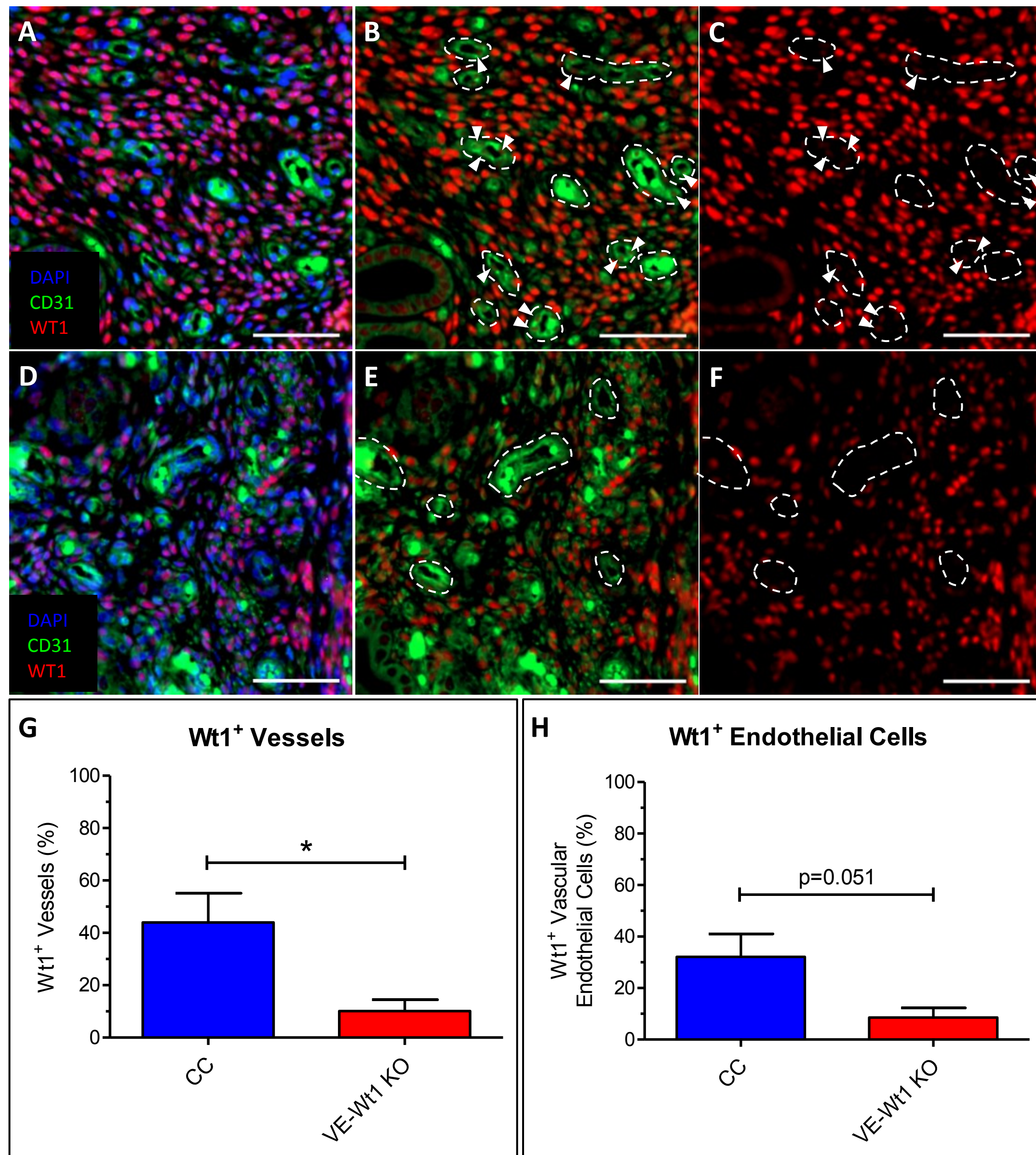

Figure 3.3.1: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) uterine tissue has less WT1$^+$ endothelial vessels than in *Cre* control (CC). CD31 (green), WT1 (red) and DAPI (blue) IHC was carried out on uterine tract tissue from **A-C)** CC and **D-F)** VE-Wt1 KO female mice. Endothelial WT1 staining (arrows), was noted in a proportion of vessels and endothelial cells in CC and VE-Wt1 KO, with less WT1$^+$ endothelial cells in VE-Wt1 KO. From this staining was quantified the proportion (%) of **G)** vessels with any WT1$^+$ endothelial cells and **H)** WT1$^+$ endothelial cells of total endothelial cells. There were significantly less WT1$^+$ vessels in VE-Wt1 KO v CC. Vessels outlined in white. Scale bars = 50µm. Columns represent mean + s.e.mean. *$p<0.05$, Student's unpaired t-test, n=5.

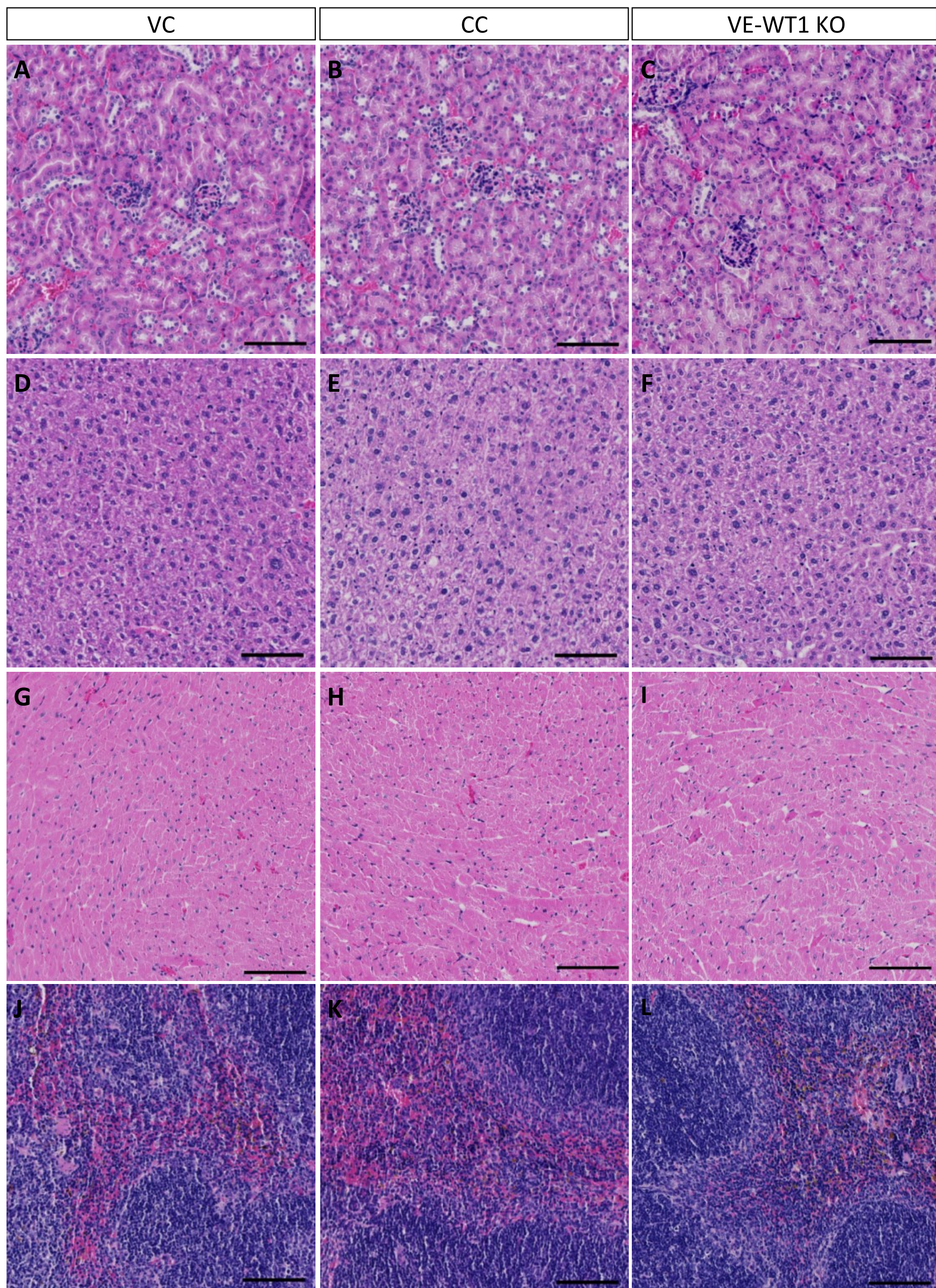

Figure 3.3.2: No morphological changes induced by vascular endothelial *Wt1* knock-out (VE-Wt1 KO) in a number of organs. H&E staining was carried out on extracted tissues 4 weeks after induction of VE-Wt1 KO in VE-Wt1 KO, vehicle control (VC) and *Cre* control (CC) mice on **A-C)** kidney, **D-F)** liver, **G-I)** heart and **J-L)** spleen. No gross morphological differences were noted in any of these tissues between groups. Representative images from n=5. VC = vehicle control, CC = *Cre* control, VE-Wt1 KO = vascular endothelial WT1 KO. Scale bars - 100μm.

3.3.1.2 **VE-Wt1 KO Mice Have Enlarged Gonads, but Reduced Testis Weight**

In VE-Wt1 KO mice, it was evident that the male gonad was visibly enlarged in comparison to CC and VC mice, which progressed with time after induction of VE-Wt1 KO. 4 weeks post-induction the weights of the epididymis, seminal vesicles and testis were recorded; it was found that there was a small, but robust reduction in male testis weight in VE-Wt1 KO in comparison to CC testis ($p<0.05$) (Figure 3.3.3 A-C).

With CD31/WT1/DAPI immunofluorescent staining, it was found that WT1 was expressed in the endothelium in a large proportion of vessels in the testis, in CC (Figure 3.3.3 D-F) and to a much lesser extent in VE-Wt1 KO (Figure 3.3.3 G-I).

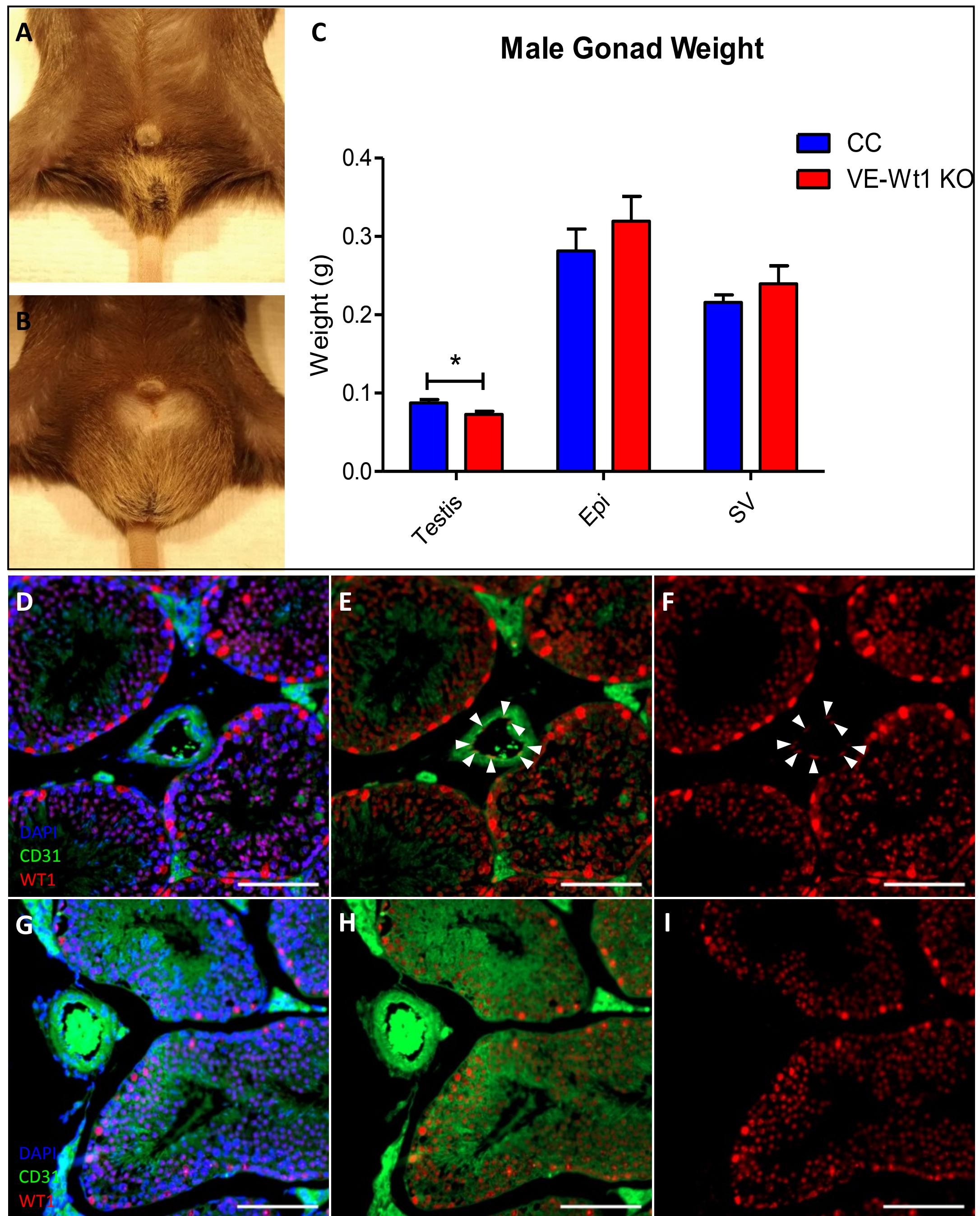

Figure 3.3.3: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) induces gross changes in gonad morphology due to reduction of endothelial testis expression. Gonad morphology in **A)** *Cre* control (CC) and **B)** VE-Wt1 KO. **C)** Testis weight was lower in VE-Wt1 KO than in *Cre* control four weeks post-induction with 33mg/kg tamoxifen. Weights of the epididymal fat (Epi), or seminal vesicles (SV) were not different. CD31/WT1/DAPI IHC in **D-F)** CC and **G-I)** VE-Wt1 KO testis. This reveals endothelial WT1 staining in CC testis in vascular endothelial cells. Scale bars = 100µm. Columns represent mean + s.e.mean; n=5, *p<0.05 (Student's unpaired t-test).

3.3.2 **Myography in C57Bl/6 Mice**

Myography was carried out on arteries from C57Bl/6 mice to establish the baseline responses of different vessels in response to contractile and relaxant drugs. Aortae and mesenteric arteries were used, either intact, or with the endothelium removed (denuded). These were assessed for viability by exposure to high (125mM) K^+ in physiological salt solution (KPSS). The type of artery and whether or not it was denuded had a significant effect on the contractile response ($p<0.05$, 2-way ANOVA) to KPSS (Figure 3.3.4 A). The responses to noradrenaline (NA) (Figure 3.3.4 B) and acetylcholine (ACh) (Figure 3.3.4 C) were assessed in intact and denuded aortae and mesenteric arteries and the response to sodium nitroprusside (SNP) (Figure 3.3.4 D) was assessed in intact and denuded aortae.

Both denuded and intact aortae were more sensitive to NA than intact mesenteric arteries and denuded mesenteric arteries and, hence, had higher PD_2 values for NA ($p<0.05$ denuded, $p<0.001$ intact, one-way ANOVA with Bonferroni post-hoc tests) (Table 3.3.1). Intact mesenteric arteries had a significantly greater maximum contractile response (Emax) to NA than intact aortae ($p<0.01$, one-way ANOVA with Bonferroni post-hoc tests) (Table 3.3.1).

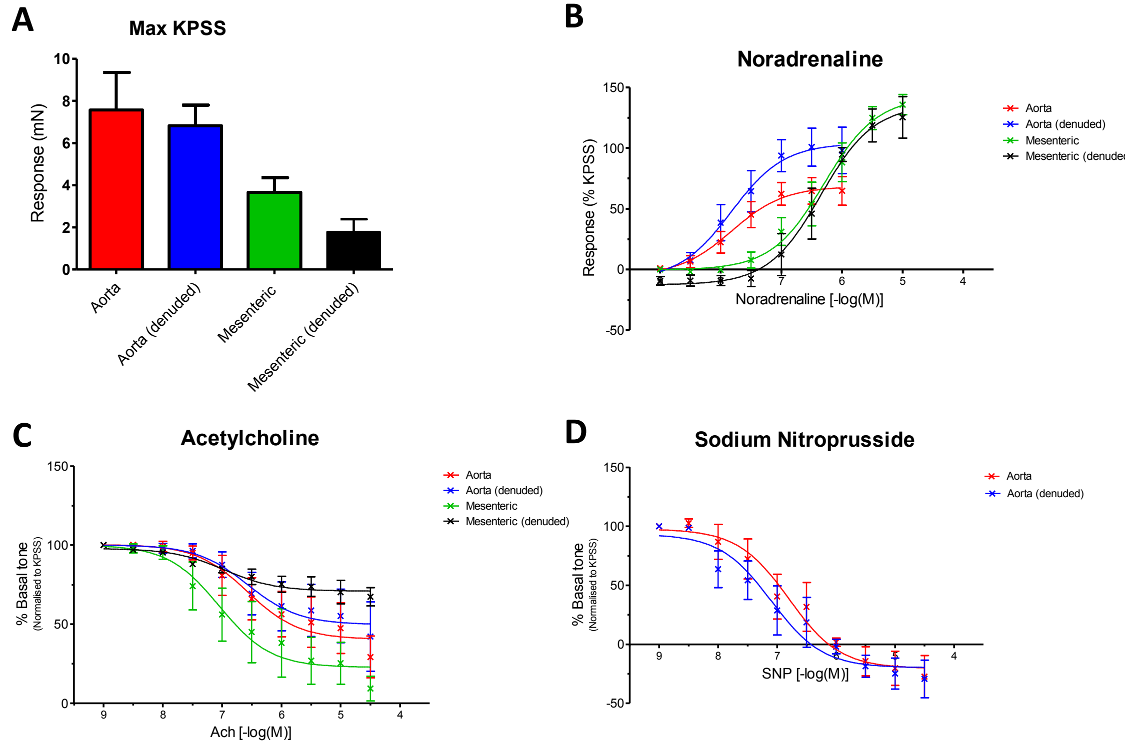

Figure 3.3.4: Analysis of normal vasoconstriction and vasodilation of C57Bl/6 arteries. Myography was carried out with C57Bl/6 aortae and mesenteric arteries, in vessels manually denuded to remove the endothelium alongside intact vessels. Vasoconstrictory response to **A)** KPSS and **B)** increasing concentrations of noradrenaline was assessed (normalised to KPSS response). Vessels were preconstricted with noradrenaline and then assessed for the vasodilatory response to acetylcholine and sodium nitroprusside, expressed as **C, D)** normalised to KPSS response and **E, F)** normalised to KPSS and expressed as % relaxation. n=5-7 for aorta. n=3-5 for mesenteric artery.

		Aortae		Mesenteric	
		ND	D	ND	D
NA	Emax: (% KPSS)	68.35 **	105.00	131.72 **	125.35
	PD_2: (-log conc.)	7.50 ***	7.42 *	6.10 ***	6.33 *
Ach	PD_2: (-log conc.)	5.50		6.38	
SNP	PD_2: (-log conc.)	7.00	7.10		

Table 3.3.1: Sensitivity (PD_2) and maximum contractile response (Emax) of C57Bl/6 aortae and mesenteric arteries. The Emax (%KPSS) (for NA only) and PD_2 (-log conc.) have been calculated for denuded (D) and non-denuded (ND) aortae and mesenteric arteries in response to NA, Ach and SNP. *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA with Bonferroni post-hoc tests (differences between boxes of same colour). n=3-5, mesenteric, n=7, aortae. NA = noradrenaline, Ach = acetylcholine, SNP = sodium nitroprusside.

3.3.3 VE-Wt1 KO Results in Increased Vasoconstriction

Myography was carried out to assess the effect of VE-Wt1 KO on general vascular function, vasoconstriction and vasodilation. There was no significant difference in contraction to KPSS between aortae from VE-Wt1 KO, VC and CC mice (Figure 3.3.5 A-B). Aortae from VE-Wt1 KO exhibited greater contraction in response to NA in intact, but not denuded aortae (Figure 3.3.5 A-B). VE-Wt1 KO aortae had a greater contractile response to NA (Figure 3.3.5 C) 5HT in intact aortae (Figure 3.3.5 E). Denuded VE-Wt1 KO aortae showed a trend toward having a greater contractile response to NA and 5HT, though this was not significant (Figure 3.3.5 D and F, respectively). There was no difference in the sensitivity between groups of aortae to NA or 5HT, as evident by a lack of difference in the PD_2 values, however, denuded VE-Wt1 KO aortae had a greater maximum contractile response (Emax) to 5HT in comparison to VC and CC aortae ($p<0.01$, one-way ANOVA) (Table 3.3.2).

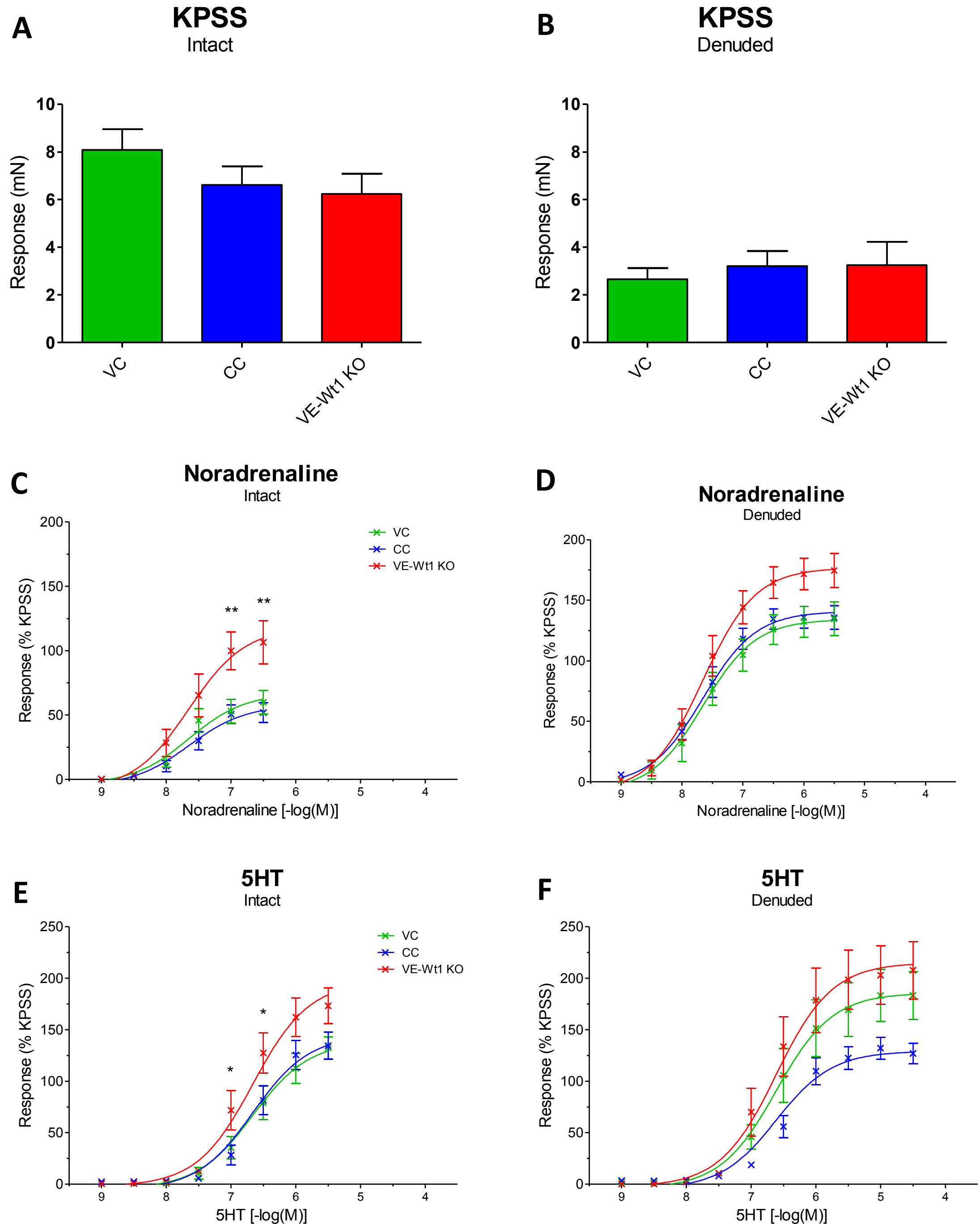

Figure 3.3.5: VE-Wt1 KO increases aortic vasoconstriction. Myography was carried out with VE-Wt1 KO, CC & VC aortae, in intact vessels and those manually denuded to remove the endothelium. There was no difference between groups in contractile response to KPSS in **A)** intact or **B)** denuded vessels. The contractile response to increasing concentrations of noradrenaline (normalised to KPSS response) was increased in **C)** intact, but not **D)** denuded VE-Wt1 KO aortae in comparison to CC and VC. The contractile response to increasing concentrations of 5HT (normalised to KPSS response) was also increased in **E)** intact VE-Wt1 KO aortae, but not **F)** denuded. *p<0.05, **p<0.01, VE-Wt1 KO v CC and VC, 1-way ANOVA with Bonferonni post-hoc test. n=7-8. VC = Vehicle control, CC = *Cre* control, VE-Wt1 KO = vascular endothelial *Wt1* KO. 5HT = 5-Hydroxtryptamine.

The vasodilatory response of VE-Wt1 KO aortae were then assessed in response to Ach and SNP in the same vessels. There was no difference in the vasodilatory response of denuded or non-denuded VE-Wt1 KO aortae in response to Ach (Figure 3.3.6 A & B) or SNP (Figure 3.3.6 C & D) in comparison to VC and CC aortae. There was no difference between groups in the PD_2 for Ach or SNP (Table 3.3.2).

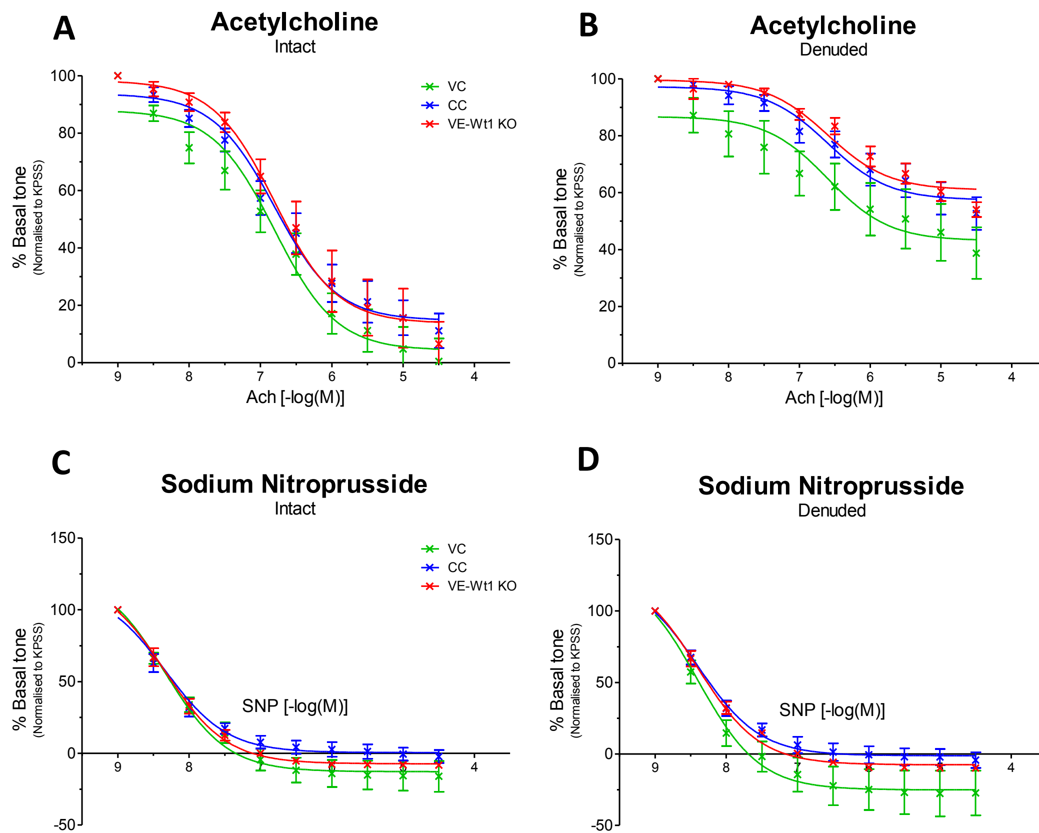

Figure 3.3.6: VE-Wt1 KO increases aortic vasoconstriction. Myography was carried out with VE-Wt1 KO, CC & VC aortae, in intact vessels and those manually denuded to remove the endothelium. There was no difference between groups in vasodilatory response to acetylcholine or sodium nitroprusside. Aortae were first pre-constricted with noradrenaline. Then vasodilatory responses to increasing concentrations of acetylcholine were assessed in **A)** intact aortae and **B)** denuded aortae and sodium nitroprusside in **C)** intact aortae and **D)** denuded aortae. Responses are normalised to KPSS and then shown as the % relaxation from starting vascular tone. There were no significant differences between groups. 1-way ANOVA with Bonferonni post-hoc test. n=7-8. VC = Vehicle control, CC = *Cre* control, VE-Wt1 KO = vascular endothelial *Wt1* KO. Ach = acetylcholine, SNP = sodium nitroprusside.

		VC		CC		VE-Wt1 KO	
		ND	D	ND	D	ND	D
NA	Emax: (% KPSS)	73.91	134.04	70.78	138.90	96.24	175.16
	PD_2: (-log conc.)	7.44	7.43	7.33	7.42	7.43	7.36
5HT	Emax: (% KPSS)	156.74	134.04	141.49	134.90	180.18	217.46 **
	PD_2: (-log conc.)	6.38	6.21	6.31	6.13	6.67	6.42
Ach	PD_2: (-log conc.)	6.71		6.5		6.14	
SNP	PD_2: (-log conc.)	8.06	8.21	8.06	8.00	8.07	8.00

Table 3.3.2: VE-Wt1 KO aortae have an increased Emax response to 5HT when denuded of endothelium. The Emax (%KPSS) (for vasoconstrictory drugs only) and PD_2 (-log conc.) have been calculated for VC, CC and VE-Wt1 KO denuded (D) and non-denuded (ND) aortae. VE-Wt1 KO denuded aortae have a greater Emax than VC and CC denuded aortae. No values for denuded aortae in response to Ach, due to lack of response. **$p<0.01$, VE-Wt1 KO, v VC and CC, 1-way ANOVA with Bonferroni post-hoc tests. n=6-8. NA = noradrenaline, 5HT = 5-hydroxytryptamine, Ach = acetylcholine, SNP = sodium nitroprusside. VC = vehicle control, CC = *Cre* control, VE-Wt1 KO = vascular endothelial WT1 KO.

3.3.4 ***Ex Vivo*** **Angiogenesis in the Aortic Ring Assay**

3.3.4.1 **Quantification of Vessel Outgrowth**

The aortic ring model was used as an *ex vivo* model of angiogenesis. Aortic rings isolated from C57Bl/6 mice were embedded in collagen and cultured in one of five different media; OptiMEM, +VEGF, +FCS, +FCS +VEGF or <3 FCS >3VEGF. In all media, multiple vessel outgrowths formed from the surface of the aortic tissue (Figure 3.3.7). From visual analysis, at Day 3 post-embedding outgrowth of cells was evident. These began to form into connected vessel

outgrowths at Day 5 and Day 7 and then continued to grow and branch up until around Day 7-10, after which point there was no clear, consistent growth or elongation.

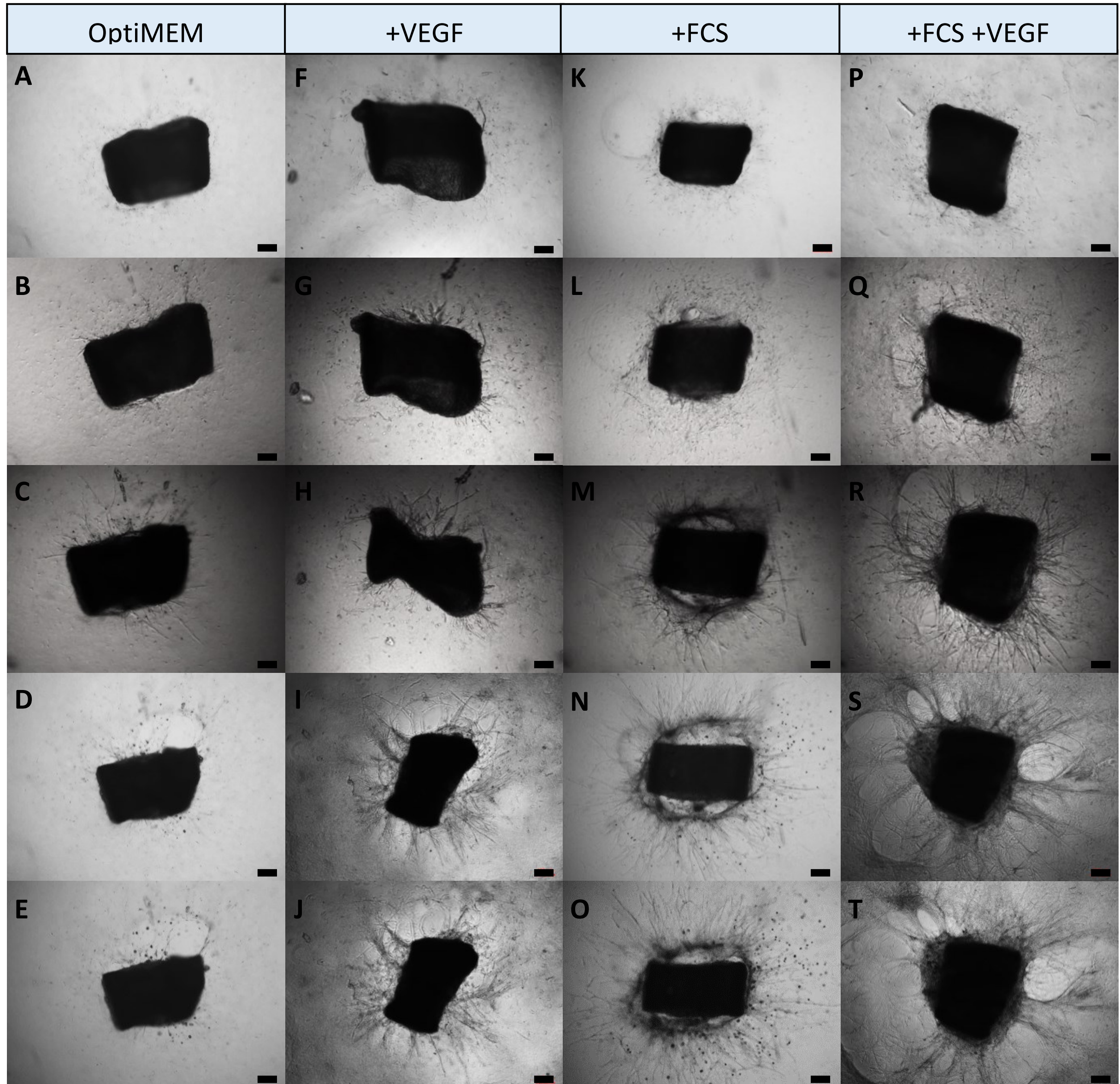

Figure 3.3.7: Angiogenesis in the aortic ring assay. Brightfield images of aortic rings from C57Bl/6 mice embedded in collagen and treated with **A-E)** unsupplemented OptiMEM medium, **F-J)** medium + 5ng/ml FCS, **K-O)** medium + 2% FCS and **P-T)** medium + 5ng/ml VEGF + 2% FCS. Images shown are at Day 3, Day 5, Day 7, Day 10 and Day 12, by row chronologically from the top to bottom. Scale bars = 200µm, representative images from n=6.

Cell and vessel outgrowth at Day 3, Day 5 and Day 7 were quantified for different media by counting under brightfield light microscopy (Figure 3.3.8). Cell outgrowths were determined as cells visible under the microscope which were evidently originating from, and still in contact with, the aortic tissue. Addition of FCS to media resulted in an increase (~75%) in the number of cellular outgrowths at Day 3 in the +FCS- and the +VEGF +FCS-treated tissue, in comparison to OptiMEM alone ($p<0.001$) (Figure 3.3.8 A). There was no significant difference at Day 3 between any of the other treatments. Culturing in +VEGF +FCS media resulted in the greatest number of vessel growths by Day 5 and all media had a significantly greater number of vessels by Day 7 in comparison to OptiMEM (+FCS & +VEGF +FCS: $p<0.05$, +VEGF & <3 FCS >3 VEGF: $P<0.001$). The maximum vessel length (used as a measure of the maximum vascularised distance) at Day 7 was also increased in all media in comparison to OptiMEM (+FCS: $p<0.05$, +VEGF & +VEGF +FCS: $P<0.001$) (Figure 3.3.8 C).

In +VEGF and <3 FCS >3 VEGF treatments vessel number continued to increase from Day 5 to Day 7, whereas in all other treatments, vessel number reduced from Day 5 to Day 7 (Figure 3.3.8 B). Vessel elongation appeared to have a biphasic growth pattern, with rapid increase in vessel length from Day 0-3 and Day 5-7, but a slow increase from Day3-5 (Figure 3.3.8 C).

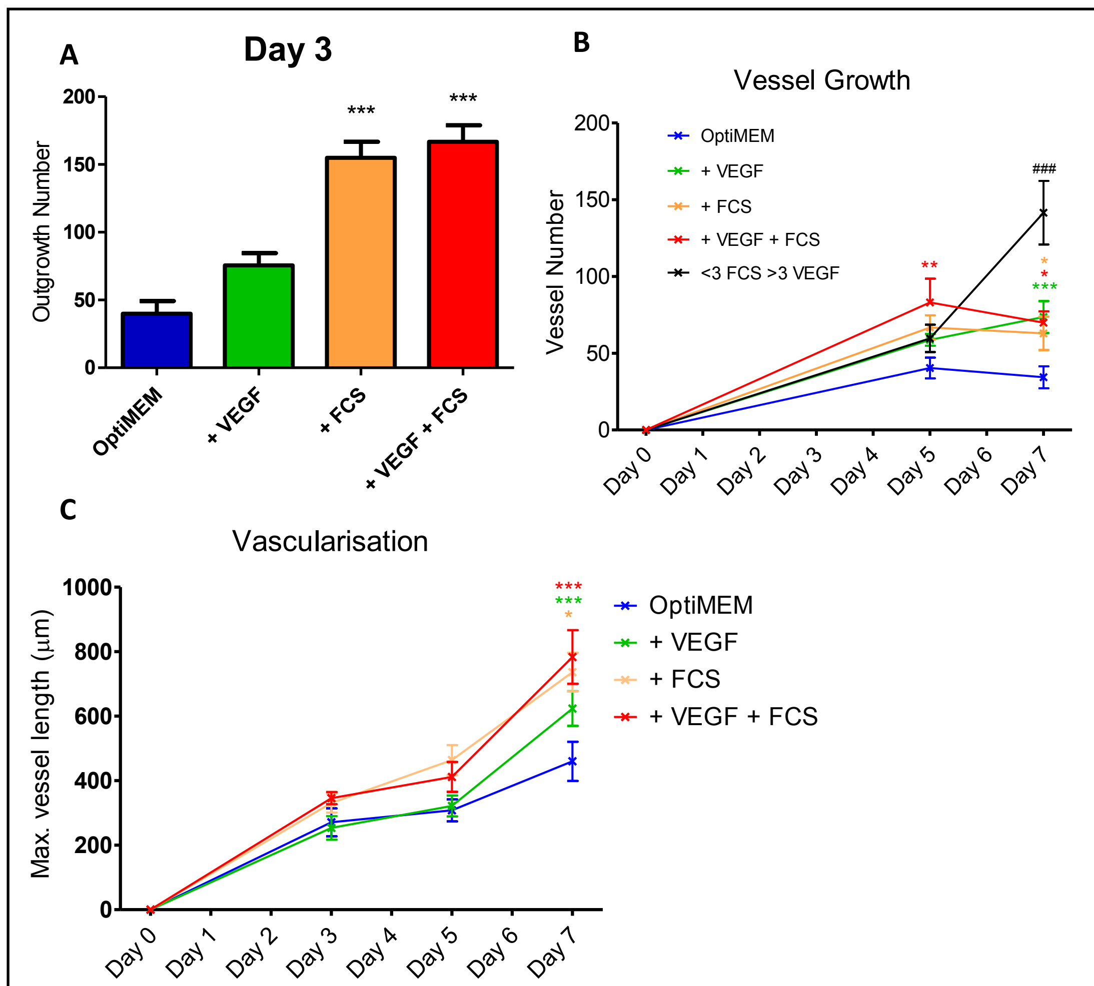

Figure 3.3.8: The effect of medium supplementation on tube-like structure formation in the aortic ring assay. Quantification of vessel outgrowth in the aortic ring assay, with different medium supplementation, assessed by brightfield microscopy. **A)** Number of vessel outgrowths/ ring at Day 3. Columns represent mean + s.e.mean. **B)** Number of vessel outgrowths/ ring at Day 5 & 7. **C)** Maximum vessel length/ ring at Day 3, 5 & 7. Symbols represent mean ± s.e.mean; I data from C57Bl/6 mice (n=5 for all graphs). Abbreviations: OptiMEM = unsupplemented OptiMEM medium; +VEGF = medium + 5ng/ml FCS; +FCS = medium + 2% FCS; +VEGF +FCS = medium + 5ng/ml VEGF + 2% FCS; <3 FCS >3 VEGF = medium + 2% FCS up to Day 3, then medium + 5ng/ml VEGF*p<0.05, **p<0.01, ***p<0.001, all vs OptiMEM & ###p<0.001 vs all other groups. 2-way ANOVA with Bonferonni post-hoc tests.

3.3.4.2 **Formation of Complex Multicellular Angiogenic Outgrowths**

Rings of aortic tissue from C57Bl/6 mice were embedded in culture for up to 7 days with OptiMEM medium + 5ng/ml VEGF. Spontaneous angiogenic vessel outgrowth occurred over this time period, with immunostaining revealing two components to these vessels; $CD31^+$/Isolectin-$B4^+$ multicellular endothelial tubes, with a supporting αSMA^+ perivascular component (Figure 3.3.9). These vessels formed branching structures radiating out from the aortic ring (Figure 3.3.9 A), with αSMA^+ cells forming a network of connections between proximate vessels Figure 3.3.9 B).

While some endothelial cells in these vessels also express αSMA in the cytoplasm, the strong cytoskeletal and cell membrane αSMA staining evident in perivascular cells is not evident (Figure 3.3.10) From this respect, two different cell populations comprising vessels can be classified for each staining. With regard to CD31; $CD31^+$/αSMA^+ cells and $CD31^-$/αSMA^+, but not $CD31^+$/αSMA^- cells. With regard to Isolectin-β4; Isolectin-$\beta 4^+$/αSMA^+ cells and Isolectin-$\beta 4^-$/αSMA^+, but not Isolectin-$\beta 4^+$/αSMA^- cells. Isolectin-β4 or αSMA in vessels. CD31 and Isolectin-β4 staining distribution appear to have a similar pattern.

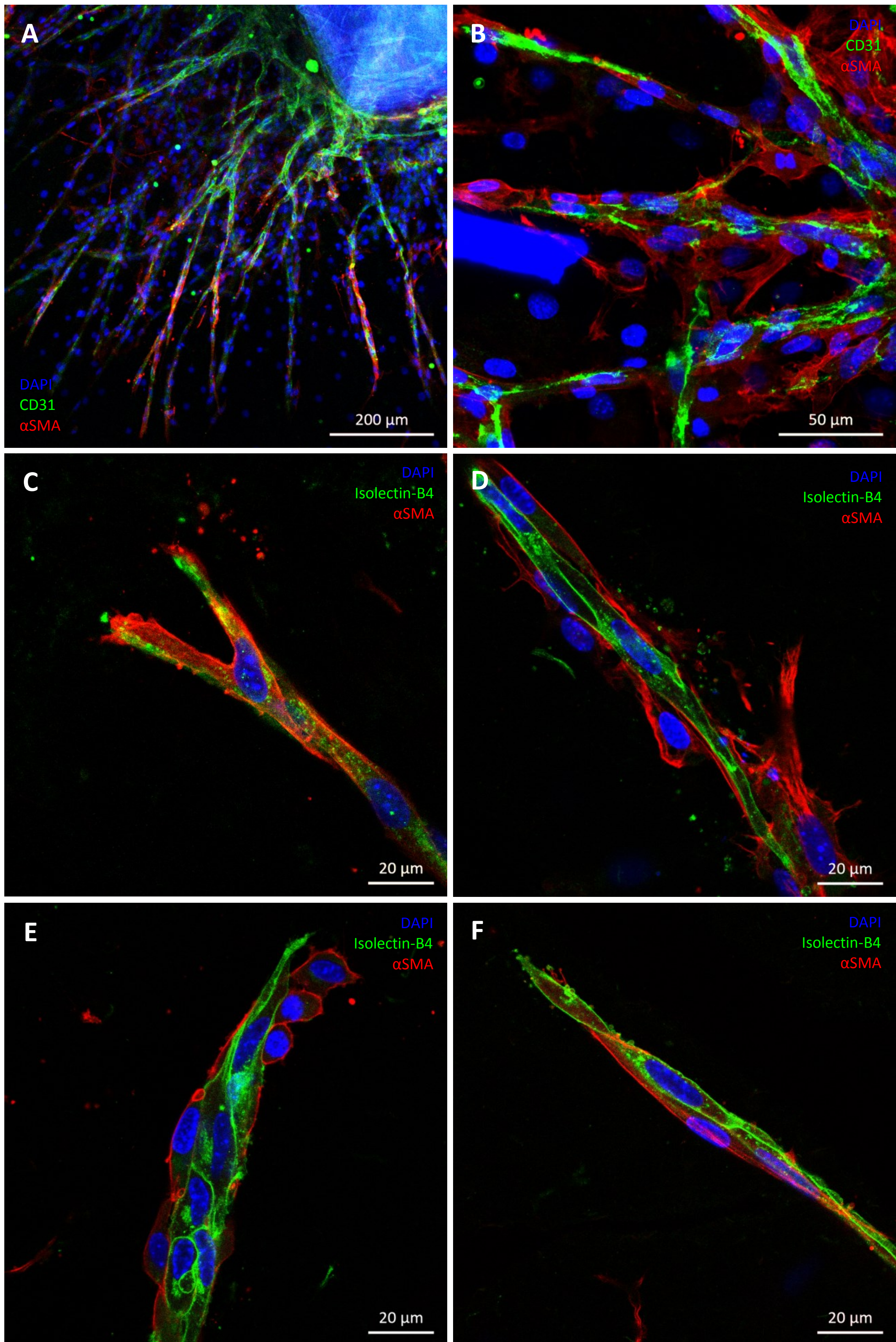

Figure 3.3.9: Immunofluorescent staining of angiogenic outgrowths in culture. Immunostaining for endothelial and perivascular markers revealed an endothelial CD31$^+$ or Isolectin-B4$^+$ tube with supporting αSMA$^+$ perivascular cells in vessels from

the aortic ring assay. Vessels were cultured with 5ng/ml VEGF. **A, B, C)** CD31/αSMA/DAPI, n=6. **D, E, F)** Isolectin-B4/αSMA/DAPI, representative images from n=9.

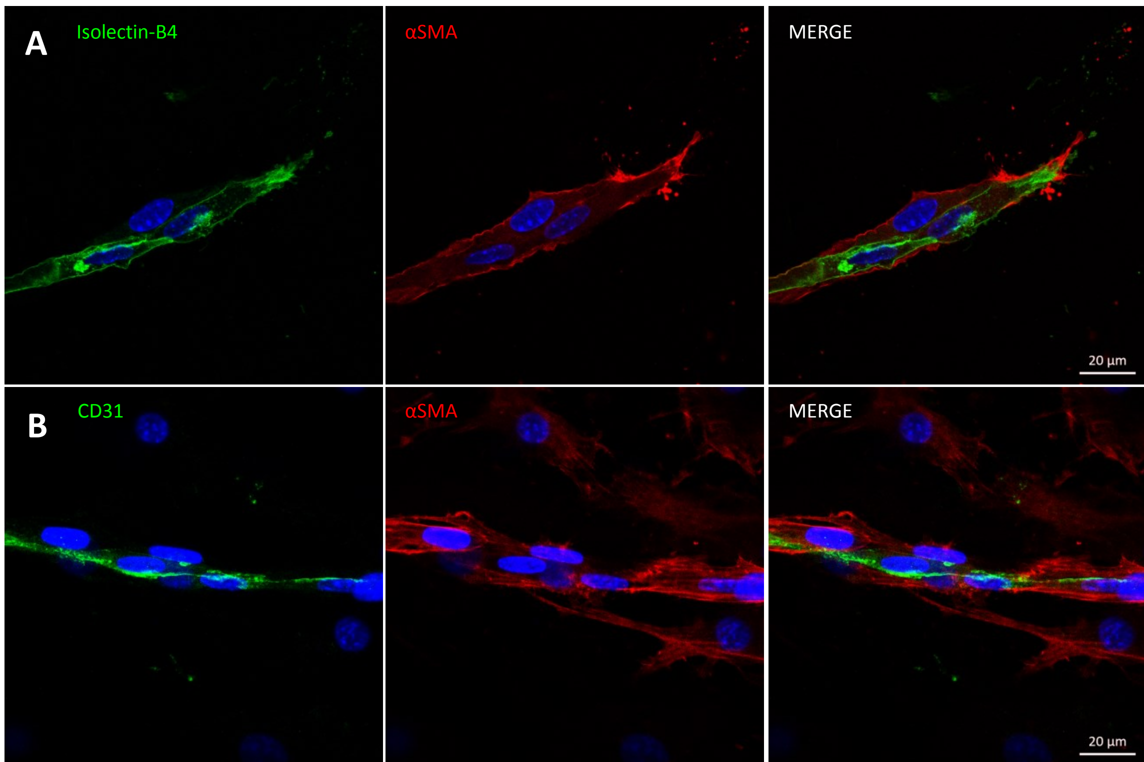

Figure 3.3.10: Endothelial cells express αSMA during *ex vivo* angiogenesis. Co-immunostaining of αSMA with **A)** Isolectin-B4 and **B)** CD31 shows endothelial cells have limited cytoplasmic αSMA expression during *ex vivo* angiogenesis, though seemingly perivascular cells do not express endothelial markers. n=6-9. Vessels were cultured with 5ng/ml VEGF.

3.3.4.3 WT1 is Expressed in Some Angiogenic Vessels

The aortic ring model has been demonstrated to be an effective model of angiogenesis, with multi-cellular angiogenic vessel formation occurring over a relatively short period of time. In order to visualise WT1 in this process, ex *vivo* aortic rings were cultured from WT1-GFP reporter mice, with GFP knock-in under the same promoter as WT1, such that GFP is synthesised upon WT1 expression ($WT1^{+/GFP}$). In preliminary experiments (n=1) nuclear GFP was ubiquitously present in Isolectin-B4^{+} and αSMA^{+} cells of certain vessel outgrowths at Day 7 (Figure 3.3.11 A) and entirely absent in others at the same time-point (Figure 3.3.11 B).

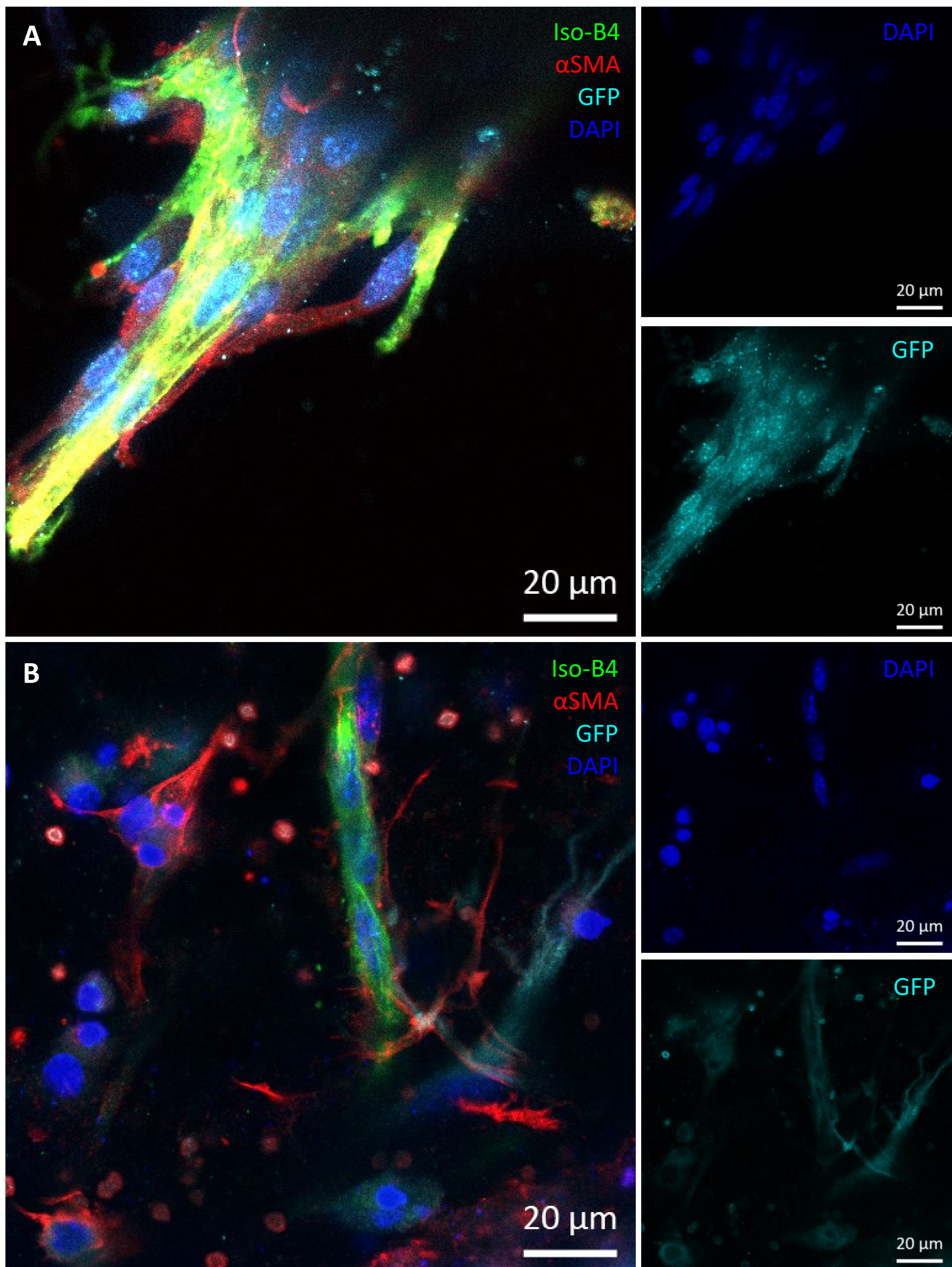

Figure 3.3.11: GFP expression is ubiquitously present in some angiogenic vessels and ubiquitously absent from others. Immunostaining against GFP in the WT1-GFP reporter mouse revealed nuclear GFP expression co-localising with DAPI in endothelial and perivascular cells of *ex vivo* angiogenic vessels **(A)**, though expression was entirely absent in most vessels **(B)**. n=1. Vessels were cultured with 5ng/ml VEGF.

3.3.4.4 **VE-Wt1 KO Transiently Inhibits Angiogenesis**

Given the early vascular expression of WT1 in angiogenic vessels, the effect of WT1 KO in endothelial cells was investigated to determine whether WT1 has a functional role in angiogenesis. Quantification of the aortic ring assay in VE-Wt1 KO mice and relevant controls revealed that KO of WT1 in endothelial cells reduced the number of vessel outgrowths present at Day 5, though there was no difference between groups by Day 7 (Figure 3.3.12 A). The lack of difference at Day 7 is attributable to a concurrent increase in vessel number in the VE-Wt1 KO group from Day 5-7 and a reduction in vessel number in the control groups over the same time period. However, when cultured in VEGF (Figure 3.3.12) or FCS for 3 days, then VEGF (Figure 3.3.12), VE-Wt1 KO had no effect on vessel number.

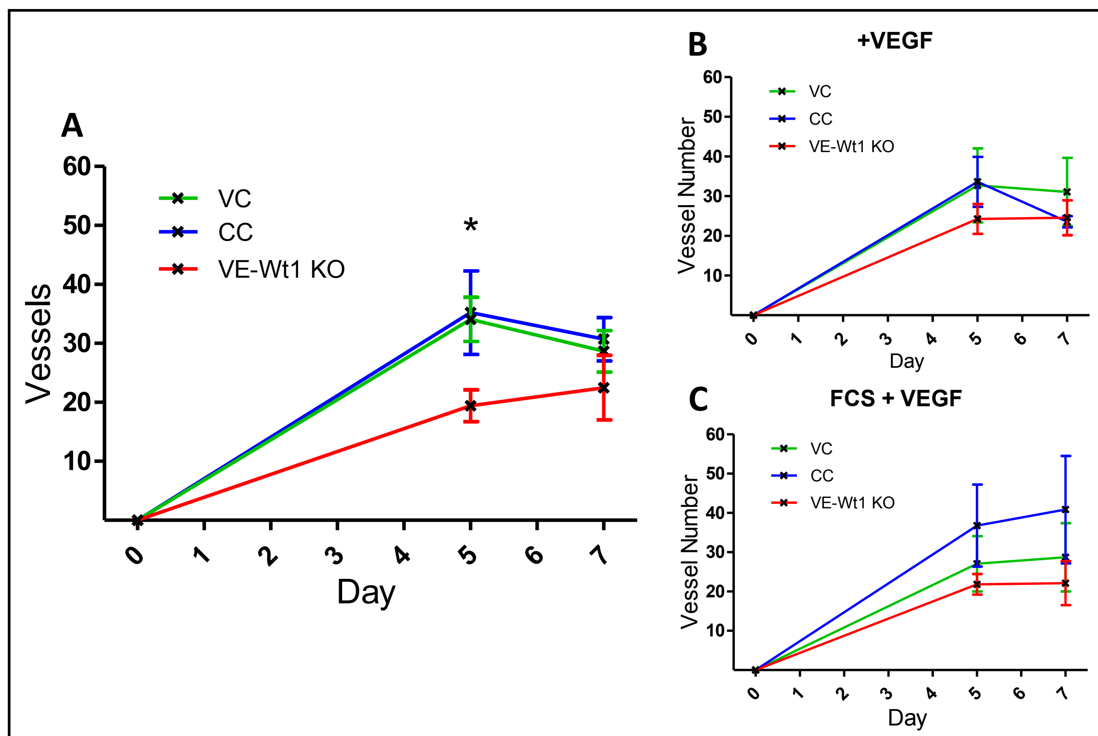

Figure 3.3.12 VE-Wt1 KO transiently impairs angiogenesis in the aortic ring assay, though VEGF counteracts this effect. Vessel counts at Day 5 and Day 7 in **A)** Un-supplemented medium, **B)** medium + 5ng/ml VEGF and **C)** medium + 2% FCS <Day 3, +5ng/ml VEGF <Day 3. Symbols repent mean ± s.e.mean; *p<0.05, VE-Wt1 KO v CC & VC, 1-way ANOVA with Bonferroni post-hoc tests. n=5-8. VC = Vehicle control, CC = *Cre* control, VE-Wt1 KO = vascular endothelial *Wt1* KO.

3.4 Discussion

The experiments in this chapter investigated the role of WT1 in vascular endothelial cells, specifically, in the context of vascular function, maintenance of vascular tone and angiogenesis. Use of the *ex vivo* aortic ring angiogenesis assay supported the hypothesis that WT1 is intrinsically involved in angiogenesis in endothelial cells; WT1 was expressed in vessel outgrowths in the aortic ring assay and selective deletion of WT1 from the endothelium (VE-Wt1 KO) transiently impaired angiogenesis. Alongside these findings, a number of intriguing and novel insights were obtained. VE-Wt1 KO increased vasoconstriction in response to NA and 5HT and while VE-Wt1 KO did not have an evident basal phenotype, it did induce testicular hypertrophy, through an unknown mechanism, with concurrent enlargement of the gonad.

3.4.1 ***Ex Vivo*** Angiogenesis

The *ex vivo* aortic ring assay is an elegant model for characterising sprouting angiogenesis, as by necessity, all new angiogenic outgrowths in culture must have originated from the vascular aortic tissue and the resident cells. Characterisation of vessel-like outgrowths in this model show that a number of typical processes associated with sprouting angiogenesis occur; a number of vascular outgrowths are formed and become elongated and branched, with pruning of vessels as angiogenesis progresses. This pruning was concurrent with rapid elongation of the remaining outgrowths.

Immunofluorescent staining of aortic ring outgrowths has been carried out previously, demonstrating two cellular components to vessel-like angiogenic outgrowths; a central endothelial structure, with supporting mural αSMA^{+} cells (Zhu and Nicosia, 2002; Zhu *et al.*, 2003; Baker *et al.*, 2012). In addition to this prior work, the experiments herein confirm the presence of these two cell populations and provide a more detailed picture of the morphology of angiogenic outgrowths. The conventional view is that endothelial cells drive angiogenesis, with pericytes and other mural cells subsequently recruited to endothelium-derived angiogenic outgrowths to provide structural support (reviewed by Carmeliet & Jain 2011). IHC staining in the aortic ring assay

confirms this to some extent, showing endothelial cells, with the filipodia and branching structure characteristic of tip cells (Jakobsson *et al.*, 2010) guiding the endothelial tube-like structures. However, contrary to this accepted wisdom, αSMA^{+} pericyte-like cells are present right up to the tip of these endothelial structures, wrapping around them, encapsulating them and often guiding expansion of angiogenic outgrowths alongside endothelial cells.

One possible function these pericyte-like cells could be facilitating is proteolysis of the extracellular matrix, which both endothelial tip cells and pericytes engage in (Karagiannis and Popel, 2006; Yana *et al.*, 2007; Augustin *et al.*, 2009; Stratman *et al.*, 2009). Given the dense collagen extracellular matrix in this assay, it is possible that pericytes are required alongside tip cells to create guidance tunnels through the matrix and this morphology is, therefore, unique to this assay. However, given the complex cross-signalling which occurs between endothelial cells and pericytes in angiogenesis (Augustin *et al.*, 2009; Gaengel *et al.*, 2009), it seems logical that pericytes would be involved throughout the process and facilitate endothelial tip cell function. It would be interesting to know whether these pericyte-like cells identified at the tip of the endothelial outgrowths have a unique function and expression profile in comparison to those further away from the tip; analogous to the unique role of endothelial tip cells are unique in their function. The histological techniques optimised in this chapter alongside FACs would be useful for investigating this relationship in the future.

Another interesting observation in the aortic ring assay is that the endothelial cells comprising the angiogenic vessel-like outgrowths express the endothelial markers CD31 and Isolectin-B4, but also express the mesenchymal marker αSMA (which is more commonly associated with mural cells and not expressed in quiescent vascular endothelial cells). Endothelial tip cells are known to have a number of properties more characteristic of mesenchymal cell types, including their high migratory capacity and proteolytic function (Jakobsson *et al.*, 2010). EMT involves transition of cells into a mesenchymal type, dissociation from other associated cells and increased migration (Kalluri and Weinberg, 2009); however, many cells are also capable of partial EMT (Revenu and Gilmour, 2009). There is preliminary evidence for EndoMT in

angiogenesis, with a number of regulators of EMT (including Snail, Slug and TGF-β; all activated by or transcriptionally activating MMP-2 and MMP-9) also apparently regulating angiogenesis (Yu and Stamenkovic, 2000; Ma *et al.*, 2007; Welch-Reardon *et al.*, 2014). However, as endothelial cells maintain cell-cell contact as outgrowths form, it has been hypothesised that a partial EndoMT occurs (Welch-Reardon, Wu and Hughes, 2015).

3.4.1 Endothelial WT1 KO Impairs *Ex Vivo* Angiogenesis

WT1 has been shown to be expressed in angiogenic vasculature in development (Duim *et al.*, 2016), cancer (Dohi *et al.*, 2010; Katuri *et al.*, 2014; Wagner *et al.*, 2014) and regenerative (Duim *et al.*, 2015) contexts. Use of the WT1-GFP reporter mouse in the aortic ring assay showed that WT1 was discretely expressed in endothelial cells and αSMA$^+$ cells of angiogenic vessel outgrowths. This confirms preliminary observation of WT1 in aortic ring outgrowths (McGregor *et al.*). Interestingly, WT1 expression was widespread in some outgrowths but completely absent from others, perhaps implying a different origin or function of these WT1$^+$ outgrowths in comparison to WT1$^-$ outgrowths. It is also possible WT1$^+$ outgrowths are at a different stage of the angiogenic process to those that are WT1$^-$ and that, therefore, this reflects temporal WT1 expression. In fact, this was seen in the adult myocardium post-MI in which WT1 was only expressed acutely after injury (Duim *et al.*, 2015). The WT1 expression in αSMA$^+$ cells is also a point of interest, as previous studies on WT1 in angiogenesis have focused on endothelial WT1 expression. These results show that if WT1 is important an important factor in angiogenesis, it may not be solely endothelial WT1 regulation which is responsible.

Unfortunately, due to the limited availability of WT1-GFP mice and the fragility of the newly formed angiogenic outgrowths during staining, images were only obtained from one mouse, limiting interpretation of these results. It would be interesting to reproduce these experiments and stain aortic ring outgrowths at different time points following the initiation of angiogenesis to assess how WT1 expression changes over time. Furthermore, lineage tracing experiments for WT1 in this model would be fascinating to determine whether WT1$^+$ vessels

have the same origins as WT1$^-$ vessels and whether WT1$^+$ endothelial cells are capable of transitioning into mesenchymal cells (and vice-versa); an element which is yet to be investigated in the aortic ring assay.

Knockdown of WT1 in the vascular endothelium (VE-WT1 KO) (confirmed to reduce endothelial WT1 expression by ~4 fold in the uterus) led to an impairment of *ex vivo* angiogenesis in the aortic ring after 5 days of culture in collagen. This demonstrates that WT1 expressed in the endothelium does indeed have a role in the angiogenic process. However, angiogenesis was not completely impaired and by day 7 vessel outgrowth number had recovered to normal levels in the VE-WT1 KO. This is interesting as outgrowths from control aortic rings exhibited pruning from day 5, whereas those from VE-WT1 KO did not; suggesting the dynamics of the angiogenic response are delayed or altered when Wt1 expression is reduced in endothelial cells. In line with the acute expression of WT1 in the endothelium post-MI (Duim *et al.*, 2015), impairment of *ex vivo* angiogenesis in the VE-WT1 KO implies that endothelial WT1 is playing an intrinsic, though non-essential, role in initiation and/or early stages of angiogenesis.

WT1 regulates Snail and Slug (Martínez-Estrada *et al.*, 2010; Takeichi *et al.*, 2013), two of the factors implicated in EndMT in angiogenesis (Yu and Stamenkovic, 2000; Ma *et al.*, 2007; Welch-Reardon *et al.*, 2014). If the hypothesis that partial EndoMT is essential to angiogenesis (Welch-Reardon, Wu and Hughes, 2015) proves to be true, it is possible this could explain the delayed angiogenesis observed in aortic rings from the VE-Wt1 KO. In this case, delayed or reduced EndoMT could hamper degradation of the basement membrane and ECM and thus, impede endothelial cell progression. It is also worth noting that αSMA$^+$ cells in this model expressed WT1 and that this expression may be sufficient to facilitate normal endothelial function through release of WT1-regulated factors from αSMA$^+$ cells into endothelial cells. Alternatively and perhaps, controversially, αSMA$^+$ cells may become endothelial cells through mesenchymal-to-endothelial transition. Additionally, the VE-Wt1 KO did not induce complete deletion of WT1 from endothelial cells and this could also explain the remaining capacity of endothelial cells to undergo angiogenesis.

It is worth noting that addition of VEGF to the assay was sufficient to overcome the suppression of angiogenesis induced by VE-WT1 KO in aortic rings. WT1 is known to regulate VEGF (Graham *et al.*, 2006; Amin *et al.*, 2011; McCarty, Awad and Loeb, 2011; Iranparast *et al.*, 2014) and the recovery of function on addition of VEGF suggests the impairment of angiogenesis seen in the VE-Wt1 KO is, at least in part, as a result of disruption to VEGF signalling. However, two alternative hypotheses are also possible; firstly, that addition of VEGF is sufficient to provide optimal angiogenic conditions and drive angiogenesis, regardless of WT1 deletion and secondly, that VEGF is activating a number of downstream targets of WT1, independently of the WT1 protein. In order to clarify the mechanism involved, the levels of VEGF protein and mRNA could be determined in VE-Wt1 KO aortic ring assays and an endothelium-selective, inducible VEGF or VEGFR KO could be used in this aortic ring model to determine whether this deletion produces a similar effect to the VE-Wt1 KO. As with WT1 KO mice, VEGF KO mice die mid-gestation (Kreidberg *et al.*, 1993; Tammela *et al.*, 2005). Given that both VEGF and WT1 are major transcriptional regulators activated by HIF-1, it would seem somewhat redundant if they were playing the same role in angiogenesis (Wagner *et al.*, 2003; Tammela *et al.*, 2005).

WT1 has been previously investigated in the aortic ring assay, using a *Tie2CreER^{T2} Wt1* KO (Wagner *et al.*, 2014). In agreement with the results described in this chapter using the VE-Wt1 KO mouse (*Wt1* KO under the control of a *VE-CadherinCreER^{T2}*), the *Tie2* WT1 KO also impaired *ex vivo* angiogenesis, though the inhibition seen with the *Tie2 Wt1* KO was more dramatic, with an almost complete inhibition of outgrowth formation (Wagner *et al.*, 2014). Alongside endothelial cells, *Tie2* is expressed in cells of a haematopoietic lineage including macrophages, B- and T-lymphocytes, natural killer cells and dendritic cells (Constien *et al.*, 2001; Kisanuki *et al.*, 2001). The greater inhibition of angiogenesis in this less selective *Wt1* KO suggests that WT1 in one or all of these other cell types is important for regulation of angiogenesis. To clarify this role, the aortic ring assay could be carried out with a ubiquitous inducible *Wt1* KO to see if the result is the same, as well as inducible *Wt1* KOs in macrophages and pericytes. All of this would

help determine the specific cell types in which WT1 expression influences angiogenesis.

The aortic ring assay has considerable unexplored potential for investigating the processes involved in sprouting angiogenesis and the role WT1 plays in these. From a pure angiogenesis line of investigation, as well as in relation to WT1, it would be interesting to stain for other cell types in this model to determine how different cellular components arising from the aorta are involved in angiogenesis and which of these are expressing WT1. Cell types that could be interesting, due to their known involvement in angiogenesis and their documented WT1 expression are monocyte-derived macrophages and dendritic cells (L W Ellisen *et al.*, 2001; Fainaru *et al.*, 2007; Jaipersad *et al.*, 2014; Li *et al.*, 2016), fibroblasts (Braitsch *et al.*, 2013; Ubil *et al.*, 2014), MSCs (Chau *et al.*, 2011; Gong *et al.*, 2017), HSCs (L W Ellisen *et al.*, 2001; Butler *et al.*, 2010) and APCs (Chau *et al.*, 2014; Cheng *et al.*, 2017). EPCs have not been shown to have WT1 expression, although, as with many WT1-expressing cells they undergo a TGF-β mediated EMT (Díez *et al.*, 2010). Regardless, investigating EPCs in the aortic ring assay could add further weight to the hypothesis of a resident vascular EPC niche involved in angiogenesis (Zammaretti and Zisch, 2005).

Finally, my group has optimised a protocol allowing RNA extraction and qPCR to be carried out in the aortic ring assay (Morgan *et al.* unpublished data). In order to provide greater insight into the mechanism underlying the function of WT1 in angiogenesis, qPCR should be carried out in the aortic ring assay with VE-Wt1 KO and ubiquitous *Wt1* KO.

3.4.2 Vasoconstriction is Altered by Endothelial WT1 KO

Myography was carried out to determine whether endothelial function was impaired by VE-Wt1 KO. WT1 is not known to be expressed in healthy adult vasculature (Chau *et al.*, 2011), so it was not envisioned that WT1 deletion would affect vascular function in non-pathological circumstances. Consistent with this hypothesis, the contractile response of aortae to potassium, was not affected by VE-Wt1 KO, suggesting calcium-mediated contraction of VSMCs.

However, surprisingly, aortae from VE-Wt1 KO mice exhibited an increased contractile response to two vasoconstrictors, 5HT (serotonin) and NA (noradrenaline). This is the first time WT1 has been shown to have any influence on vasoconstriction. This could prove to be a novel and exciting area of investigation. At this point, it is worth noting that the effect of VE-Wt1 KO was restricted to vasoconstriction, with no effect on vasodilation in response to the two drugs assessed (ACh and SNP). There are a number of factors to consider in relation to these novel findings.

Serotonin binds to 5HT receptors in the endothelium and VSMCs, with counterbalancing effects. In the endothelium, binding of serotonin to $5HT_{1B}$, $5HT_{1D}$ and $5HT_{2B}$ receptors induces vasodilation, which is in part NO-dependent, while binding of serotonin to 5HT receptors in VSMCs induces vasoconstriction (Nagatomo *et al.*, 2004; Watts and Davis, 2011). Noradrenaline binds to the α- and β-adrenergic receptors in endothelial cells and VSMCs, to induce vasoconstriction, though it also has the potential to induce EDRF-dependent endothelial relaxation (Chotani *et al.*, 2000; Tanoue *et al.*, 2002; Dessy *et al.*, 2004). The simplest explanation for the increased contractile response seen in VE-Wt1 KO aortae to these drugs is that VE-Wt1 KO has affected the number of 5HT and adrenergic receptors in the vasculature and that WT1 normally plays a role in modulating these receptors. As the maximum contractile response to 5HT is increased in denuded VE-Wt1 KO aortae, this suggests changes in receptors both in the endothelium and VSMCs. If WT1 is expressed at low levels in some endothelial cells and therefore, VE-Wt1 KO affected serotonin and noradrenaline signalling post-induction of KO, it is possible these receptor changes could occur as a compensatory response to physiological changes induced by VE-Wt1 KO. Transcription factors, such as WT1, are capable of producing surprisingly rapid transcriptional changes in response to stimuli (Kahlert *et al.*, 2000) and so, alternatively, as WT1 is not thought to be ordinarily expressed in the endothelium the differences seen could reflect a rapid transcriptional response by WT1 in the endothelium. A final, explanation is that VE-Wt1 KO could produce systemic effects that alter NA and 5-HT-mediated signalling;

concordantly, the levels of circulating NA and 5-HT could be measured in these mice.

A role of WT1 in vasoconstriction would be very relevant to cardiovascular disease in which hypertension increases risk of myocardial infarction, stroke and heart failure. In Denys-Drash syndrome and some other glomerulopathies resulting from *Wt1* mutations hypertension is evident (Little *et al.*, 1993; Wagner *et al.*, 2008; Lipska *et al.*, 2014). The increased vasoconstriction in VE-Wt1 KO observed here raises the possibility that WT1 could be important in regulating vascular tone and that WT1 mutations are causative of hypertension. This is further supported by previous observations that WT1 regulates NO signalling (Johannesen *et al.*, 2003; Mazzei *et al.*, 2010). However, selective endothelial KO of WT1 does not inhibit endothelium-dependent (ACh-mediated) relaxation, which suggests that impaired angiogenesis in this model is not due to a generalised impairment in the endothelial cell NO pathway. In order to further investigate the possible role of Wt1 in vascular tone, the blood pressure of VE-Wt1 KO mice should be assessed *in vivo* to determine if they are hypertensive and are therefore, predisposed to cardiovascular disease.

Furthermore, regulation of vascular tone is connected with angiogenesis and some factors can influence both processes (Coletta *et al.*, 2012). In fact, serotonin and noradrenaline signalling are intertwined; in sympathetic nerves, activation of the $5HT_{1D}$ receptor (also present in endothelial cells) inhibited release of noradrenaline (Molderings *et al.*, 1990). Excitingly, given the focus of this thesis on angiogenesis, serotonin and noradrenaline signalling influence angiogenesis. Serotonin has been shown to be pro-angiogenic, inducing endothelial cell proliferation and migration and *in vitro* 2D-tube formation and also promoting angiogenesis *in vivo* by reducing Thrombospondin-1 levels (Qin *et al.*, 2013). In a colon cancer model, serotonin deficiency affected MMP-12 release by macrophages and hence, reduced angiogenesis and tumour growth (Nocito *et al.*, 2008). Moreover, in a murine ischaemic limb model, stimulation of endothelial $5HT_{1B}$ by inhibiting VSMC $5HT_{2A}$, activated the eNOS/Akt pathway and increased *in vivo* angiogenesis (Iwabayashi *et al.*, 2012). Similarly, the β_3-adrenergic receptor

was shown to influence retinal endothelial cell proliferation and migration with potential involvement of MMP-2, MMP-9, PI3K and MEK (Steinle *et al.*, 2003). Furthermore, *in vivo* noradrenaline upregulated VEGF, MMP-2 and MMP-9 (Yang *et al.*, 2006). This all raises the exciting possibility that WT1 may be controlling vascular tone and angiogenesis simultaneously through regulation of noradrenaline and serotonin signalling pathways, though the mechanism is, as yet, unclear.

3.4.3 Endothelial WT1 has a Role in the Adult Testis and Male Gonad

Histological assessment of a number of organs was carried out in the VE-Wt1 KO mouse and found to be grossly normal. As a result, it was initially assumed that the VE-Wt1 KO mouse had no phenotype, which was to be expected, given the assumed absence of endothelial WT1 expression in the adult (except in vessels of the uterine wall and mammary glands). However, following induction of VE-Wt1 KO, it was observed that the male gonad became visibly enlarged. This was shown to occur despite a reduction in testis weight and with no change in the weight of the epididymal fat or seminal vesicles. Immunofluorescent staining in the testis revealed a large number of endothelial $WT1^+$ vessels in the testis of adult CC mice; this staining appeared to be reduced in VE-Wt1 KO mice, consistent with the reduction in WT1 expression in the vessels of the uterus in this line. This is the first time a role for endothelial WT1 in the testis has been shown has been shown and it should henceforth be assumed that WT1 is normally expressed in the vascular endothelium of the adult testis.

In relation to WT1 in the testis, endothelial WT1 expression should be assessed in completely wildtype mice, however, to confirm this is not specific to these mice and in mice of different ages to confirm this is not specific to our examined time point or the age of these mice. While it is possible that WT1 expression in the testis is induced by the activation of *VE-Cadherin Cre* in these mice, the fact that the gonadal enlargement was seen in VE-Wt1 KO

and not CC means it is not likely this is just a *Cre* effect. As the testis continues to grow in mice throughout early adulthood (Sharpe *et al.*, 2003) it is not clear from our results whether the reduced testis size is a result of impaired testicular growth, testicular atrophy, or a combination of the two. Induction of VE-Wt1 KO in older mice should be carried out and the change in testis weight assessed over time post-induction of VE-Wt1 KO to determine if they reduce in size or have impaired growth. How VE-Wt1 KO is specifically affecting testicular morphology could be assessed by histology to determine if there is any structural alterations in Leydig or Sertoli cells.

The visible enlargement of the gonad cannot be attributed to increased size of the testis, epididymal fat or seminiferous vesicles, as these were ruled out. Therefore, two possible hypotheses for the enlarged gonad are that it is occurring as a result of inflammation, or of fluid accumulation. It appears unlikely to be due to inflammation, as this would likely result in the components of the gonad becoming enlarged and inflamed themselves. When WT1 was ubiquitously deleted in adult mice, it rapidly resulted in generalised oedema and atrophy of the spleen and pancreas (Chau *et al.*, 2011). It would appear this is occurring locally to the testis in the VE-Wt1 KO mouse. The endothelial WT1 observed in testicular vessels of CC mice, make it reasonable to assume this is attributable to a reduction in endothelial WT1 expression in the testicular vasculature. This may result in poorer perfusion of the testis and increased permeability of the vasculature, in turn leading to fluid leaking into the surrounding tissue and localised oedema. WT1 regulates VEGF in the endothelium (Wagner *et al.*, 2003) and VEGF has an essential role in regulation of vascular permeability (Nagy, Dvorak and Dvorak, 2007). The reduction in testis size is in line with the reduction in tumour size observed following *Tie2 Cre Wt1* KO, attributable to impaired tumour angiogenesis (Wagner *et al.*, 2014). If a similar mechanism is occurring in the testis, the reduced size observed may be as a result of impaired angiogenesis limiting normal testis grow in early adulthood.

3.4.4 **Conclusions**

The experiments contained in this chapter provided greater insight into the role of WT1 in the vascular endothelium *ex* vivo. This supported the previously accepted paradigm and our hypothesis that WT1 is intrinsically involved in angiogenesis in endothelial cells. However, it is also clear that WT1 is also expressed in other vascular cells in angiogenesis and that endothelial WT1 is not indispensable to the progression of angiogenesis. There were also a number of novel and exciting findings in relation to the vascular function of endothelial WT1 outside of the context of angiogenesis; namely that endothelial WT1 influences vasoconstriction and potentially vascular permeability alongside angiogenesis in the adult testis. This raises the possibility that WT1 is a broad regulator of endothelial and vascular function.

CHAPTER 4:

The Role of WT1 in *In Vivo* Angiogenesis

4.1 Introduction

Cardiovascular disease (CVD) is still the leading cause of death worldwide; in 2013 17.3 million people died of CVD, equivalent to 31.5% of all deaths (Naghavi *et al.*, 2015). Ominously, unlike the majority of other preventable diseases, deaths from CVD have risen over the last 20 years or so, as the global population moves towards a more sedentary, lifestyle and increasingly unhealthy diet (Naghavi *et al.*, 2015). In Europe, the picture is somewhat brighter, with deaths from CVD steadily dropping (Townsend *et al.*, 2016) and in 2012, for the first time, cancer overtook CVD as the leading cause of death for men in the UK (Bhatnagar *et al.*, 2015). First amongst these CVDs for fatality was coronary heart disease, which can result in heart attack (myocardial infarction), with cerebrovascular stroke the second most common cause of death (Bhatnagar *et al.*, 2015). In the case of critical limb ischaemia, while there is no appropriate treatment for some patients, it rarely results in death. It is worth considering that even sub-lethal cardiovascular events, such as often occur with critical limb ischaemia, can have debilitating long term impact on patient health and welfare. Given the persistent global burden of CVD, further incremental advances in our treatment of CVD will be necessary if we are to continue to reduce its impact. The work in this chapter addresses the role of WT1 *in vivo* in relation to CVD, particularly regenerative, ischaemic angiogenesis and to the potential of WT1 as a therapeutic target in CVD.

In ischaemic cardiovascular diseases such as myocardial infarction, stroke and critical limb ischaemia /peripheral arterial disease, damage is induced in tissues by deprivation of oxygen as a result of disruption to normal blood flow. Most commonly, ischaemia is induced as a result of plaque build-up in the arterial wall, also known as atherosclerosis. In atherosclerosis, accumulation of lipids in the arterial wall forms an atherosclerotic plaque, which narrows the lumen of the artery and reduces downstream blood flow (reviewed by Lusis 2000). This can be sufficient to induce ischaemia in affected tissues; however, acute ischaemic attacks are induced when the atherosclerotic plaque becomes unstable and ruptures, accompanied by thrombosis, blocking proximal arteries and capillaries. Which vessels are affected determine which

tissues become ischaemic and, hence, the symptoms that develop; blockage of the coronary arteries results in coronary artery disease and myocardial infarction, blockage of the cerebral microvasculature induces stroke, and blockage of the peripheral limb microvasculature results in peripheral artery disease which can progress into critical limb ischaemia. Atherosclerosis is accompanied, and worsened, by endothelial cell dysfunction, wherein the normal anti-inflammatory and anti-thrombotic function of the vascular endothelial cells becomes impaired. In addition there is dysregulation of vascular tone and permeability (in part due to decreased NO), and increased expression of adhesion molecules by the endothelium (Sitia *et al.*, 2010).

While the symptoms atherosclerosis depend on the site of lesion formation, the underlying pathophysiology is broadly similar; ischaemic pathophysiology in myocardial infarction, stroke and critical limb ischaemia has been reviewed by Buja (2005), Durukan & Tatlisumak (2007), and Varu *et al.* (2010), respectively. Initial ischaemic damage occurs as a result of oxygen deprivation and, hence, energy deprivation as mitochondria are unable to generate sufficient ATP. This progresses particularly rapidly in tissues such as the brain and heart which have a high-energy demand. The damage in ischaemic cells, including endothelial cells and inflammatory cells (such as leukocytes), results in increased free radical formation and increased cytosolic Ca^{2+}. The endothelial cell dysfunction seen in an atherosclerotic milieu is also observed in ischaemic tissues, in which endothelial dysfunction also occurs (Viehman *et al.*, 1991; Sitia *et al.*, 2010). Ischaemia induces upregulation of a number of pro-inflammatory factors and cytokines, such as MCP-1, NF-kB, TNF-α, interleukins, and the complement pathway, which results in recruitment of inflammatory cells and induction of an inflammatory response (Frangogiannis, Smith and Entman, 2002). Neutrophils rapidly migrate to the affected tissue, with macrophages, mast cells and leukocytes then recruited, with seemingly both beneficial and detrimental effects (Frangogiannis, Smith and Entman, 2002).

Fibrosis also occurs in myocardial infarction and peripheral artery disease, driven by resident and recruited fibroblasts. While fibrosis can often be detrimental, in these diseases fibroblasts also have a regenerative and

reparatory role. Post-myocardial infarction, fibroblasts deposit extracellular matrix and collagen to maintain structural integrity in the infarct area as myocardial cells die-off (Shinde and Frangogiannis, 2014). They also interact with inflammatory cells by secreting pro-inflammatory cytokines and release pro-angiogenic factors (van Nieuwenhoven and Turner, 2013). Both the heart and skeletal muscle have resident progenitor populations capable of regenerating muscle, or epicardial cells and satellite cells, respectively. Interestingly, in skeletal muscle regeneration, fibroblasts and inflammatory cells regulate proliferation and differentiation of satellite cells into mature myocytes (Murphy *et al.*, 2011; Wang and Rudnicki, 2011). In the heart, cardiac fibroblasts are generated from the epicardium post-MI (Braitsch *et al.*, 2013; Ellison *et al.*, 2013) and appear to have the capacity to generate myocardial cells (Jayawardena *et al.*, 2012; Qian *et al.*, 2012).

If ischaemia is maintained, its deleterious impact can become irreversible with widespread cell apoptosis; however, activation of angiogenesis occurs early after onset of ischaemia in myocardial infarction, stroke and peripheral artery disease and has the potential to re-perfuse damaged tissues to induce repair and regeneration (Carmeliet and Jain, 2011). In all of these contexts, angiogenesis and arteriogenesis simultaneously occur to increase the extent of reperfusion. Interventions which increase *in vivo* angiogenesis have been shown to improve function post-ischaemia in animal models of stroke (Wang *et al.*, 2004; Fan *et al.*, 2010; Jin *et al.*, 2014), MI (Hao *et al.*, 2007; Garbern *et al.*, 2011; Meloni *et al.*, 2013) and critical limb ischaemia (Emanueli *et al.*, 2002; Iwase *et al.*, 2005).

In the context of CVD, WT1 has primarily been investigated *in vivo* in myocardial infarction. As previously mentioned, the outer layer of the heart, the epicardium, has the capacity to partially regenerate the heart post-MI and, while all other cell populations in the heart lose WT1 expression in adulthood, epicardial cells are $WT1^+$ and *Wt1* is overexpressed in the murine epicardium post-MI (Duim *et al.*, 2015). After injury, activated epicardial cells generate epicardial-derived-cells (EPDCs) which differentiate into cardiomyocytes and other cell types through EMT (van Wijk *et al.*, 2012). In fact, post-MI, EPDCs, cardiomyocytes, VSMCs and endothelial cells proximal to the infarct area all

express WT1 (K. Wagner *et al.*, 2002; Smart *et al.*, 2011; Duim *et al.*, 2015). In other models of cardiac injury, including ischaemia-reperfusion, WT1 was also expressed in areas of fibrosis (Braitsch *et al.*, 2013). In these *in vivo* models, WT1 directly regulated adult epicardial cell EMT through direct transcriptional regulation of *Snail*, *VCAM-1* and *Pdgfrα* (Braitsch *et al.*, 2013).

WT1 is known to play a role in developmental angiogenesis in the heart (Martínez-estrada *et al.*, 2010; Zhou and Pu, 2012) and this is replicated post-MI where WT1 is expressed in the vascular endothelial cells and supporting cells of vessels proximal to the infarct area, co-localising with VEGF and PCNA (K. Wagner *et al.*, 2002; Duim *et al.*, 2015). In line with the important role of WT1 in the epicardium, a proportion of $WT1^{+}$ vascular endothelial cells arise from the epicardium and EPDCs (S. Chen *et al.*, 2013). Finally, in the subcutaneous sponge implantation model of *in vivo* angiogenesis, preliminary data suggested that induction of vascular endothelial *Wt1* KO, under the control of *VE-Cadherin Cre*, resulted in a ~8 fold reduction in blood vessel formation (McGregor et al. 2014, Wagner *et al.* unpublished data).

As it has long been evident that WT1 is involved in the response to ischaemia in cardiovascular disease and could, through angiogenesis and other beneficial processes, have regenerative potential, it is surprising it has not been investigated more thoroughly in this context. Given the immense global burden of CVD and that modern medicine is currently incapable of averting ischaemic damage, new regenerative treatments are necessary. WT1 appears to be a promising candidate given its potential role in ischaemic angiogenesis, though *in vivo* experiments are thought ultimately necessary in order to effectively determine this.

4.1.2 **Hypothesis**

This chapter aims to address the hypothesis that:

> *"Wt1, particularly Wt1 in endothelial cells, is key to ischaemic angiogenesis and hence, VE-WT1 KO will impair in vivo angiogenesis."*

4.1.3 **Aims**

The specific aims addressed in this chapter were to determine:

1. The expression of WT1 in vascular and non-vascular cells in two models of *in vivo* angiogenesis.
2. Whether VE-Wt1 KO inhibits angiogenesis in the subcutaneous sponge implantation model.
3. Whether VE-Wt1 KO inhibits angiogenesis in the hindlimb ischaemia model.

First, the expression of WT1 in normal C57Bl/6 mice will be determined in two murine models of *in vivo* angiogenesis; the subcutaneous sponge implantation model and the hindlimb ischaemia (HLI) model of critical limb ischaemia. This will provide a greater insight into the role of WT1 in angiogenesis and ischaemia, as the role of WT1 has not been only minimally investigated in the sponge model and not at all in the hindlimb ischaemia model.

Then, in both these models, *Wt1* will be knocked-out in the vascular endothelium using the VE-Wt1 KO mouse (Monvoisin *et al.*, 2006; Wagner *et al.*, 2014) and angiogenesis assessed, to determine the role of endothelial WT1 in *in vivo* angiogenesis and ischaemia.

4.2 Materials and Methods

For full materials and methods refer to Chapter 2.

4.2.1 Animals

4.2.1.1 C57Bl/6 Mice

C57Bl/6 mice were used as wildtype controls in both the subcutaneous sponge implantation (SSI) and hindlimb ischaemia (HLI) models, to characterise the models and determine the role of WT1 in control mice. All C57Bl/6 mice were C57Bl/6J males, 10-18 weeks of age at the start of experiments.

4.2.1.2 Endothelial WT1-KO Mouse

The endothelial WT1-KO mouse was generated by Kay-Dietrich Wagner and Nicole Wagner (Université de Nice) and SSI was carried out in this model at Université de Nice by this group in mice treated with tamoxifen (KO) or vehicle (VE-Wt1 KO). This mouse used the *Cre-Lox* system to induce an inducible, endothelial specific, WT1-KO with *VE-Cadherin Cre*. This mouse is analogous to the VE-Wt1 KO mouse (Section 2.1.1) and work with this mouse line has been previously published (Wagner *et al.*, 2014). Sponges were extracted 21 days post-implantation for histology and RT-qPCR and samples were kindly provided by Kay-Dietrich Wagner and Nicole Wagner (Université de Nice). All mice used were male and 12-18 weeks of age at start of experiments (n=6).

4.2.1.3 Vascular Endothelial ***Wt1***-KO (VE-Wt1 KO) Mouse

The generation and genotype of the VE-Wt1 KO mouse is described in full in Section 2.1.1. This mouse was generated at The University of Edinburgh and is analogous to the mice generated in Nice (Section 4.2.1.2). Tamoxifen injection was carried out as previously described (Section 2.1.1). Controls used were; vehicle controls (VC), with identical genotype to VE-Wt1 KOs, injected with oil only and *Cre* controls (CC), with only *VE-Cadherin* $CreER^{T2}$ in the absence of $Wt1^{lox/lox}$, injected with tamoxifen to control for the effect of tamoxifen and *Cre*.

Subcutaneous sponge implantation (SSI):

VE-Wt1 KO, VC and CC mice used were all male and 11-16 weeks at start of experiment (n=8). Sponges were extracted 21 days post-implantation, then either fixed in 10% formalin and wax embedded prior to staining, or snap frozen on dry ice and stored at -80°C prior to RNA extraction and qPCR (Section 2.10).

Hindlimb ischaemia (HLI):

VE-Wt1 KO, VC and CC mice used were all male and 11-16 weeks at start of experiment (n=8). Gastrocnemius muscle of ischaemic (ligated) and non-ischaemic (contralateral, non-ligated) limbs were extracted 28 days post-implantation, then either fixed in 10% formalin and wax embedded prior to staining, or snap frozen on dry ice and stored at -80°C prior to RNA extraction and qPCR (Section 2.10).

4.2.2 **Histology**

All histological staining was carried out using the techniques outlined in Sections 2.11 & 2.12. The details of antibodies used for immunofluorescent staining and their concentrations are given below (Table 4.2.1) where not previously provided in Section 2.11 & 2.12.

H&E (Section 2.11.2) and PSR staining (Section 2.11.3) were carried out as previously described. Immunoprecipitation staining of CD31 (rabbit v mouse anti-CD31 (1/300) (Abcam, ab28364)) and WT1 (rabbit v mouse anti-WT1 (1/300) (Abcam, ab28364)) were carried out as per the protocol in Section 2.11.4. Immunoprecipitation staining of F4.80 (rat v mouse anti-F4.80 (1/300) (eBioscience, 14-4801) was carried out by the Histology department (Shared University Research Facilities (SURF), University of Edinburgh) at The Queen's Medical Research Institute, University of Edinburgh.

1º Antibody	2º Antibody
Rabbit v mouse anti-WT1 (1/300) (Abcam, ab28364)	**Goat v rabbit IgG Peroxidase** (1/200) (Vector, PI-1000) **TSA Tyramide Fluorescein** or **Cy3** or **Cy5** (1/50) (PerkinElmer, NEL701A001KT or SAT704A001EA or SAT705A001EA)
Rabbit v mouse anti-CD31 (1/300) (Abcam, ab28364)	**Goat v rabbit Alexa 488** (1/200) (Molecular Probes, GAR-A488)
Isolectin-B4 (1/500) (Invitrogen, 1110271)	**Streptavidin - Alexa 488** (1/400) (Invitrogen, A-32354)
Rabbit v mouse anti-αSMA (1/500) (C6198, Sigma)	Directly conjugated to Cy3
Goat v mouse anti-SNAIL (1/300) (Abcam, ab53519)	**Donkey v goat Alexa 555** (1/200) Molecular probes (A11056)
Goat v mouse anti-PCNA (1/300) (Santa Cruz, sc-9857)	**Donkey v goat Alexa 555** (1/200) Molecular probes (A11056)
Goat v mouse anti-VEGFR2 (KDR) (1/300) (Abcam, ab10972)	**Donkey v goat Alexa 555** (1/200) Molecular probes (A11056)
DAPI (1/1000) (Sigma, D9542)	N/A

Table 4.2.1: Antibodies used for immunofluorescent staining. Primary antibodies are provided with the relevant secondary antibodies used in the adjacent column. Antibody description/name, concentration used, provider and catalogue number are all provided.

4.2.3 Quantification of Histology

For quantification, images were taken on an Axioscan Z1 slide scanner (Zeiss) and four random regions of interest (ROIs) 500μm x 500μm were selected on Zen 2012 Blue software (Zeiss). Images were extracted and analysed using

Image J software (V1.47, National Institute of Health, USA). The mean of the values for these four regions of interest was then calculated.

For sponges, due to the sponges being more vascularised and cellularised around the periphery, alongside selecting ROIs at random (referred to as 'Total'), ROIs were also randomly selected from the outer circumference of sponge up to a depth of 1000μm from the edge (referred to as 'Outer') and the centre of the sponge (referred to as 'Inner') (Figure 4.2.1).

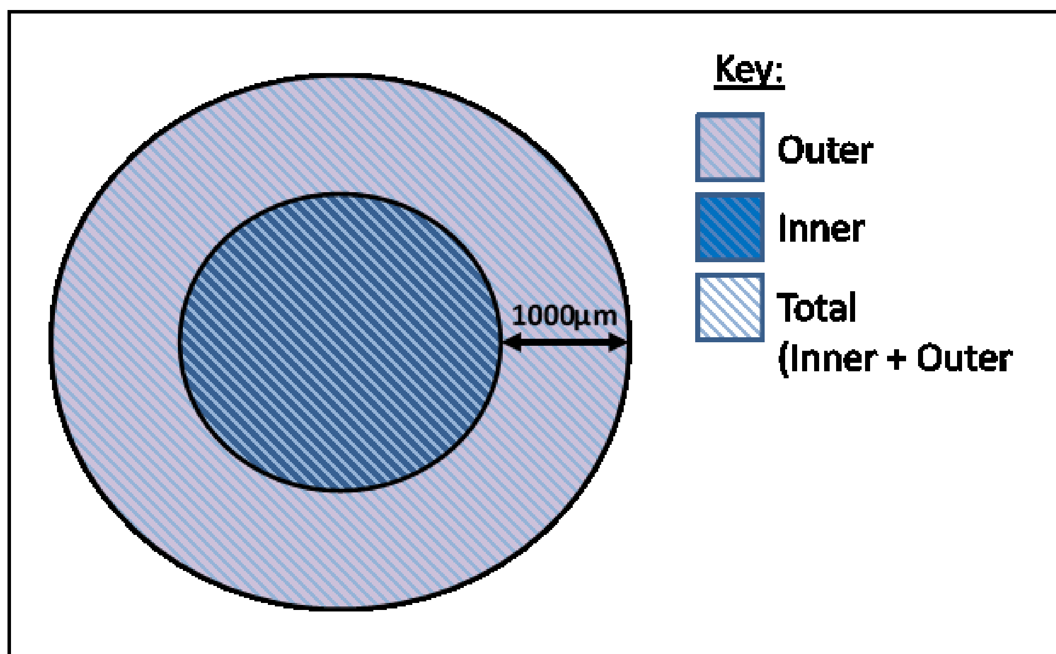

Figure 4.2.1: Quantification of histology in sponge sections. The Total cross sectional area of the sponge was broken up for quantification of histology into a section comprising the outer 1000μm (Outer) and the remaining central area of the sponge (Inner). This was necessary due to the uneven vascular and cellular infiltration into the sponge.

4.2.3.1 Vessel Density

Vessels were identified by luminal structures with a $CD31^+$ endothelial layer and counted within the selection ROIs. This was quantified as vessel density by vessel number divided by area.

4.2.3.2 WT1 Positive Nuclei

Nuclei were identified by DAPI staining. The number of $WT1^+$ nuclei within each ROI was counted and expressed as the number of $WT1^+/DAPI^+$ nuclei divided by the total number of $DAPI^+$ nuclei.

4.2.3.3 **Percentage Positive Area**

ROIs were thresholded by colour in Image J to convert them to black and white images such that the stain of interest was converted to black pixels and the rest of the image was converted to white pixels. The percentage positive area (for the stain of interest) was then calculated as the number of black pixels divided by the total number of pixels in the ROI (black pixels + white pixels). The same parameters were used to threshold each ROI.

	Immunoperoxidase (DAB):	PSR:
Hue:	0-40	220-255
Saturation:	50-255	0-255
Brightness:	0-255	0-255

4.2.4 **RT-qPCR**

RNA extraction, CDNA synthesis and RT-qPCR was carried out as described in Section 2.10.

4.3 Results

4.3.1 The Subcutaneous Sponge Implantation (SSI) Model of Angiogenesis

In wildtype control (C57Bl/6) mice, numerous cell types infiltrated into implanted sponges, along with angiogenesis providing a blood supply into the sponge matrix through new blood vessel formation (Figure 4.3.1). There was a visible difference in sponge appearance at Day 3 (Figure 4.3.1A) compared with Day 21 (Figure 4.3.1B), with the colour of sponges changing as cells and vessels infiltrated. It was also evident that cutaneous vessels had been recruited to, and grown into, the sponges to provide them their own blood supply (Figure 4.3.1B). However, H&E staining revealed cells and blood vessels only infiltrated a limited distance into the sponges by Day 21, with the majority of the sponge still largely uncolonised (Figure 4.3.1C).

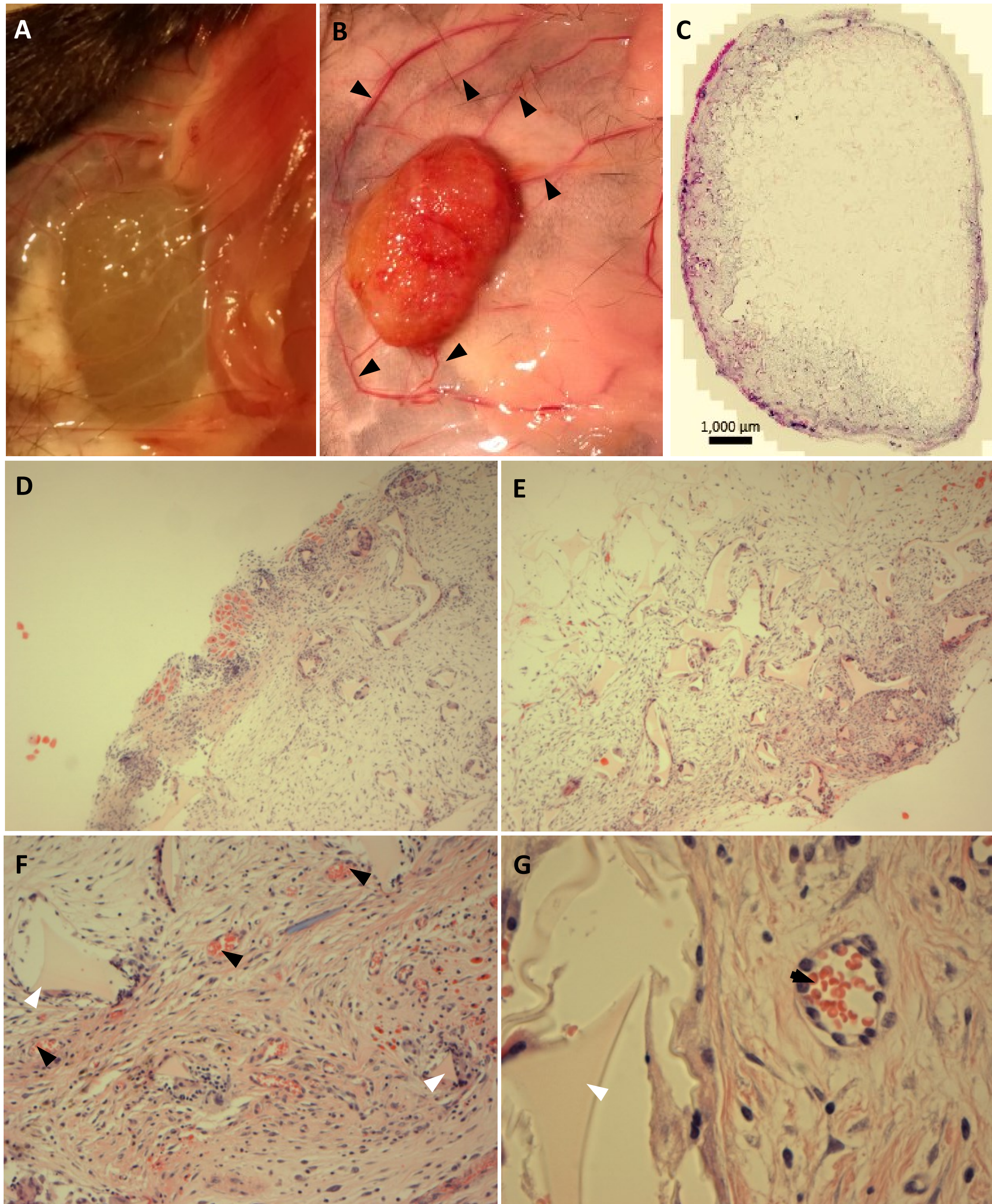

Figure 4.3.1: Cell and vessel infiltration in the subcutaneous sponge model. Subcutaneous sponge implantation was carried out in C57Bl/6 mice. Subcutaneous sponges *in situ* at **A)** Day 3 and **B)** Day 21, post-implantation (blood vessels indicated with black arrows). **C)** A cross-section through an H&E stained sponge at Day 21, shows the depth of cell infiltration into the sponge. **D, E)** Representative H&E images at Day 21 (50x magnification). **F, G)** Representative H&E images at Day 21 (100x & 200x magnification, respectively), with blood vessels clearly visible (black arrows) and areas of sponge indicated by white arrows. Representative images from n=3.

4.3.2 **WT1 Expression in the Subcutaneous Sponge Model (SSI)**

C57Bl/6 mice, were examined for WT1 expression in normal mice in response to subcutaneous sponge implantation. Isolectin-B4/WT1/DAPI IF IHC staining was carried out on these tissues to visualise WT1 expression and determine if there was vascular WT1 expression. Isolectin-B4 stained the cytoplasm of vascular endothelial cells of vessels. There were also a large number of cells within the sponges that expressed Isolectin-B4 that were not associated with blood vessels and other non-vascular cells which did not express Isolectin-B4.

WT1 staining was observed in sponges; a proportion of this was due to expression in cells of the vascular wall but some was also evident in non-vascular cells proximal to vessels and some was in other non-vascular cells. Whilst many blood vessels had no $WT1^+$ cells, WT1 staining was present in a proportion of Isolectin-$B4^+$ endothelial cells of newly formed blood vessels, with staining occurring in a granular distribution within the nucleus (Figure 4.3.2 C-E). Many non-vascular Isolectin-$B4^+$ cells and Isolectin-$B4^-$ cells also expressed nuclear WT1 (Figure 4.3.2).

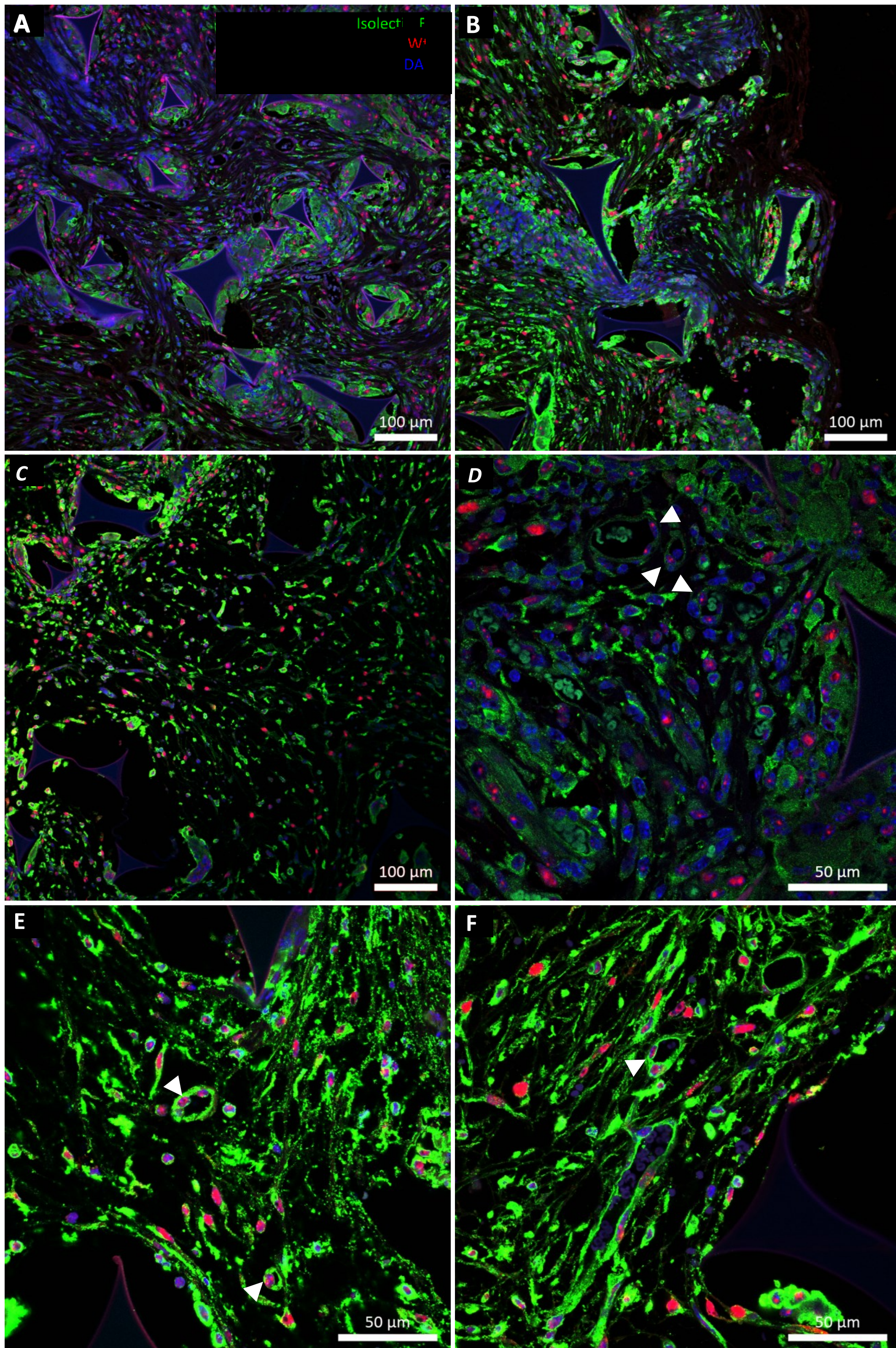

Figure 4.3.2: WT1 is widely expressed in vascular and non-vascular cells of the subcutaneous sponge implantation model. Representative images from Isolectin-β4 (green), WT1 (red) and DAPI (blue) staining in sponges from the SSI model from C57Bl/6 mice. Isolectin-β4 is expressed in a large proportion of cells in the sponge. WT1⁺ cells are widely evident throughout the sponge, including in

vascular-associated endothelial cells (white arrows). Representative images from n=3.

4.3.3 Endothelial WT1 KO in the Subcutaneous Sponge Implantation Model

Sections from sponges implanted (21 days) in mice with endothelium-selective WT1 KO mice were kindly provided by Drs Kai & Nicole Wagner (University of Nice). This KO was generated using a VE-Cadherin Cre system as described in Section 2.1.1 and is analogous to the VE-Wt1 KO mouse described previously and used in aortic ring models (Section 3.3.4.4). However, as the generation of the KO and the surgeries were carried out in Nice, and there is only a vehicle control (no *Cre* control), these will be referred to separately to avoid confusion. Preliminary data from this work has been published (Wagner *et al.* 2014, McGregor *et al.* unplublished data).

4.3.3.1 Endothelial WT1 KO Has No Effect on Vessel Density in SSI

Sections of sponges provided by the Wagner group were stained to determine whether angiogenic vessel growth was altered by selective deletion of WT1 from endothelial cells. Sections were assessed using three histological approaches: H&E; CD31 IP IHC; and CD31/αSMA/DAPI IF. Vessel density was not altered in mice with selective deletion of WT1 from the endothelium (Figure 4.3.3 & 4.3.4). The extent of CD31 positivity (IP (Figure 4.3.3)) and CD31, αSMA and DAPI intensity (IF (Figure 4.4)) was not significantly different between groups.

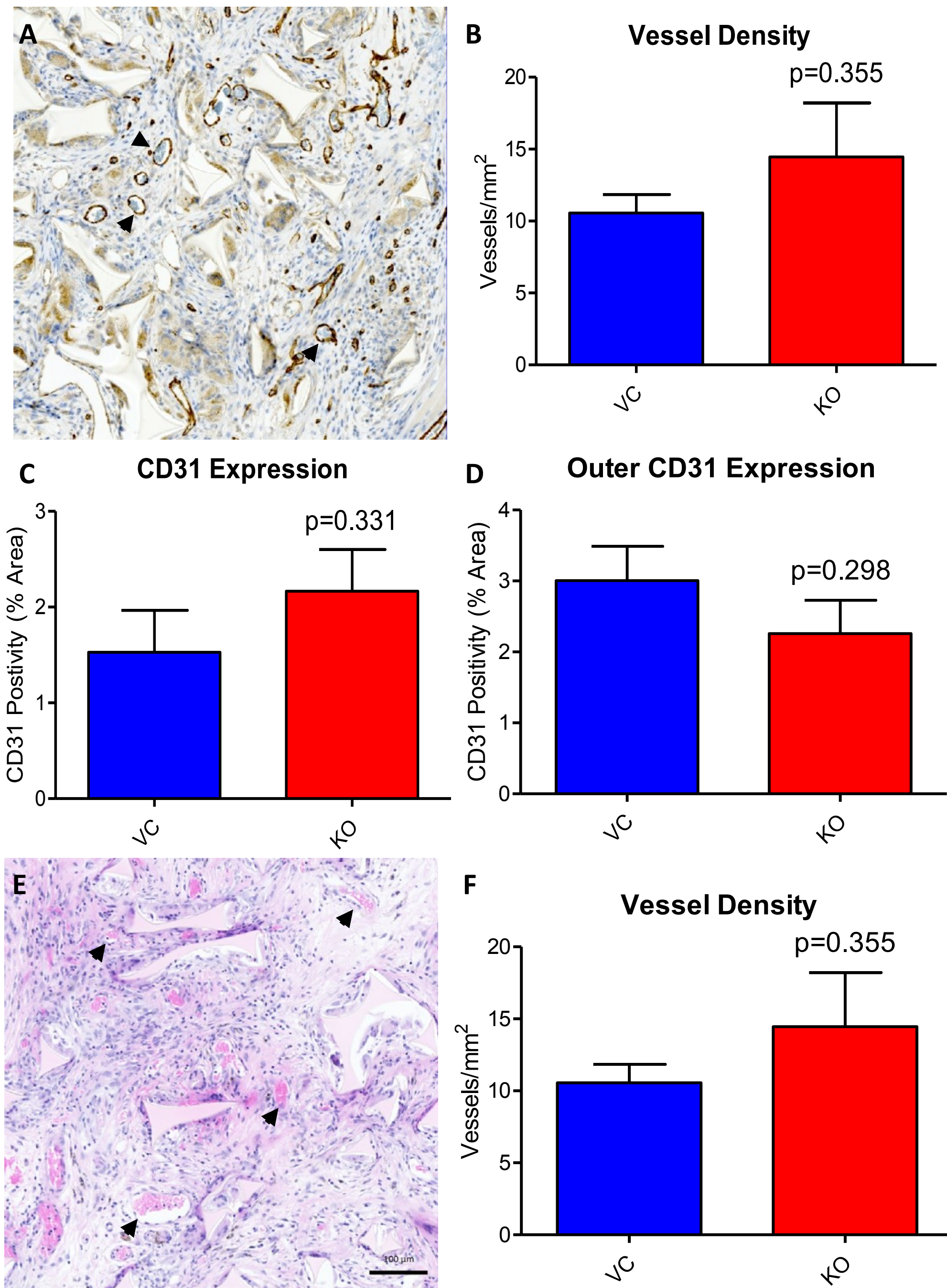

Figure 4.3.3: Vessel density is not affected by endothelial WT1 KO in SSI. Vessels were visualised using CD31 and H&E histological staining in endothelial WT1 KO mice (KO) and vehicle controls (VC). **A)** Representative CD31 IP IHC in VC sponge (examples of CD31⁺ vessels indicated with arrows). **B)** Manual quantification of CD31⁺ vessels. Quantification of CD31 expression **C)** across the whole cross-section of the sponge (cellularised and non-cellularised area) and **D)** in the outer 1000µm of the sponges (cellularised area only). **E)** Representative H&E staining in VC sponge (examples of vessels indicated with arrows). **F)** Manual quantification of vessels from H&E staining. VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. P-values shown, unpaired Student's t-test. n=5.

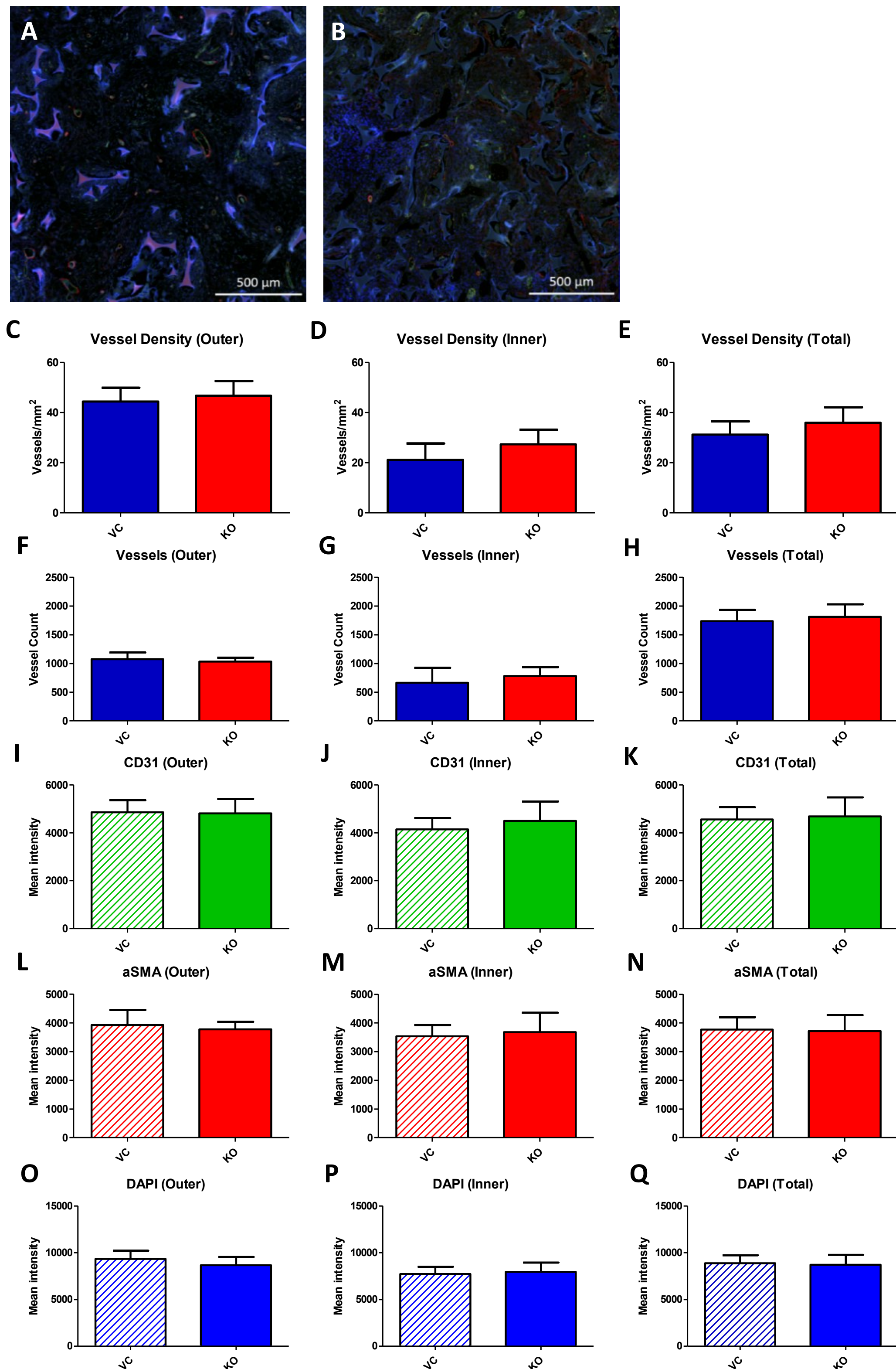

Figure 4.4: Vessel density is not affected by endothelial WT1 KO in SSI. Vessels were visualised using CD31/αSMA/DAPI IF IHC. Representative staining in **A)** VC and **B)** KO sponges (green = Isolectin-B4, red = WT1, blue = DAPI). Quantification of **C-E)** vessel density and **F-G)** total vessel number. Mean fluorescence intensity of staining for **I-K)** CD31, **L-N)** αSMA and **O-Q)** DAPI. Outer= outer 1000µm of the sponges (cellularised area only), Inner = central section of sponge excluding outer 1000µm (predominately non-cellularised), Total = whole cross-section of the sponge

(cellularised and non-cellularised area). VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. $p>0.05$, unpaired Student's t-test. n=5.

4.3.3.2 Endothelial WT1 KO Has No Effect on WT1 Expression in SSI

WT1 staining in VC and KO sponges from SSI was visualised by WT1 IP IHC. This revealed WT1 expression in a number of the cells which had infiltrated into the sponge in VC and KO sponges (Figure 4.3.5). From what could be identified as vessels, while there appeared to be some vascular cells which were WT1$^+$, the majority of WT1$^+$ cells were non-vascular. WT1$^+$ cells were present around the periphery of implanted sponges and in the less densely colonised area at the centre of sponges. WT1$^+$ cells occasionally seemed to be infiltrating into the sponge between skeletal muscle cells present at the periphery of the sponges (Figure 4.3.5 A).

WT1 staining in sponges was quantified in VC and KO sponges as the number of WT1$^+$ nuclei (Figure 4.3.5 F) and the total area stained positive for WT1 (Figure 4.3.5 G). There was a trend towards a reduction in WT1 staining when quantified by both of these measures (15.80% vs 4.57%, p=0.073 and 2.25% vs 1.30%, p=0.099, respectively), though this did not reach significance.

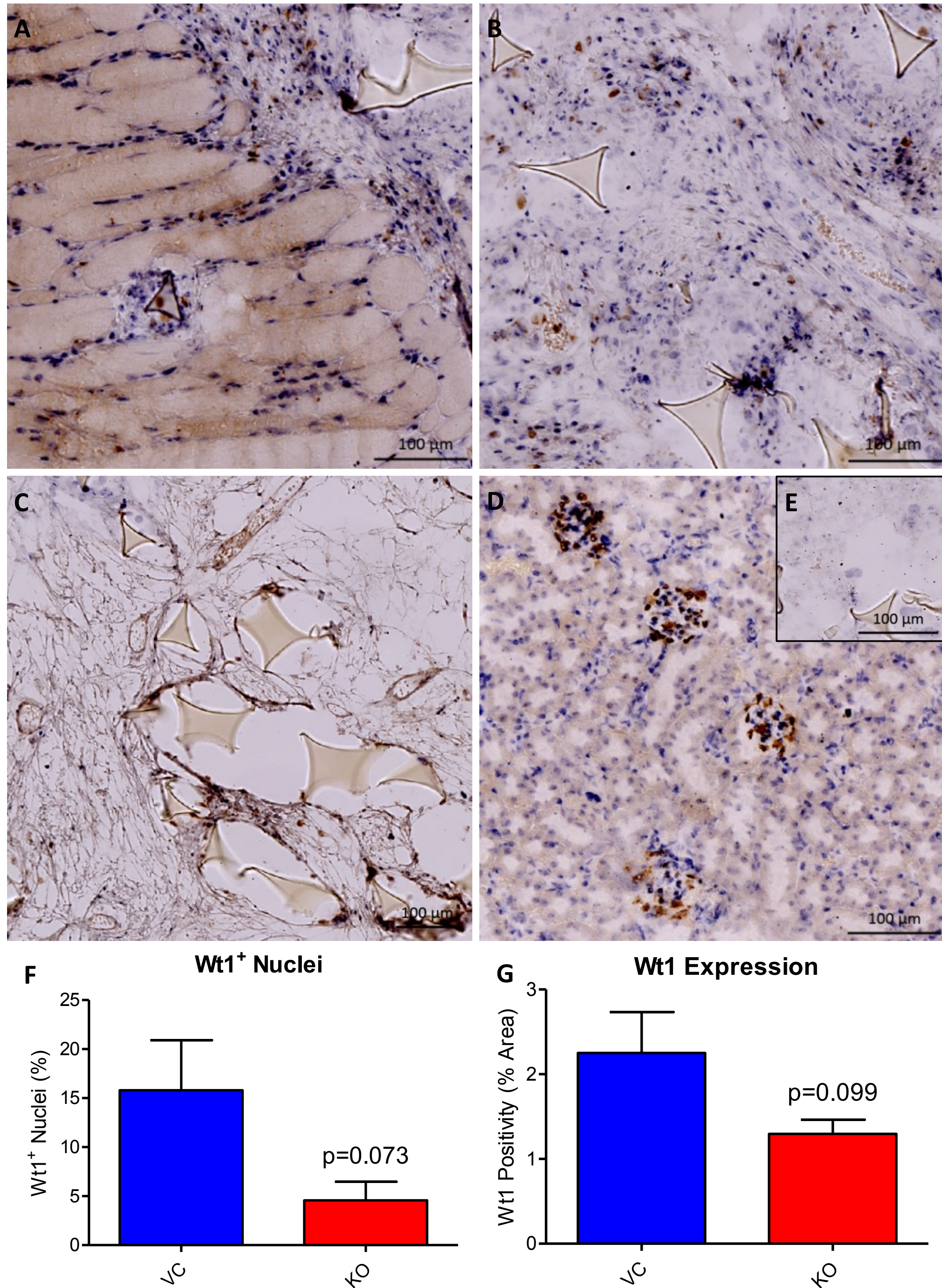

Figure 4.3.5: Total WT1 expression is not significantly affected by endothelial WT1 KO in SSI. A-C) Representative WT1 IP IHC in VC sponges (sponge boundary indicated by dashed line). **D)** Kidney tissue, positive control for WT1 staining and **E)** Negative control IgG. **F)** Manual quantification of WT1⁺ nuclei in VC and KO. **G)** Quantification of WT1 expression in VC and KO. VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. P-values shown, unpaired Student's t-test. n=5.

4.3.3.3 Increased Macrophage & Collagen Infiltration in Endothelial WT1 KO Sponges

To determine the effect of endothelial WT1 KO on *in vivo* fibrosis and inflammation, collagen deposition (PSR) and macrophage infiltration (F4.80) was assessed by histology. Images were thresholded and the % area of staining quantified. There were no significant differences in macrophage (Figure 4.3.6) or collagen (Figure 4.3.7) density in sponges from WT and KO groups. However, endothelial-specific WT1 KO sponges had a greater mean depth of collagen infiltration than controls (3088μm vs 1806μm ($p<0.005$)) and, concurrently, a greater mean depth of macrophage infiltration (3219μm vs 1746μm ($p<0.0005$)). Increased depth of cell migration into sponges was also evident with CD31/αSMA/DAPI staining (Figure 4.3.8). This revealed a pattern of sponge colonisation where αSMA+ cells had infiltrated deepest into the sponge, with CD31+ cells adjacent distally; this was the same between genotypes.

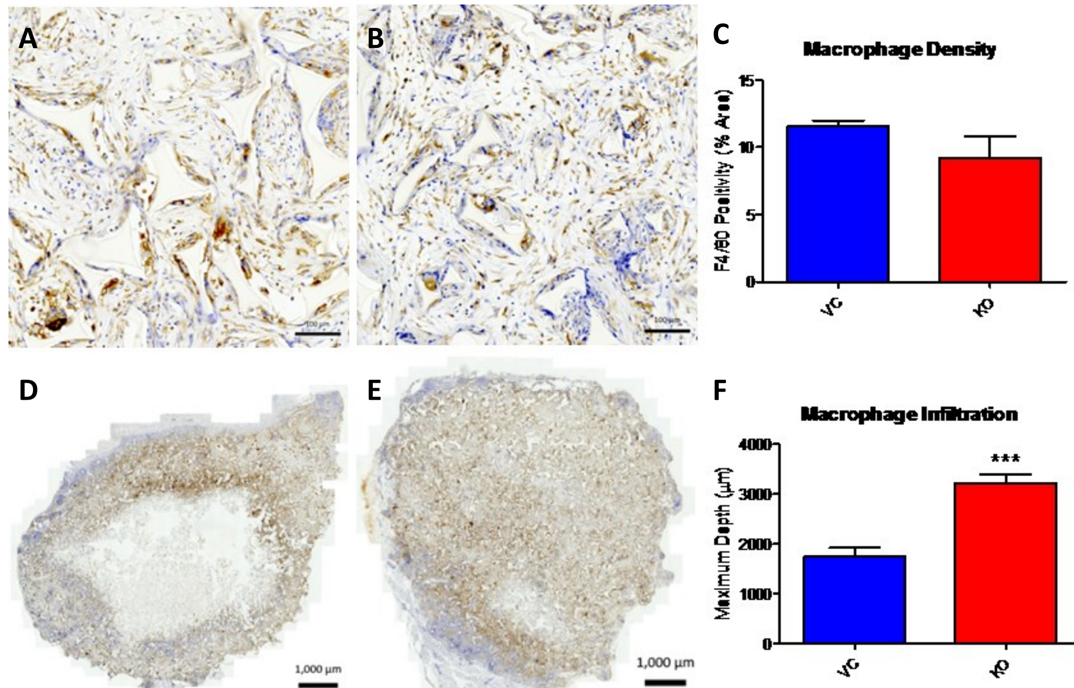

Figure 4.3.6: Depth of macrophage infiltration, but not macrophage density, increased in sponges from endothelial-specific WT1 KO mice. Sponges were stained for the macrophage marker F4.80. Representative F4.80 images from **A, D)** VC and **B, E)** KO sponges. **C)** Quantification of F4.80 staining, for F4.80 positivity by area. **F)** Quantification of maximum depth of macrophage infiltration revealed

significantly greater depth of macrophage infiltration in KOs. VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. ***p<0.005, unpaired Student's t-test. n=5.

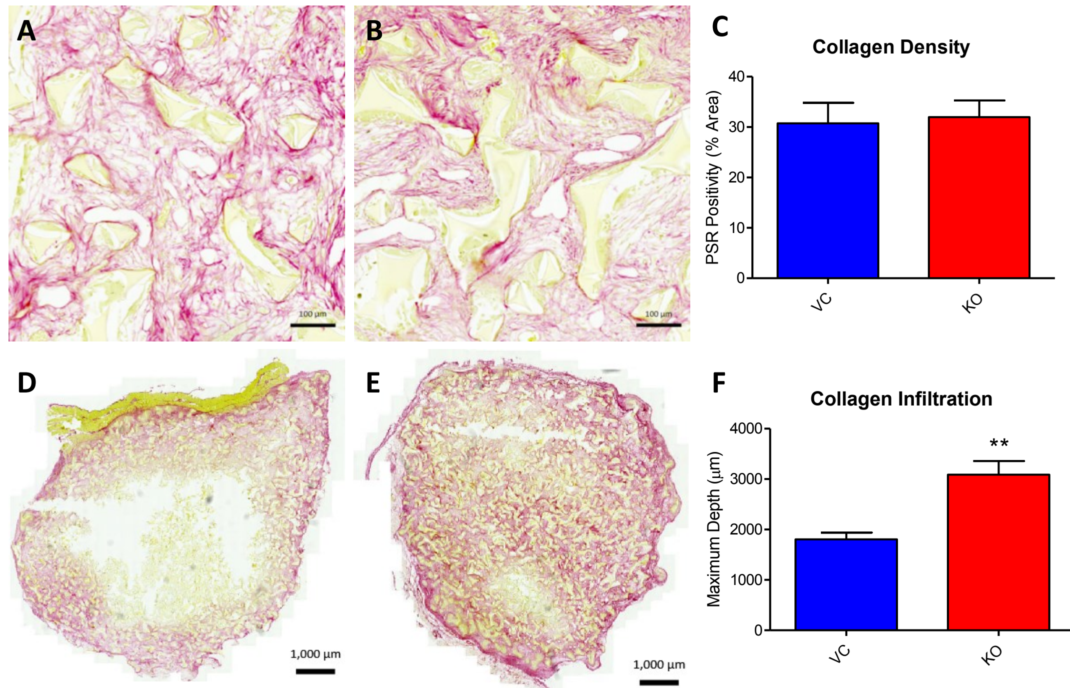

Figure 4.3.7: Depth of collagen infiltration, but not collagen deposition increased in endothelial WT1 KO sponges. Sponges were stained using PSR to visualise collagen. Representative PSR images from **A, D)** VC and **B, E)** KO sponges. **C)** Quantification of PSR staining, for PSR positivity by area. **F)** Quantification of maximum depth of collagen infiltration revealed greater depth of collagen infiltration in KOs. VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. **p<0.01, unpaired Student's t-test. n=5.

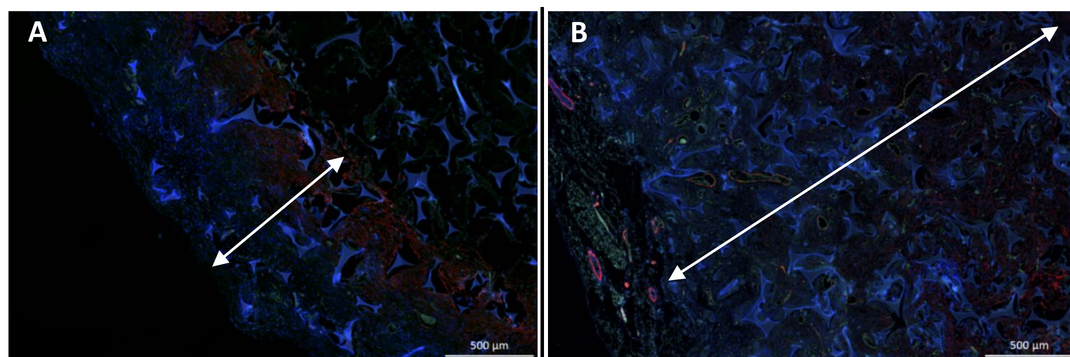

Figure 4.3.8: Endothelial WT1 KO sponges have greater depth of cellular infiltration. CD31/αSMA/DAPI IF IHC revealed a greater depth of cellular infiltration in **B)** KOs compared to **A)** VCs. $αSMA^+$ cells have visibly infiltrated more deeply than $CD31^+$ cells. Depth of cell infiltration indicated by arrows. n=5.

4.3.3.4 Endothelial WT1 KO Has No Effect on Expression of Genes Associated with Angiogenesis or EMT

RNA was extracted from sponges and gene expression was quantified by RT-qPCR for selected genes associated with angiogenesis (HIF-1a, VEGF-A) or EMT (Snail, Slug). First, 'housekeeper' genes were selected, to which the expression of all other genes were normalised. β-Actin and GAPDH were selected, as while linear regression of β-Actin v GAPDH was not significantly non-zero, their expression was not different between groups (Figure 4.3.9 A-D).

After normalising to β-Actin and GAPDH, expression of HIF-1a, VEGF-A, Snail and Slug did not significantly differ between VC and KO tissue (Figure 4.3.9 E).

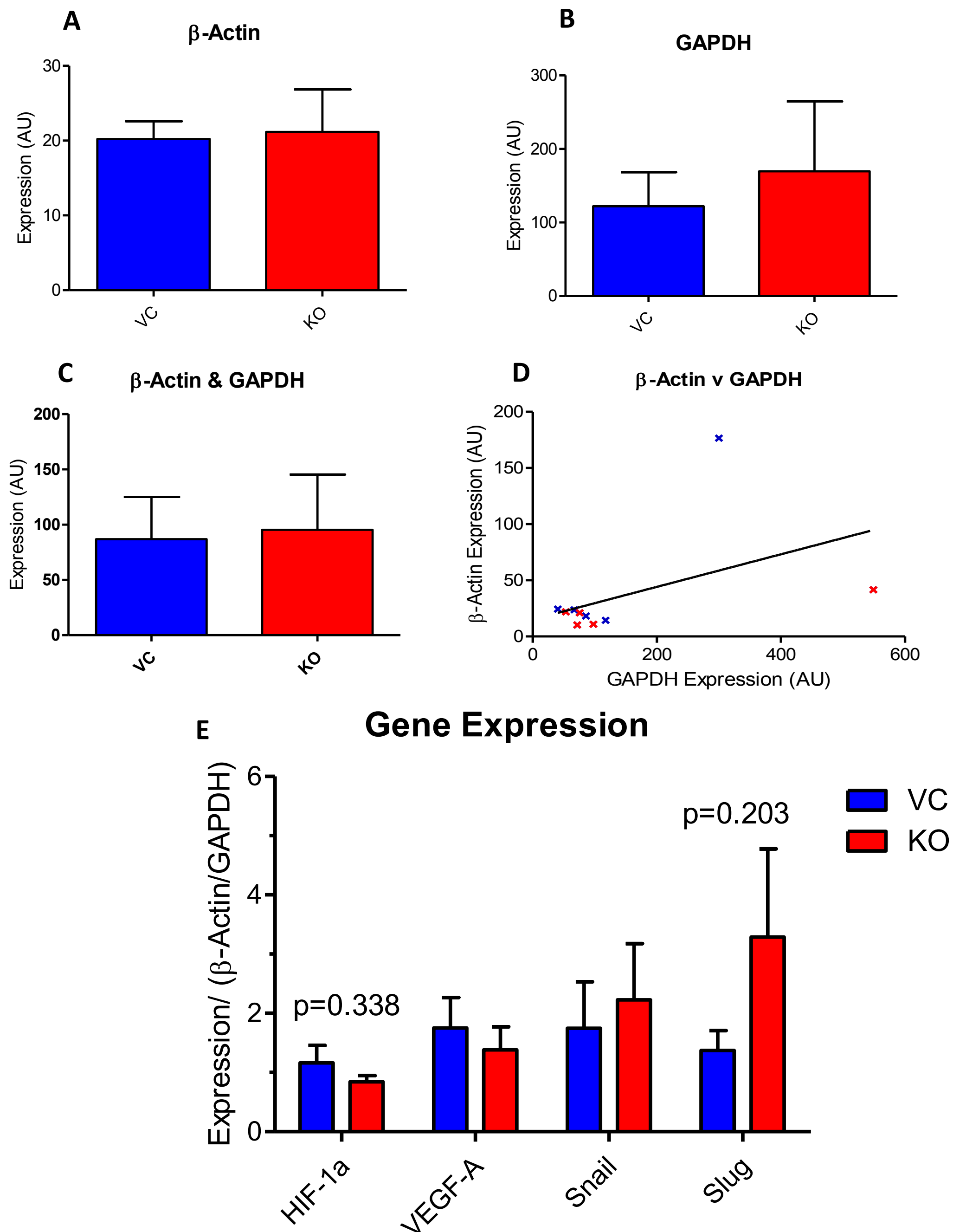

Figure 4.3.9: Endothelial WT1 KO does not affect global gene expression of angiogenic or EMT genes. Global gene expression was assessed by RT-qPCR in extracted sponge tissue. β-Actin and GAPDH were chosen as housekeeper genes as **A, B, C)** expression did not differ significantly between groups and **D)** Linear regression of β-Actin v GAPDH in all samples was not significantly non-zero. All other genes were normalised to β-Actin and GAPDH expression. **E)** Expression of HIF-1a, VEGF-A, Snail and Slug in VC and KO sponge tissue as assessed by RT-qPCR. There was no difference between groups in expression of these.VC = vehicle control, KO = endothelial WT1 KO. All data mean +/-s.e. mean. P-values shown, 2-way ANOVA with Bonferroni post-hoc tests. n=5.

4.3.4 VE-Wt1 KO in the Subcutaneous Sponge Model

Demonstrating the effect of endothelial WT1 KO on *in vivo* angiogenesis and other processes in sponges obtained from experiments performed in France (Section 4.3.3) was limited by the absence of a *Cre* control (CC). Therefore, this experiment was repeated to control for the potential effects of Cre and ensure any differences between VC and KO groups was not solely due to the action of *Cre*. In the following section, an inducible endothelial WT1 KO (VE-Wt1 KO) was generated and the subcutaneous sponge implantation model was applied to it, alongside VC and CC groups. Sponges from SSI were removed from the mice after 21 days and assessed by IHC and RT-qPCR.

4.3.4.1 VE-Wt1 KO Does Not Reduce Vascular Density

Angiogenesis in the SSI model was assessed by IHC for CD31 and VE-Cadherin. No significant difference was found between VC, CC and VE-Wt1 KO for CD31 positivity as measured by percentage area immunopositive for CD31 (Figure 4.3.10 A, B). Concurrently, vessel number was quantified in sponges from VE-Wt1 KO, CC and CC mice and there was no significant difference between groups in $CD31^+$ (Figure 4.3.10 C, D) or VE-$Cadherin^+$ (Figure 4.3.10 E, F) vessel density.

4.3.4.2 VE-Wt1 KO Does Not Affect Cell Infiltration

VC, CC and VE-Wt1 KO sponges were H&E stained. There were no evident differences in sponge morphology between groups. Cell infiltration into sponges was quantified and there was found to be no effect of VE-Wt1 KO on depth of cell infiltration in sponges when measured by maximum depth of cell infiltration, area of cell infiltration and area of cell infiltration as a percentage of total sponge area (Figure 4.3.11).

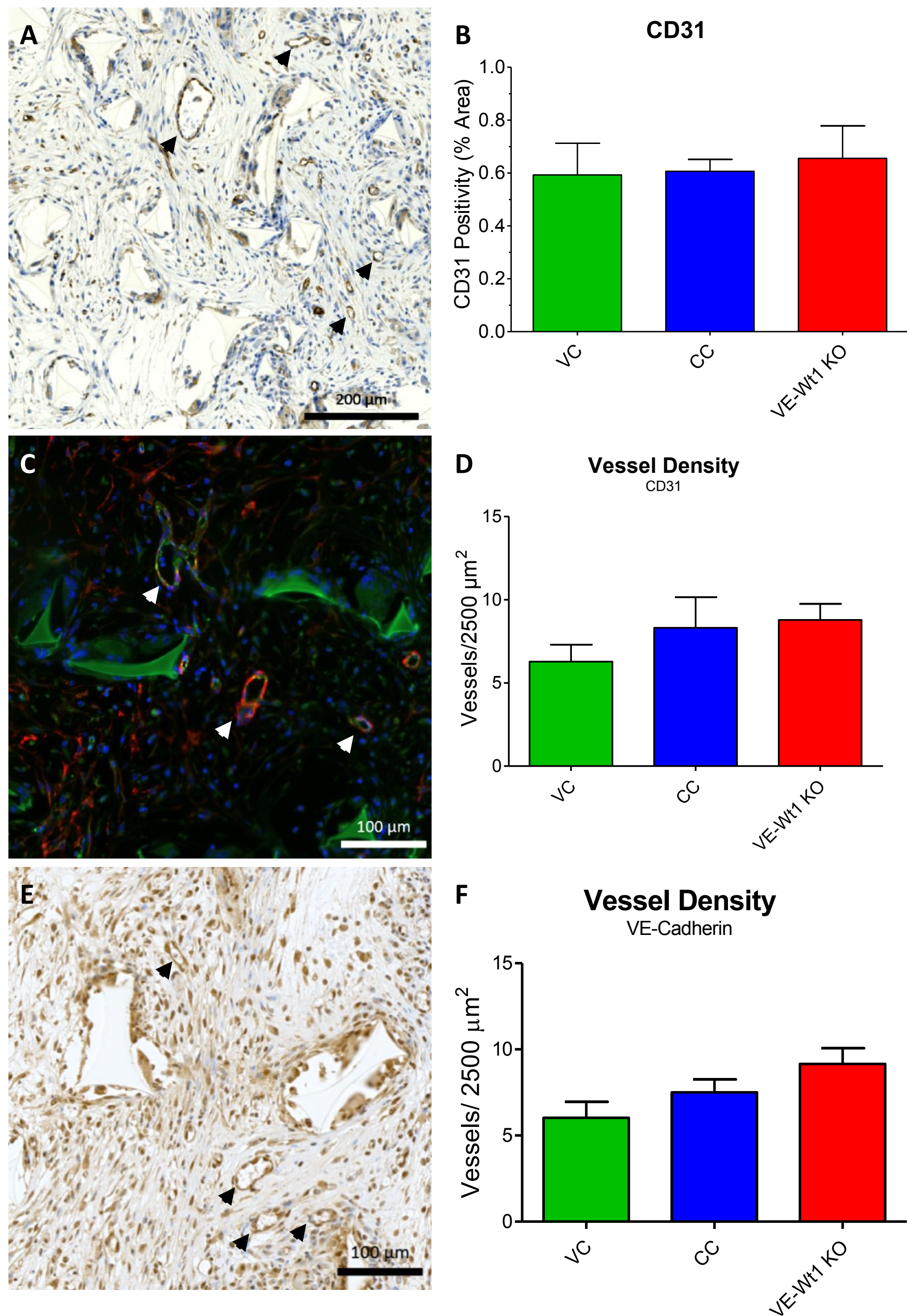

Figure 4.3.10: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) has no effect on vascular density in SSI. Vascular density was quantified in VE-Wt1 KO, *Cre* control (CC) and vehicle control (VC) sponges from SSI at 21 days post-implantation. It was found that VE-Wt1 KO had no effect on vascular density. **A)** Representative CD31 IP IHC staining in VC and **B)** quantification of CD31 positivity from thresholding this staining. **C)** Representative CD31/αSMA/DAPI staining in VC and **D)** quantification of $CD31^{+}$ vessel density. **E)** Representative VE-Cadherin IP IHC staining in VC and **F)** quantification of VE-$Cadherin^{+}$ vessel density. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. Arrows indicate examples of vessels. All data mean +/-s.e. mean. P>0.05, 1-way ANOVA with Bonferroni post-hoc tests. n=8.

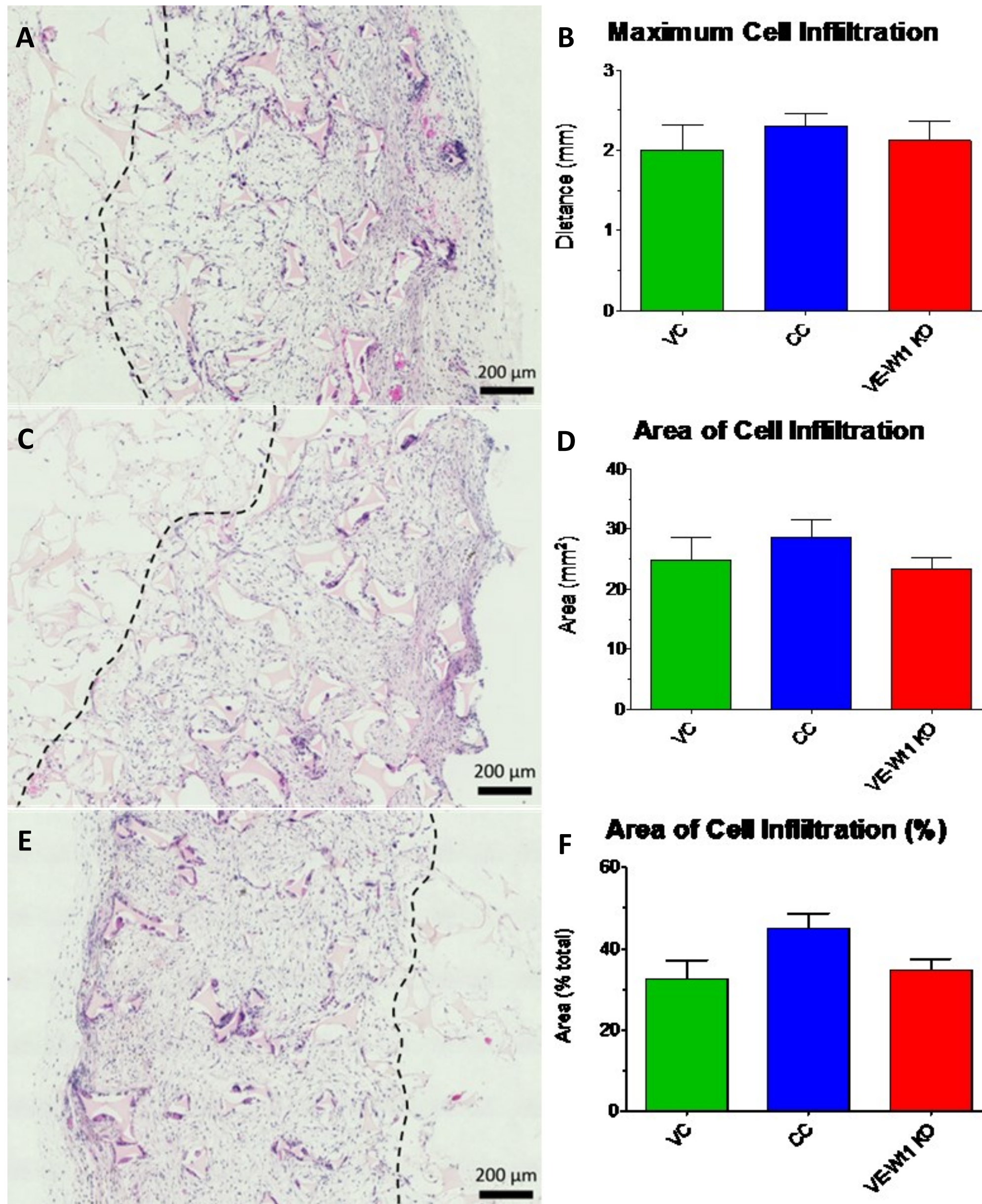

Figure 4.3.11: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) has no effect on cell infiltration in SSI. At Day 21 post-implantation sponges were removed and depth of cell infiltration was determined by thresholding images from H&E staining to visualise all cells. **A, C, D)** Representative H&E images demonstrating depth of cell infiltration into sponges in vehicle control (VC), *Cre* control (CC) & VE-Wt1 KO, respectively. Limit of cell infiltration indicated by dashed line. Cell infiltration was assessed by **B)** maximum depth of cell infiltration, **D)** cross-sectional area of total cell infiltration and **F)** cross-sectional area of cell infiltration as a percentage of the total cross-sectional area of the sponge. There was no significant difference between groups in any of these parameters. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data mean +/-s.e. mean. P>0.05, 1-way ANOVA with Bonferroni post-hoc tests. n=8.

4.3.4.3 **VE-Wt1 KO Does Not Affect Macrophage or Collagen Infiltration**

To determine the effect of VE-Wt1 KO on *in vivo* fibrosis and inflammation, collagen deposition (PSR) and macrophage infiltration (F4.80) was assessed by histology. The pattern of PSR and F4.80 staining was broadly comparable to that observed in the sponges provided by our collaborators in Nice (Section 4.3.3; Figures 4.3.5 & 4.3.7). Images were thresholded and the percentage area of staining quantified. There was increased F4.80 staining in sponges from VE-Wt1 KO mice vs VC mice, but not vs CC mice which had a trend towards increased F4.80 staining vs VC (VC = 4.72%, CC = 8.58%, VE-Wt1 KO = 10.52%, $p<0.01$ VE-Wt1 KO vs VC, 1-way ANOVA with Bonferroni post-hoc tests) (Figure 4.3.12). There was no difference collagen (Figure 4.3.13) density between VE-Wt1 KO, CC and VC.

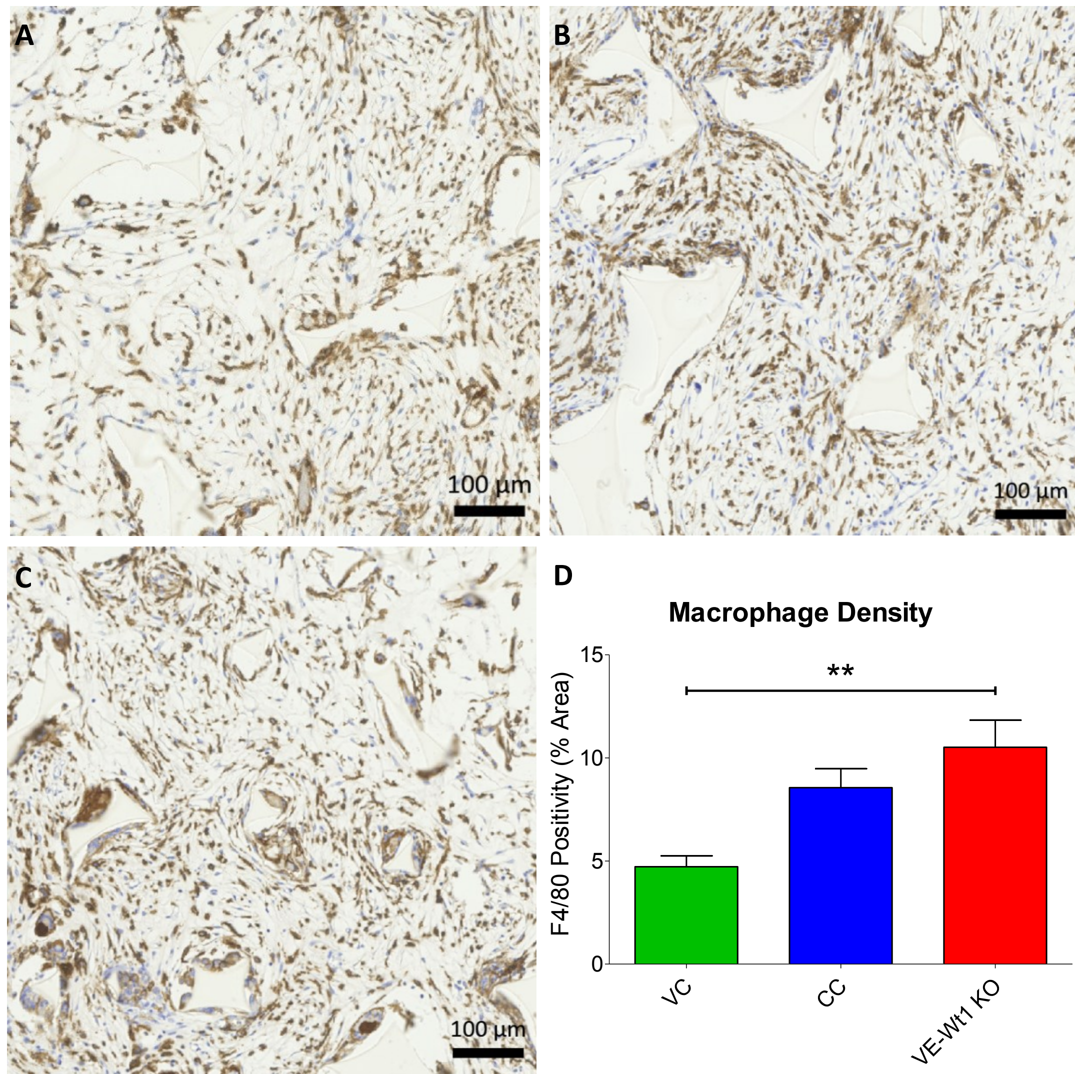

Figure 4.3.12: Macrophage infiltration into sponges is increased by vascular endothelial *Wt1* knock-out (VE-Wt1 KO). Sponges were stained for the macrophage marker F4.80. **A, B, C)** Representative F4.80 images from vehicle control (VC), *Cre* control (CC) and VE-Wt1 KO, respectively. **D)** Quantification of F4.80 staining, for F4.80 positivity by area, revealed greater macrophage infiltration in VE-Wt1 KO vs VC, but not vs CC. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. **p<0.01, 1-way ANOVA with Bonferroni post-hoc tests. n=8.

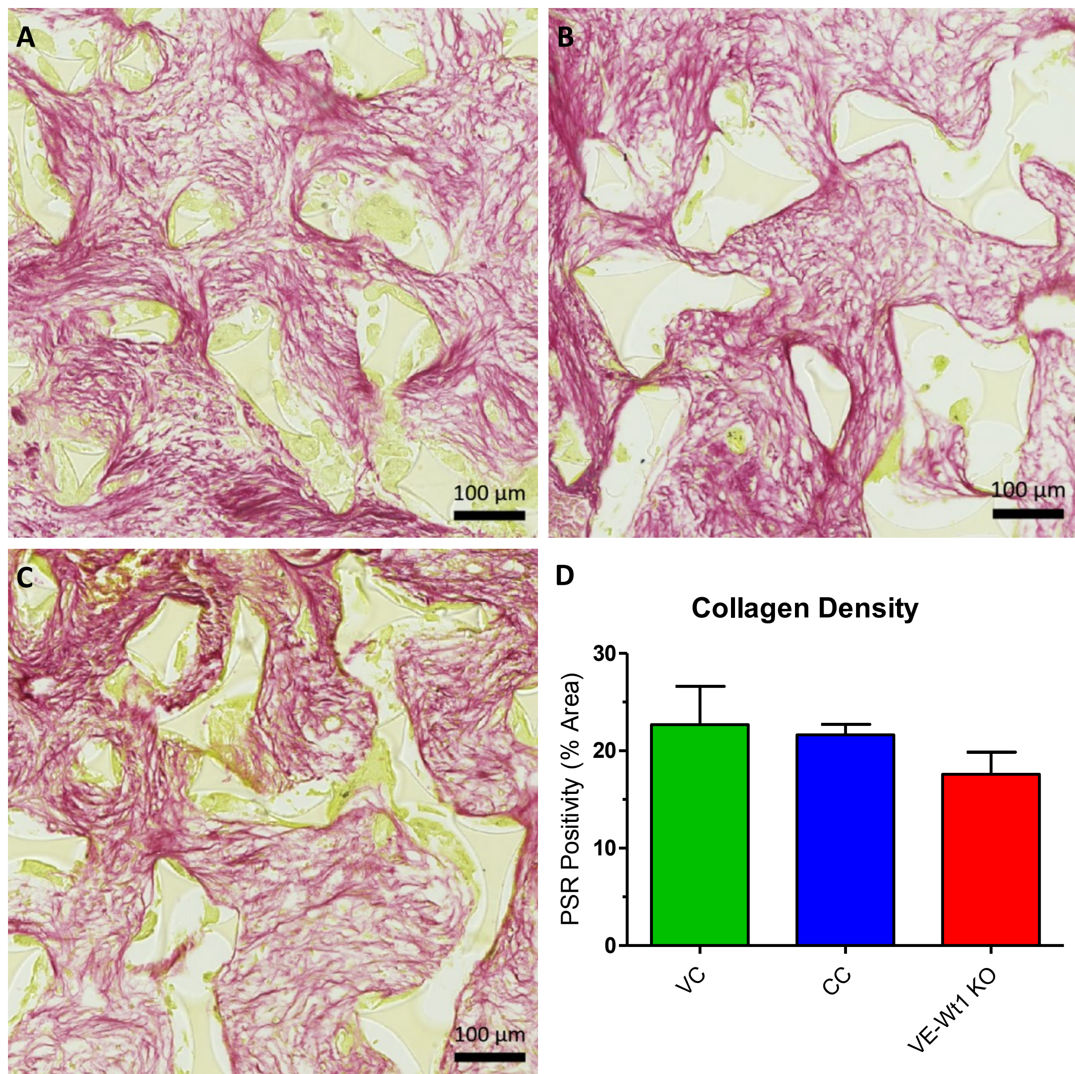

Figure 4.3.13: Collagen deposition within sponges is not affected by vascular endothelial *Wt1* knock-out (VE-Wt1 KO). Collagen in sponges was visualised by PSR staining. **A, B, C)** Representative PSR images from vehicle control (VC), *Cre* control (CC) and VE-Wt1 KO, respectively. **D)** Quantification of PSR staining, for PSR positivity by area, revealed no significant difference between groups. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. 1-way ANOVA with Bonferroni post-hoc tests. n=8.

4.3.4.4 VE-Wt1 KO Has No Effect on Vascular, Angiogenic or EMT Gene Expression

RNA was extracted from sponge tissue and gene expression was quantified by RT-qPCR for selected vascular cell markers (CD31, αSMA, VE-Cadherin, PDGFRβ), genes associated with angiogenesis (HIF-1a, VEGF-A) and genes associated with EMT (Snail, Slug). First, 'housekeeper' genes were selected,

to which the expression of all other genes were normalised. β-Actin and GAPDH were selected, as their expression did not differ significantly between groups and there was a significant correlation between their expression values ($p<0.0001$, linear regression) (Figure 4.3.14A-D).

After normalising to β-Actin and GAPDH, expression of CD31, αSMA, VE-Cadherin, PDGFRβ, HIF-1a, VEGF-A, Snail and Slug did not significantly differ between VE-Wt1 KO, VC and CC tissue (Figure 4.3.14). While the mean expression of CD31 was lower in VE-Wt1 KO tissue, this was not significant due to the large variation in CD31 expression in VC and CC tissue ($p=0.699$, VE-Wt1 KO vs VC, unpaired Student's t-test).

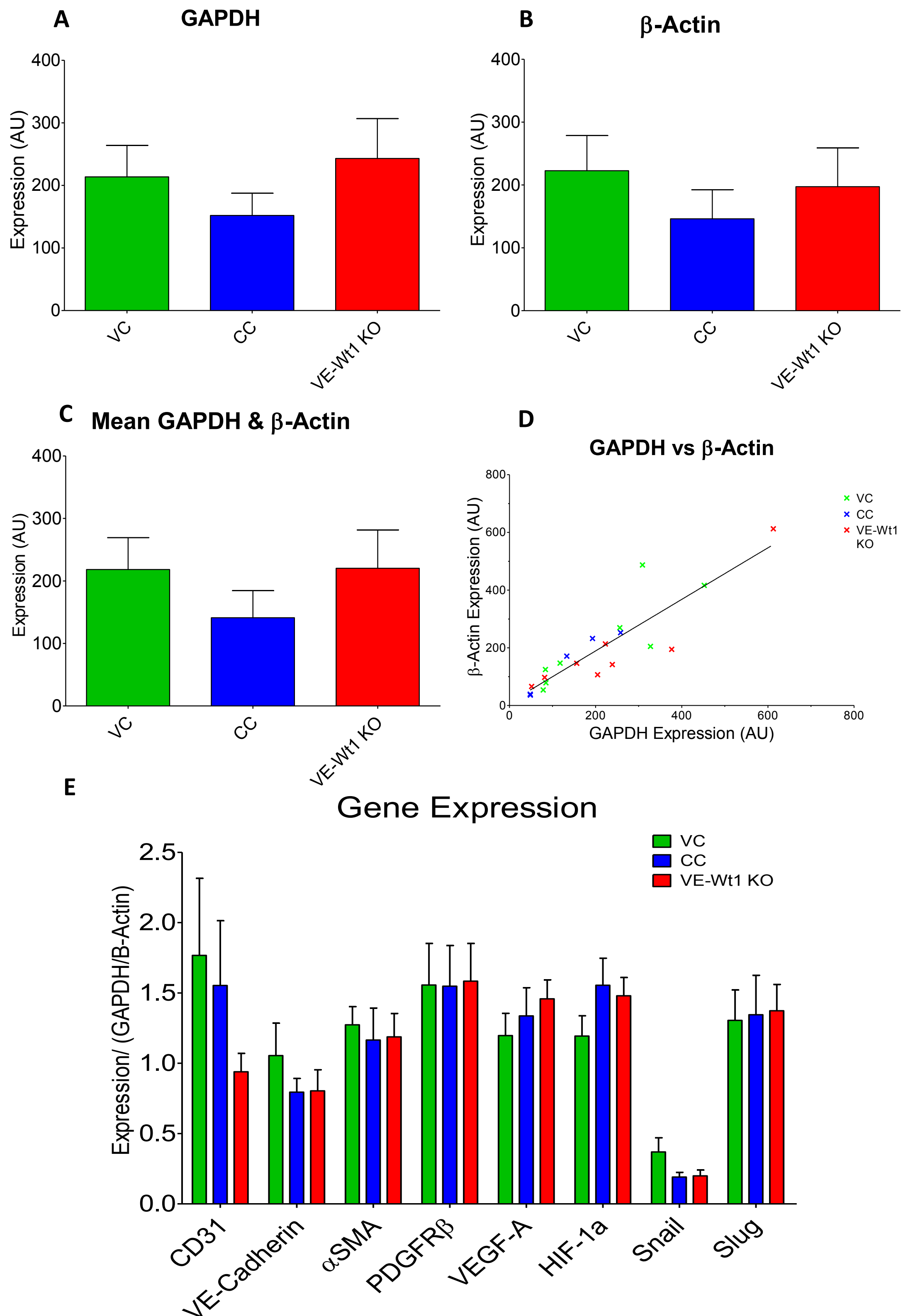

Figure 4.3.14: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) does not affect global gene expression of vascular, angiogenic or EMT genes. Global gene expression was assessed by RT-qPCR in RNA extracted from sponge tissue from vehicle control (VC), *Cre* control (CC) and VE-Wt1 KO mice. β-Actin and GAPDH were chosen as housekeeper genes as **A, B, C)** expression did not differ significantly between groups and **D)** there was a good correlation between their expression (linear regression, $p<0.0001$). All other genes were normalised to β-Actin and GAPDH

expression. **E)** Expression of CD31, αSMA, VE-Cadherin, PDGFRβ, HIF-1a, VEGF-A, Snail and Slug in VE-Wt1 KO, CC and VC sponge tissue was assessed by RT-qPCR. There was no difference between groups in expression of these. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data mean +/- s.e. mean. P>0.05, unpaired Student's t-test and 1-way ANOVA with Bonferroni post-hoc tests. n=8.

4.3.5 The Hindlimb Ischaemia (HLI) Model of Angiogenesis

Having examined WT1 in a simple model of *in vivo* angiogenesis, the hindlimb ischaemia (HLI) model was employed as a model which simulates critical limb ischaemia seen in humans(Limbourg *et al.*, 2009). This allowed study of the role of WT1 *in vivo* in a more pathophysiological model of cardiovascular disease involving ischaemia and angiogenesis. In C57Bl/6 mice, initial characterisation of this model was carried out prior to examining the role of WT1 in these disease conditions.

4.3.5.1 Blood Flow Reperfusion Following Femoral Artery Ligation

Following induction of HLI by proximal and distal ligation of a length of femoral artery (Section 2.7), partial ablation of arterial blood flow, alongside a degree of vascular remodelling was visible at day 28 post-ligation (Figure 4.3.15). Assessment of hindlimb blood flow by laser Doppler revealed almost complete ablation of blood flow in the distal hindlimb immediately following ligation, with a degree of reactive hyperaemia in the non-surgical limb (Figure 4.3.16 A & B). However, laser Doppler visualisation from day 3-28 showed a steady and substantial reperfusion in the surgical hindlimb over time.

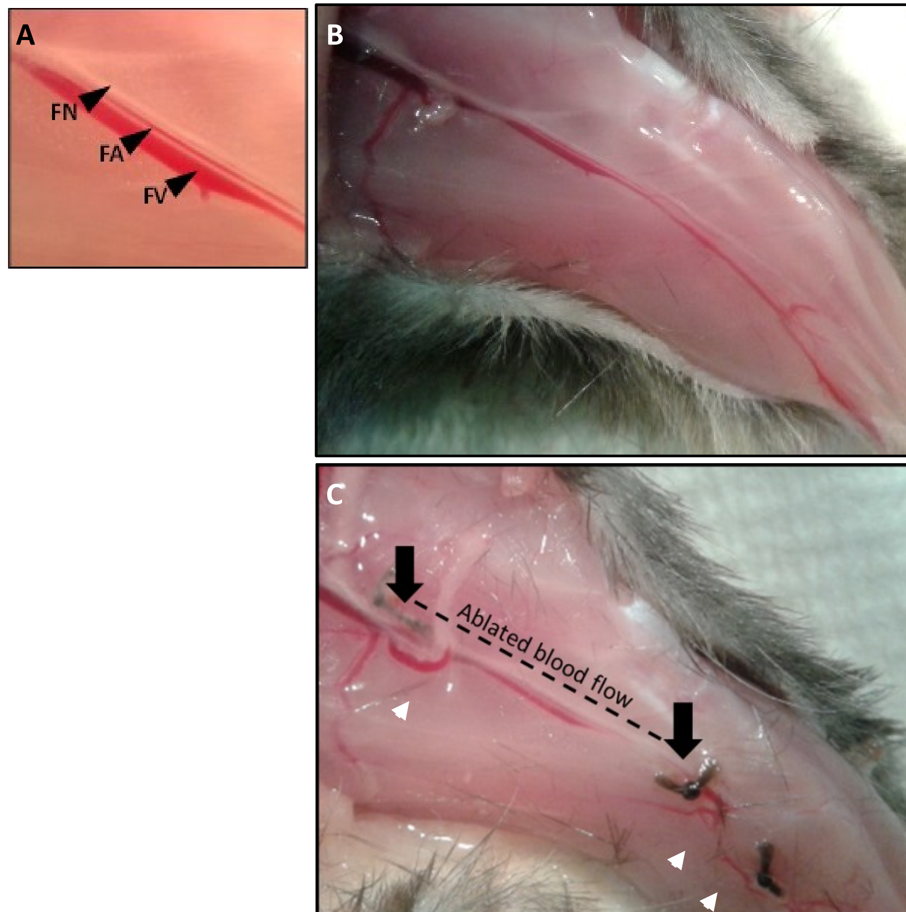

Figure 4.3.15: Vascular architecture in the surgical (ischaemic) and non-surgical (non-ischaemic) hindlimb. Images of surgical (ischaemic) and non-surgical (non-ischaemic) hindlimb at day 28 in the HLI model in C57Bl/6 mice. **A)** Normal vascular architecture. FN = femoral nerve, FA = femoral artery, FV = femoral vein. **B)** Non-ischaemic hindlimb at day 28. **C)** Ischaemic hindlimb at day 28. Ligatures applied to the femoral artery are visible, with the section of femoral artery in which blood flow was ablated indicated (dashed line). A degree of vascular remodelling is visible in the areas close to the ligature, with perfusion of small blood vessels not evident in the non-surgical limb (indicated by white arrows).

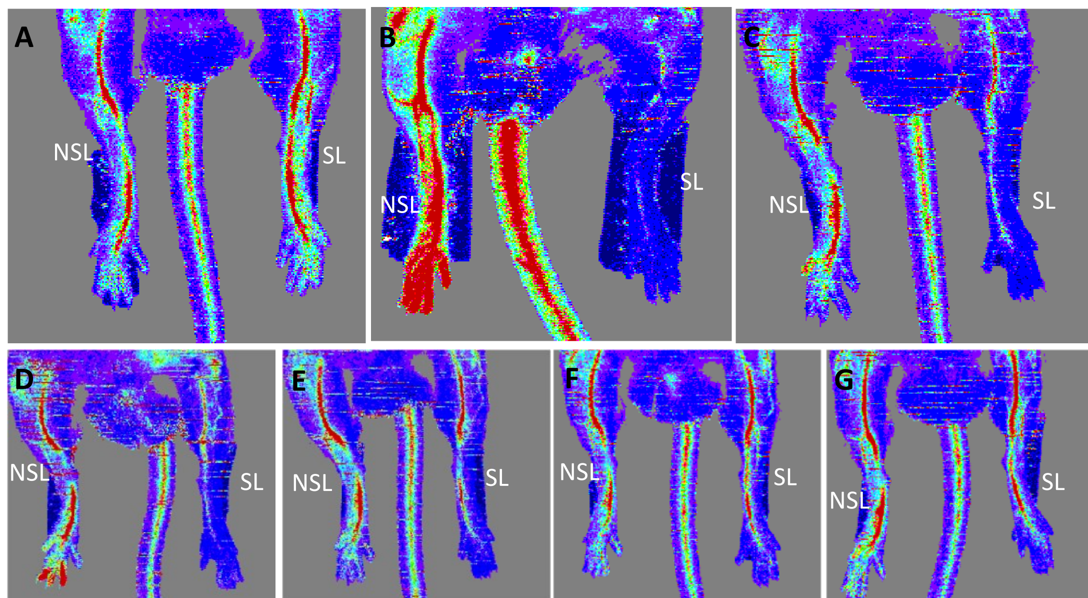

Figure 4.3.16: Reperfusion in the HLI model. Visualisation of blood flow in the C57Bl/6 hindlimb over time, on a colourimetric scale, where red is high blood flow and blue is low blood flow. Images were generated by laser Doppler imaging of murine hindlimbs over the course of recovery from hindlimb ischaemia surgery. **A)** day 0, pre-op, **B)** day 0, post-op, **C)** day 3, post-Op, **D)** day 7, post-op, **E)** day 14, post-op, **F)** day 21 post-op and **G)** day 28, post-op. SL = surgical limb, NSL = surgical limb.

Quantification of laser Doppler images using MoorLDI software confirmed a significant reduction in blood flow in the ischaemic hindlimb, immediately following femoral artery ligation ($p<0.05$) (Figure 4.3.17). In the ischaemic hindlimb, a biphasic reperfusion response was evident, with an initial relatively rapid recovery of blood flow between day 0 and day 7 and a slower recovery of blood flow from day 21 onwards. In the non-ischaemic hindlimb, there was a small, but non-significant increase in blood flow immediately following femoral artery ligation, which decreased by day 3 and remained relatively constant from day 3 to day 7.

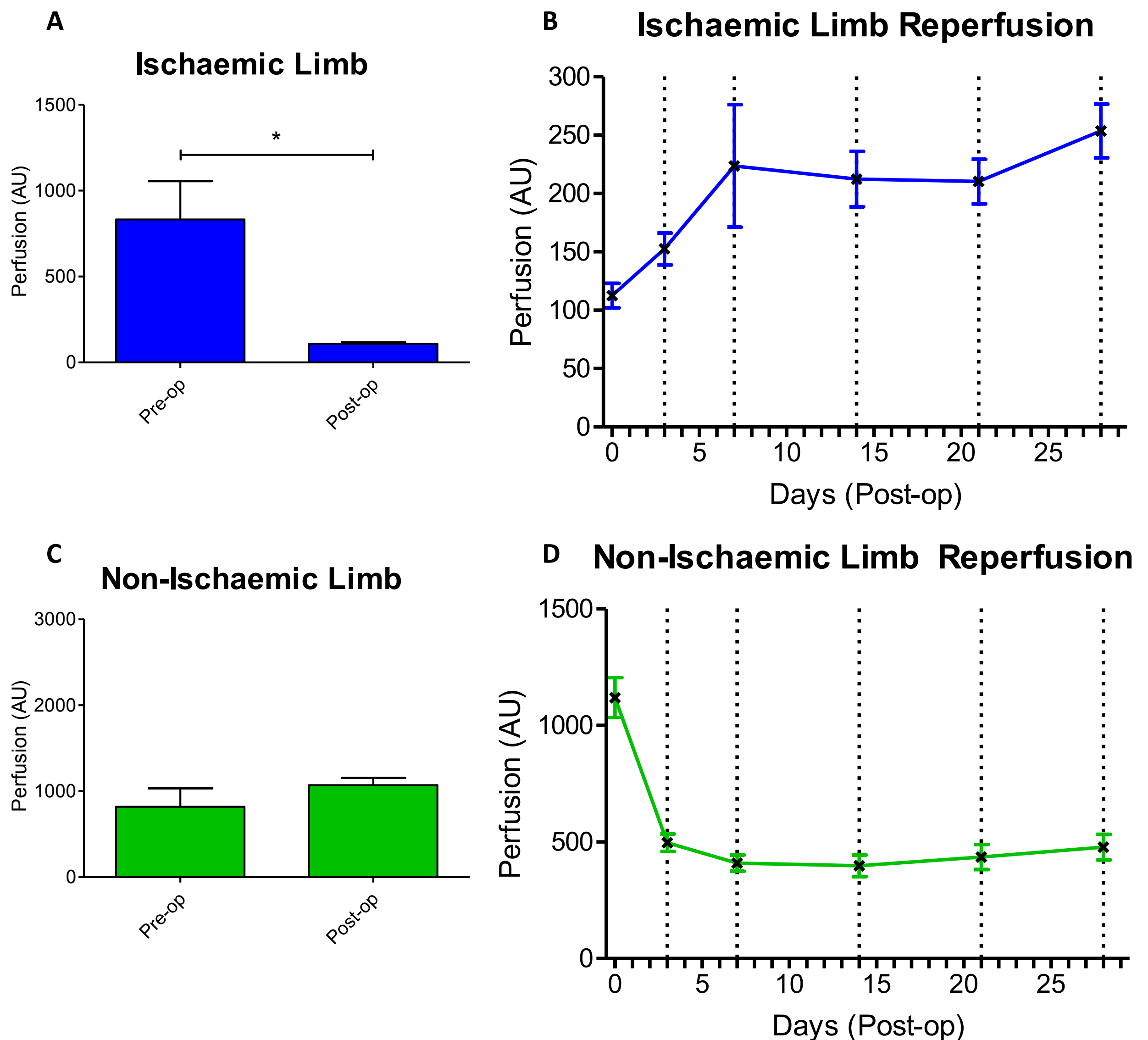

Figure 4.3.17: Femoral artery ligation in the HLI model results in a rapid decrease in hindlimb blood flow, followed by a biphasic reperfusion response. Quantification of laser Doppler images from HLI model immediately pre-op, immediately post-op and at days 3, 7, 14, 21 and 28 post-op. **A)** pre-op and post-op blood flow in the ischaemic (surgical) hindlimb, showing a significant reduction in blood flow immediately post-op (unpaired t-test). **B)** Blood flow quantified up to 28 days post-op in the ischaemic hindlimb, showing a biphasic reperfusion response. **C)** pre-op and post-op blood flow in the non-ischaemic (control) hindlimb. **D)** Blood flow quantified up to 28 days post-op in the non-ischaemic hindlimb, showing blood flow decreased and remained steady from day 3. All data mean +/- s.e. mean. *p<0.05, unpaired Student's t-test. n=8.

4.3.6 **WT1 Expression in the HLI Model**

Femoral artery ligation to induce HLI was carried out in C57Bl/6 mice and tissues were harvested at different time points in order to examine the role of WT1 in the response of normal, healthy mice to ischaemia.

4.3.6.1 **Vascular WT1 Expression Following Induction of Ischaemia**

C57Bl/6 HLI ischaemic and non-ischaemic gastrocnemius was isolated at day 3, day 7 and day 28 post-op. Isolectin-B4/WT1/DAPI immunofluorescent staining was carried out on these tissues to visualise vascular WT1 expression. As Isolectin-B4 stains endothelial cells, it is possible to identify arteries and veins as large luminal structures with an inner Isolectin-B4$^+$ layer. Capillaries are smaller, Isolectin-B4$^+$ luminal structures. As it is not consistently possible to distinguish capillaries from other, non-vascular Isolectin-B4$^+$ cells WT1 expression was initially examined in arteries and veins.

In gastrocnemius from non-operated C57Bl/6 mice, there was no evident WT1staining in vessel associated cells. However, at day 3, day 7 and day 28 following femoral artery ligation, the majority of Isolectin-B4$^+$ vessels had no WT1$^+$ cells associated with them, though there were vessels with Isolectin-B4$^+$/WT1$^+$ and Isolectin-B4$^-$/WT1$^+$ cells. Vessels were also visible with only Isolectin-B4$^+$/WT1$^+$ or only Isolectin-B4$^-$/WT1$^+$ cells (Figure 4.3.18 A-H). The localisation of vascular associated Isolectin-B4$^-$/WT1$^+$ cells external to the endothelial layer suggests they are either smooth muscle or perivascular cells. In a few cases, there were Wt1$^+$ cells associated with the vasculature external to the endothelial layer which were Isolectin-B4$^+$ and had a morphology suggestive of some type of immune cell (Figure 4.3.18 F).

Single WT1 staining in the same tissues confirmed that there were no WT1$^+$ vascular cells in completely uninjured, C57Bl/6 gastrocnemius, in ischaemic gastrocnemius, vessels with WT1$^+$ endothelial cells and vessels without any WT1$^+$ cells were present at day 3, day 7 and Day28 (Figure 4.3.18 I-O). In these tissues, visualisation of WT1 by both methods suggested there were more WT1$^+$ vessels at day 3 and day 7 in comparison to at day 28, though this

was not quantified due to the relatively small proportion of vessels with WT1 expressing cells. All WT1 staining was nuclear and in a granular pattern, similar to that observed in sponges (Section 4.3.2).

In C57Bl/6 ischaemic gastrocnemius $WT1^+$ and $WT1^-$ vascular endothelial cells and the vessels they comprised often had noticeably different morphologies, as revealed by Isolectin-B4/αSMA/WT1/DAPI. In Figure 4.3.19, two examples are shown. Here, in comparison to $WT1^-$ cells (Figure 4.3.19 B, C), $WT1^+$ endothelial cells (Figure 4.3.19 D, E) have a more cuboidal shape, as opposed to their normal squamous morphology and are seemingly more numerous and tightly packed. There is also an interesting Isolectin-$B4^-$ $WT1^+$ cell of unknown identity within the lumen of the vessel, integrated into the endothelial layer. Further, the vessel itself, exhibits disruption of the endothelial and smooth muscle layer (also seen in Figure 4.3.18 C, F), with Isolectin-$B4^+$ cells passing though and integrated into the $αSMA^+$ layer.

Apelin staining was carried out following previous observations that the expression of Apelin in vascular cells may denote these vessels are undergoing angiogenesis and distinguish them from non-angiogenic vessel in the same tissue (Liu *et al.*, 2015). This was carried out in sections of gastrocnemius taken 3, 7 or 28 days following femoral artery ligation and from non-ischaemic, non-operated controls (Figure 4.3.20). However, the staining showed Apelin in the majority of vessels in both ischaemic and non-ischaemic tissue and stained a large number of non-vascular cells, including the majority of skeletal myocyte nuclei. It was therefore decided that it was not a useful marker of angiogenesis and further co-staining for Apelin and WT1 was not carried out.

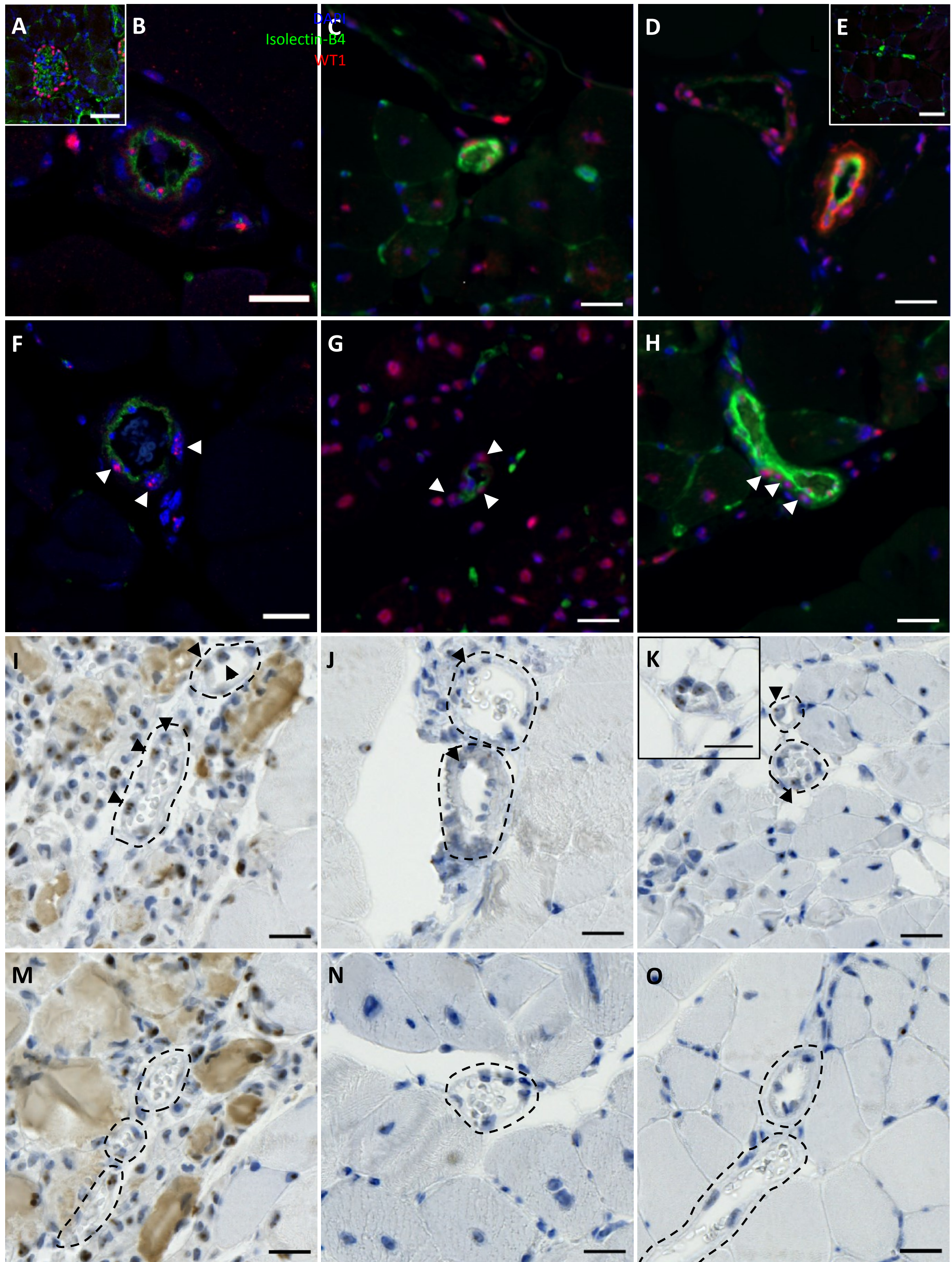

Figure 4.3.18: WT1 is expressed in endothelial and non-endothelial cells of vessels in ischaemic gastrocnemius *in vivo*. IHC revealed that, in the HLI model, WT1 was expressed in a proportion of vessels *in vivo* in the ischaemic gastrocnemius. Representative images of Isolectin-β4 (green), WT1 (red) and DAPI (blue) staining in **A)** positive control kidney tissue and C57Bl/6 ischaemic gastrocnemius at **B, F)** day 3, **C, G)** day 7 and **D, H)** Day 28 (white arrows show non-endothelial, vascular WT1^{+}

cell nuclei). **E)** Completely uninjured C57Bl/6 gastrocnemius. Representative WT1 IP IHC images from C57Bl/6 ischaemic gastrocnemius at **I)** day 3, **J)** day 7 and **K, L)** day 28 showing WT1^{+} vessels and (dashed lines) and WT1^{+} endothelial cells (examples indicated with arrows) and at **M)** day 3, **N)** day 7 and **O)** day 28 showing WT1^{-} vessels (dashed lines). Scale bars = 50µm. n=3-4.

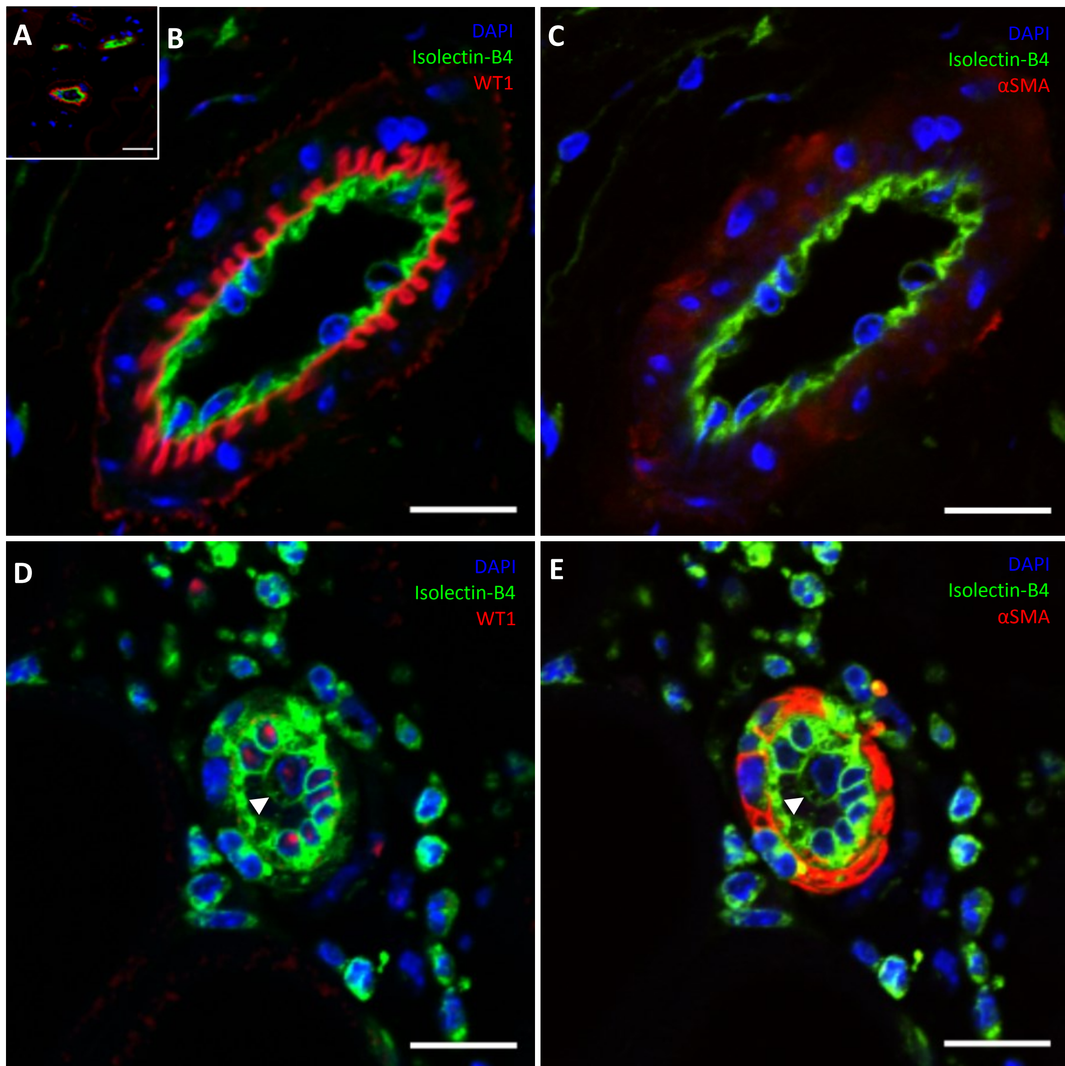

Figure 4.3.19: WT1^{+} vessels are morphologically distinct from WT1^{-} vessels. IF IHC for Isolectin-B4/αSMA/WT1/DAPI in C57Bl/6 ischaemic gastrocnemius. **A)** Negative control with IgG antibody (red) instead of anti-WT1 antibody demonstrating background WT1 staining of endothelial basement membrane of large vessels. Representative image of a WT1^{-} vessel stained for DAPI (blue), Isolectin-B4 (green) and **B)** WT1 (red) or **C)** αSMA (red). Representative image of a WT1^{+} vessel from the same tissue stained for DAPI (blue), Isolectin-B4 (green) and **D)** WT1 (red) or **E)** αSMA (red). Unidentified WT1^{+} cell indicated by arrow. Scale bars = 50µm. n=3-4.

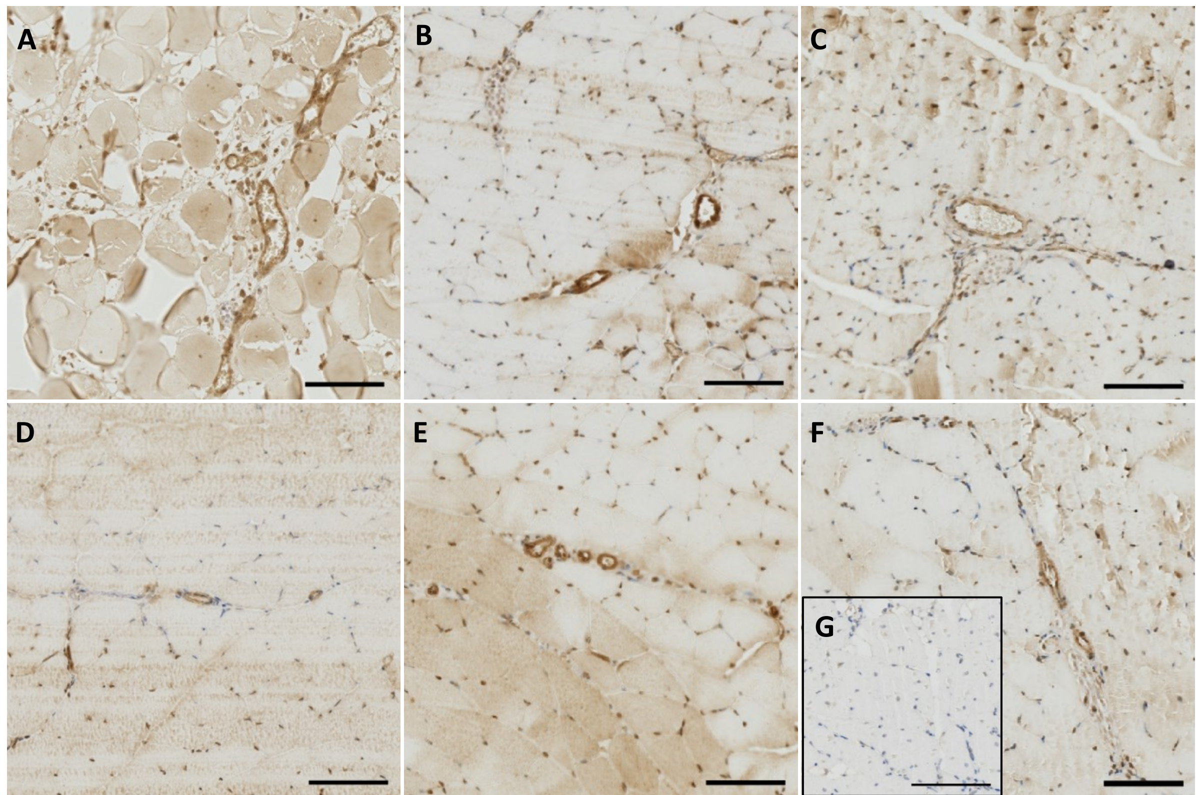

Figure 4.3.20: Apelin staining in C57Bl/6 ischaemic and non-ischaemic gastrocnemius. Apelin stains the majority of vessels in ischaemic and non-ischaemic tissue and a number of other cell types. Apelin IP IHC staining in C57Bl/6 ischaemic gastrocnemius at **A)** day 3, **B)** day 7, **C)** day 28 and non-ischaemic gastrocnemius at **D)** day 3, **E)** day 7, **F)** day 28. **G)** Negative control with IgG antibody instead of anti-Apelin antibody. Scale bars = 100μm. n=3-4.

4.3.6.2 Non-Vascular WT1 Expression Following Induction of Ischaemia

While there was vascular endothelial and non-endothelial WT1 expression, the majority of WT1 expression in ischaemia was observed in cells not visibly associated with vascular structures. In completely uninjured, C57Bl/6 gastrocnemius there were non-vascular $WT1^+$ nuclei (Figure 4.3.21A). Quantification of immunofluorescent staining of ischaemic and contralateral non-ischaemic gastrocnemius revealed that WT1 expression was elevated at day 3 post-op in ischaemic tissue, in comparison to ($p<0.01$, one-way ANOVA with Bonferroni post-hoc tests vs uninjured non-operated gastrocnemius) (Figure 4.3.21 I). WT1 expression at day 7 and day 28 was not significantly higher than expression in uninjured, non-operated gastrocnemius. WT1

expression in the contralateral, non-ischaemic gastrocnemius was not significantly higher than uninjured at day 3, day7 or day 28 (Figure 4.3.21 H).

However, Isolectin-B4/WT1/DAPI immunofluorescent staining revealed that while the highest levels of tissue WT1 expression in ischaemic gastrocnemius was at day 3, there was still visibly elevated WT1 expression in ischaemic gastrocnemius at day 7 and day 28 in relation to uninjured gastrocnemius. At day 3 there was infiltration of a large number of Isolectin-B4$^+$ cells into the interstitial space between skeletal myocytes. These persisted to some extent at day 7, but only a small population was still present by day 28. Many of these Isolectin-B4$^+$ cells, as well as some Isolectin-B4$^-$ interstitial cells expressed WT1 (Figure 4.3.21 B-D).

At day 3, day 7 and day 28 skeletal myocytes were found to express nuclear WT1 (4.3.21 E-G). Nuclear WT1 was almost always expressed in smaller myocytes with at least one (often multiple) central nuclei and to a lesser extent in larger myocytes with peripheral nuclei. These WT1$^+$ myocytes with central nuclei occurred in distinct patches. Within these areas, there was an evident increase in the Isolectin-B4$^+$ capillaries and larger vessels and this vasculature frequently had WT1$^+$ endothelial cells.

Undamaged skeletal muscle has a resident population of satellite cells which are activated in damaged muscle to facilitate repair (Wang and Rudnicki, 2011; Berberoglu *et al.*, 2017). Cells expressing WT1 were co-stained with Pax7 (a marker of satellite cells) in completely uninjured gastrocnemius and gastrocnemius at day 7 post-op (Figure 4.3.22). The anti-Pax7 antibody produced a high degree of background staining, particularly in injured muscle, but lack of background in the negative control suggests this is likely due to off target anti-Pax7 antibody binding. As previously described, there was sporadic nuclear WT1 staining in uninjured gastrocnemius muscle tissue and it appeared that the majority of these WT1$^+$ nuclei co-expressed Pax7. At day 7 in the HLI model damaged myofibres with central nuclei appeared to express WT1 and Pax7 simultaneously.

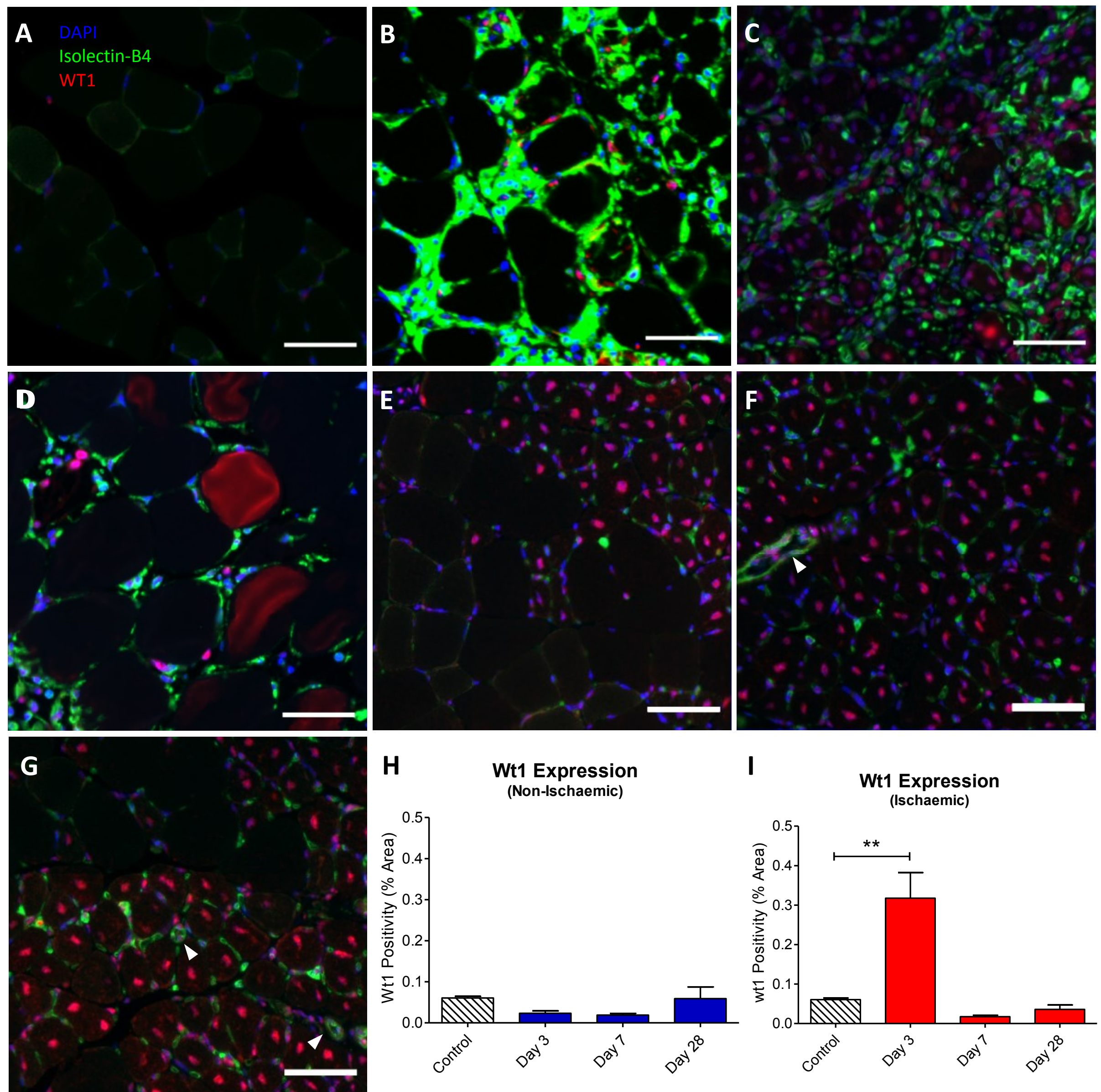

Figure 4.3.21: WT1 is expressed in infiltrating cells and skeletal myocytes in ischaemia. WT1 is expressed in a number of non-vascular cells in ischaemic muscle tissue, in a time dependent manner. Isolectin-B4/WT1/DAPI immunofluorescent staining in C57Bl/6 gastrocnemius in **A)** completely uninjured tissue and in HLI at **B, E)** day 3, **C, F)** day 7 and **D, G)** Day 28 following femoral artery ligation. WT1 is expressed in infiltrating Isolectin-B4^{+} cells, endothelial and non-endothelial vascular cells and skeletal muscle with central nuclei. Arrows indicate examples of vessels expressing WT1. Quantification of WT1 staining by percentage area over time at different time points in comparison to control (completely uninjured) tissue in **H)** the non-ischaemic contralateral limb and **I)** ischaemic gastrocnemius. **$p<0.05$, unpaired t-test. Scale bars = 50µm. n=3-4.

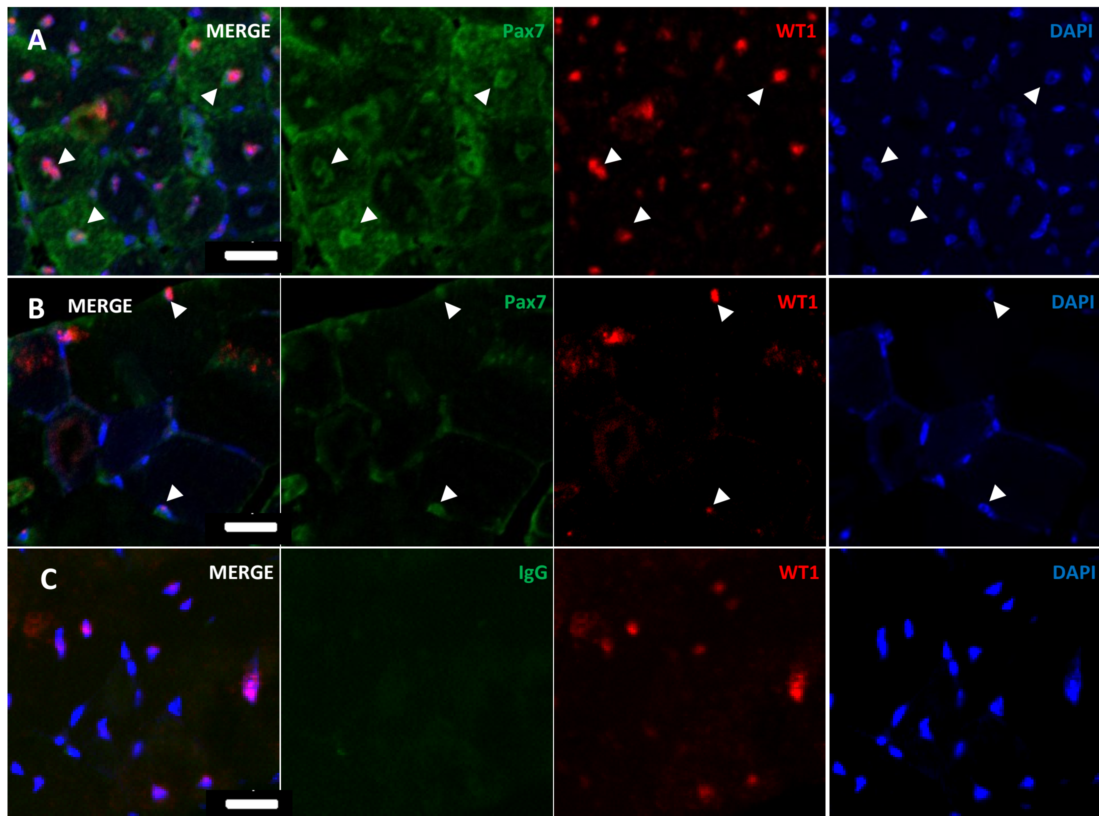

Figure 4.3.22: WT1 and Pax7 appear to partially overlap in preliminary IHC. Immunostaining for WT1 (red), Pax7 (green) and DAPI (blue) in **A)** C57Bl/6 gastrocnemius Day 7 post HLI and **B)** uninjured gastrocnemius. WT1/Pax7 co-expression indicated with arrows. **C)** Pax7 negative control. Scale bars= 20µm. n=3-4.

4.3.6.3 WT1 Co-expresses with Snail in the HLI model

IF IHC was carried out in ischaemic gastrocnemius from C57Bl/6 28 days following femoral artery ligation to examine the extent to which WT1 was co-expressed with VEGFR2, PCNA and Snail. There was found to be no overlap between WT1 and VEGFR2 expression which were both expressed discretely in nuclei of different cells (Figure 4.3.23 A-C). However, there was some co-expression of WT1 and PCNA which are both expressed in cell nuclei, though WT1+/PCNA- and $PCNA^+/WT1^-$ nuclei were also present (Figure 4.3.23 D-F). As expected, anti-Snail staining was predominately cytoplasmic and, while there were $Snail^+/WT1^-$ cells, almost all WT1+ cells also expressed Snail (Figure 4.3.23 G-I). The identity of $WT1^+/PCNA^+$ and $WT1^+/Snail^+$ cells was not identified; however these cells were not evidently associated with local vasculature.

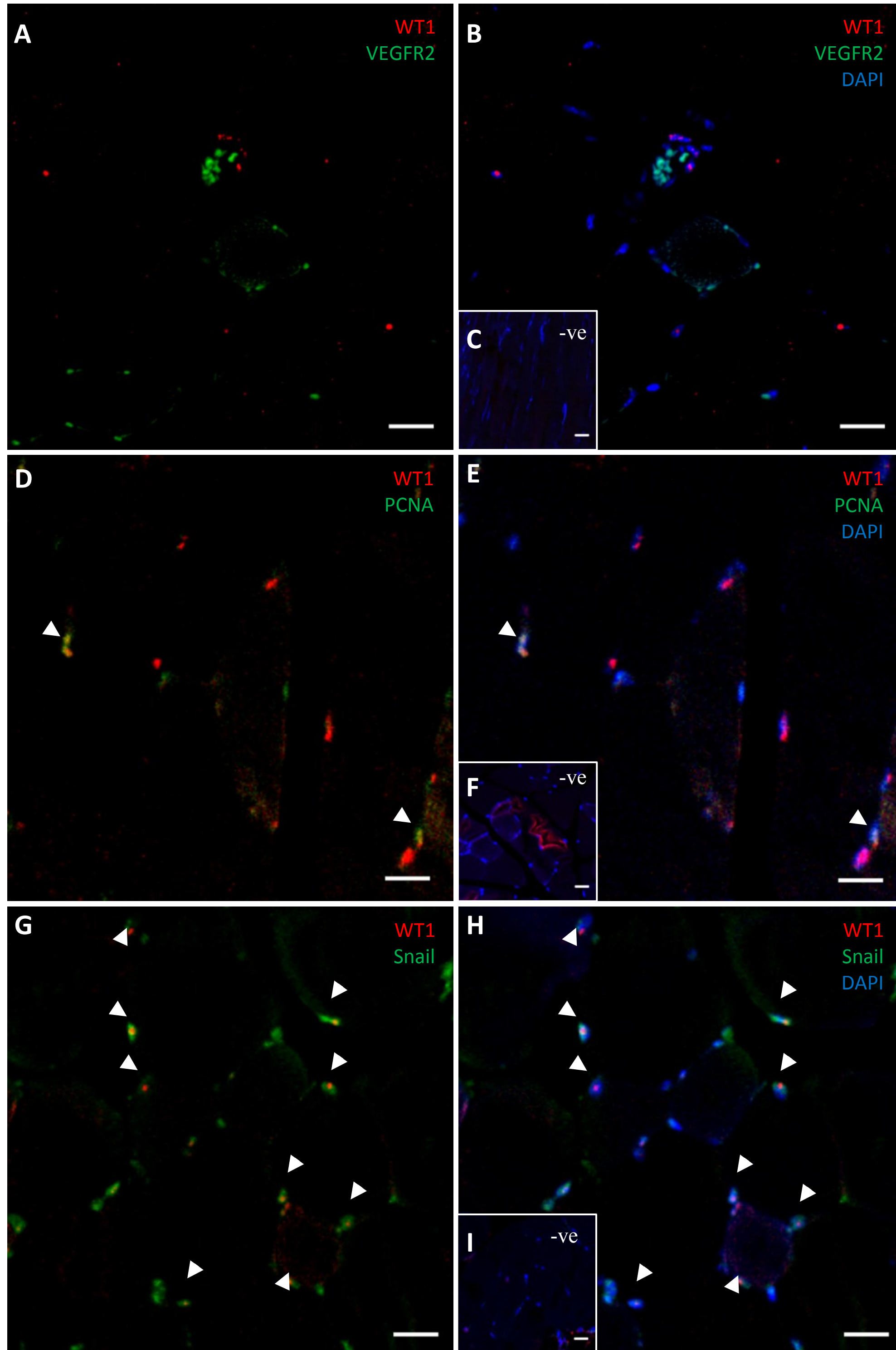

Figure 4.3.23: WT1 co-expresses with snail and to a lesser extent PCNA, but not with VEGFR2. IF IHC staining for WT1 (red), DAPI (blue) and **A, B)** VEGFR2 (green), **D, E)** PCNA (green) and **G, H)** Snail (green). Arrows indicate co-staining. Negative controls for anti-WT1 and **C)** anti-VEGFR2, **F)** anti-PCNA and **I)** anti-Snail. Scale bars = 50µm. n=3.

4.3.7 **VE-Wt1 KO in the HLI Model**

Having observed increased WT1 expression in ischaemic tissue following induction of HLI, including in endothelial cells of the ischaemic vasculature (Section 4.3.6), the effect of WT1 in angiogenesis and other processes of relevance was investigated. In order to examine the role of endothelial WT1, the VE-Wt1 KO mouse described previously, was subjected to HLI and tissues harvested after 28 days were assessed for any effect of VE-Wt1 KO.

4.3.7.1 **VE-Wt1 KO has no Effect on Reperfusion Following Femoral Artery Ligation**

Femoral artery ligation resulted in visible hyperaemia in the ischaemic hindlimb and reactive hyperaemia in the non-ischaemic hindlimb in all groups (VC, CC & VE-Wt1 KO) (Figure 4.3.24). Pre-op blood flow in both limbs was not the same between groups. As seen by laser Doppler imaging, over 28 days progressive, incremental reperfusion occurred in the ischaemic hindlimb, similarly to the response seen following HLI in C57Bl/6 mice (4.3.5). There was no visible difference in the extent or pattern of reperfusion between groups.

Quantification of these laser Doppler images revealed ablation of blood flow in the ischaemic hindlimb and increase in blood flow in the non-ischaemic hindlimb following femoral artery ligation. HLI surgery was as effective in VC, CC and VE-Wt1 KO and there was no difference in pre- or post-op blood flow between groups. Blood flow in the non-ischaemic limb had recovered to approximately pre-op levels by day 7 in all groups and remained constant. There was no difference in non-ischaemic hindlimb blood flow over 28 days between groups.

Quantification of reperfusion in the ischaemic hindlimb revealed that VE-Wt1 KO perfusion was not lower at any time points, though there was a trend towards reduced blood flow at day 7 in the VE-Wt1 KO. There was no difference in the perfusion of the ischaemic hindlimb between VC, CC or VE-Wt1 KO at any time quantified over the 28 days post-op during HLI.

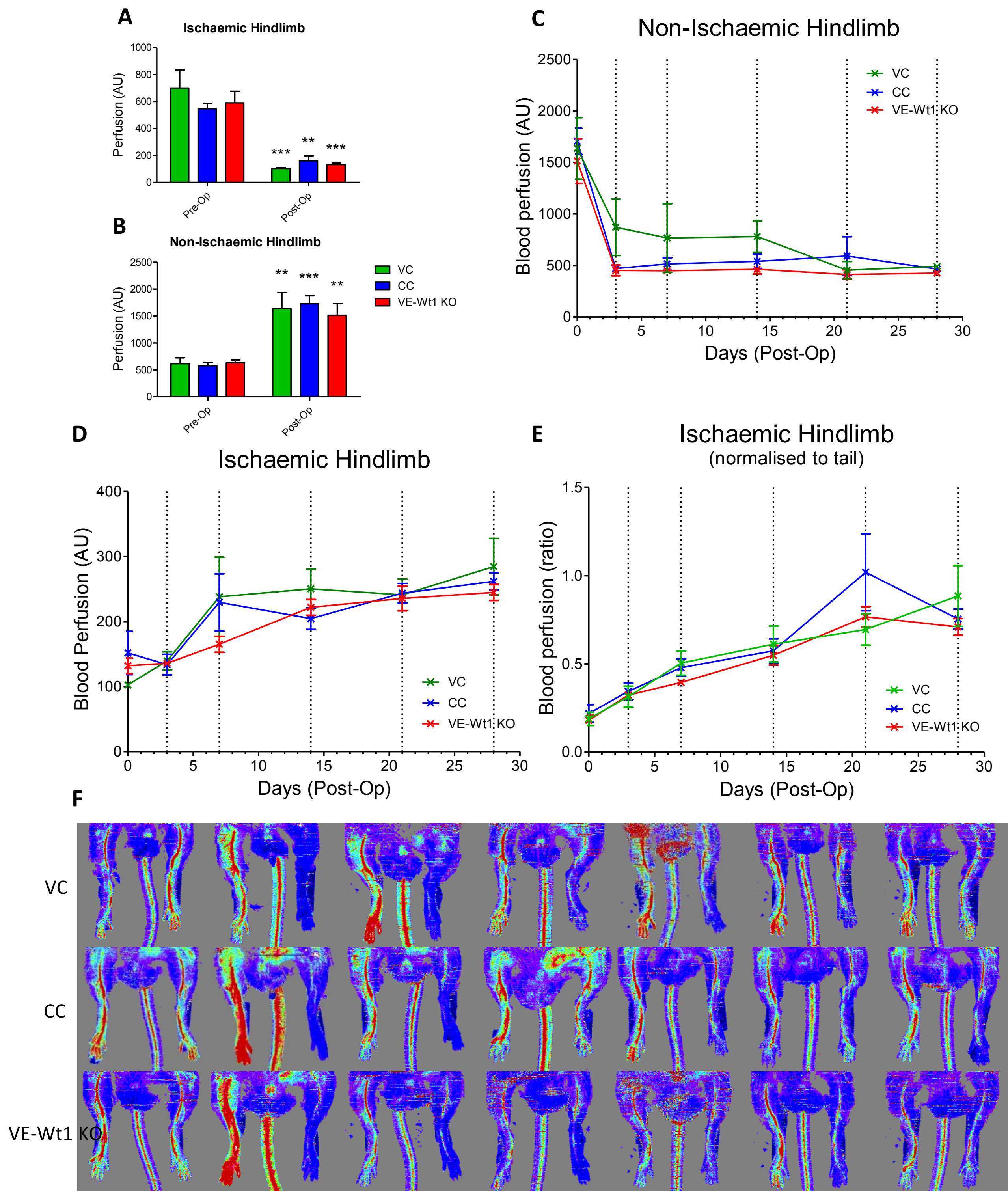

Figure 4.3.24: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) has no effect on hindlimb reperfusion following femoral artery ligation. Visualisation and quantification of laser Doppler images from the HLI model showed no difference between vehicle control (VC), *Cre* control (CC) and VE-Wt1 KO in hindlimb reperfusion. Quantification of hindlimb perfusion immediately pre- and post-op in **A)** the ischaemic and **B)** non-ischaemic hindlimb. Quantification of perfusion in the **C)** non-ischaemic and **D)** ischaemic hindlimb and **E)** ischaemic perfusion normalised to

tail perfusion. **F)** Representative laser Doppler images (from left to right) immediately pre- and post- op, at day 3, 7, 14, 21 and 28. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. **p<0.005, ***p<0.0005, pre-op v post-op perfusion, paired Student's t-test. n=8.

4.3.7.2 VE-Wt1 KO has no Effect on Vessel Density at Day 28 in HLI

Hindlimb reperfusion in the HLI model is a result of angiogenesis and arteriogenesis. In order to isolate the effect of VE-Wt1 KO on angiogenesis, gastrocnemius muscle from HLI day 28 was isolated and vascularisation was quantified from histological staining of blood vessels.

CD31 IP IHC effectively stained arteries, veins and capillaries in all groups (Figure 4.3.25 A-C). Quantification of CD31 expression by percentage area of staining in ischaemic gastrocnemius revealed significantly less CD31 expression in VE-Wt1 KO than VC, but not CC, which had slightly reduced CD31 expression in comparison to VC (Figure 4.3.25 D). This is suggestive of a *Cre* effect. However, there was no significant difference in $CD31^+$ vessel density between groups (Figure 4.3.25 E). VE-Cadherin IP IHC appeared to primarily stain larger vessel rather than capillaries (Figure 4.3.25 G-I). It was not practical to assess the extent of VE-Cadherin staining due to high background staining. Quantification of VE-$Cadherin^+$ vessel density showed no significant difference between groups.

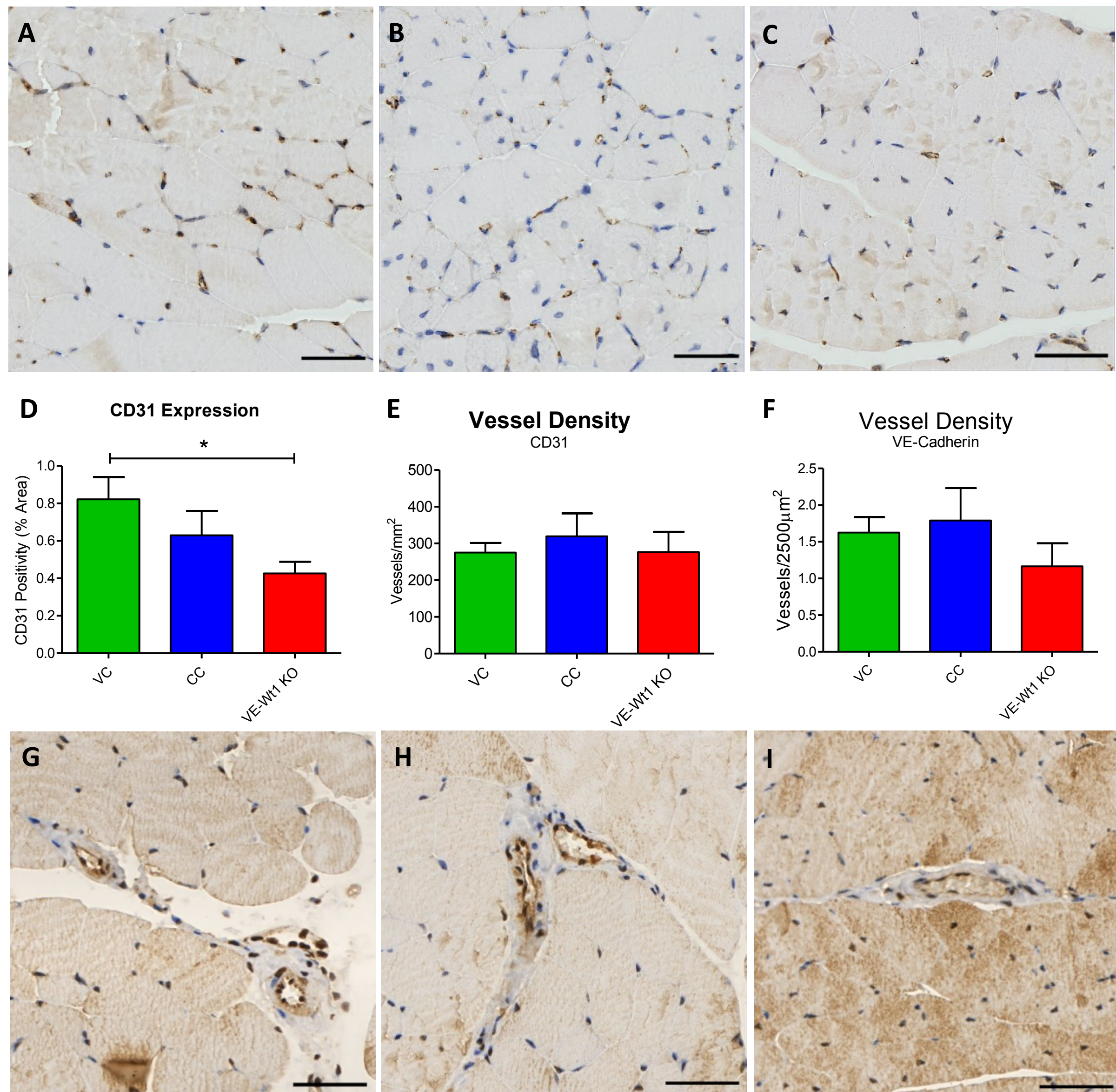

Figure 4.3.25: Vascular endothelial *Wt1* knock-out VE-Wt1 KO had no effect on vessel density in HLI at day 28. Representative images of CD31 IP IHC in **A)** vehicle control (VC), **B)** *Cre* control (CC) and **C)** VE-Wt1 KO. **D)** Quantification of CD31 expression (% area). *p<0.05, unpaired t-test, VC v VE-Wt1 KO. **E)** Quantification of CD31$^+$ vessel density. **F)** Quantification of VE-Cadherin expression (% area). Representative images of VE-Cadherin IP IHC in **A)** VC, **B)** CC and **C)** VE-Wt1 KO. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. *p<0.05 1-way ANOVA with Bonferroni post-hoc tests. Scale bars = 50μm. n=8.

4.3.7.3 VE-Wt1 KO has no Effect on Whole Tissue WT1 Expression in HLI at Day 28

As previously shown (Sections 4.3.6.1 & 4.3.6.2), endothelial WT1 expression makes up a small proportion of WT1 expression in the ischaemic hindlimb in

HLI. Concurrently, quantification of WT1 staining in ischaemic gastrocnemius at HLI day 28 revealed that deletion of WT1 from the endothelium did not reduce the total WT1 staining or the number of WT1 positive cells in the gastrocnemius (Figure 4.3.26).

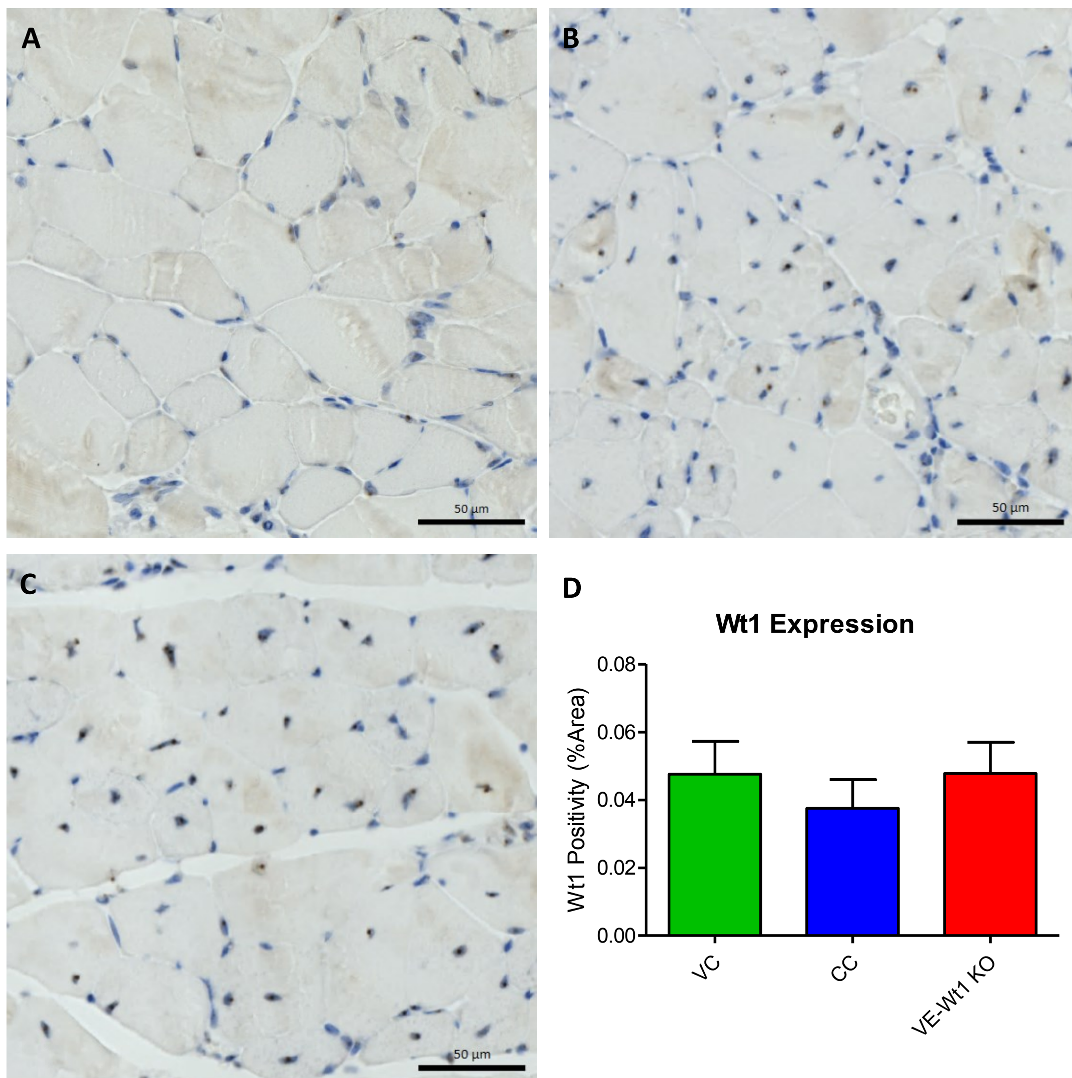

Figure 4.3.26: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) had no effect on total WT1 expression in HLI at day 28. Representative images of WT1 IP IHC in **A)** vehicle control (VC), **B)** *Cre* control (CC) and **C)** VE-Wt1 KO. **D)** Quantification of WT1 staining (% area) in VC, CC and VE-Wt1 KO. Nuclear WT1 staining is visible as brown nuclei. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. Scale bars = 50µm. n=8.

4.3.7.4 VE-Wt1 KO has no Effect on Collagen Deposition in HLI

PSR staining in ischaemic gastrocnemius at HLI day 28 was carried out to visualise fibrosis in the HLI model. PSR staining revealed collagen localised primarily between myofibres (Figure 4.3.27 A-C). VE-Wt1 KO tissues had significantly less collagen than VC tissues and appeared to have less than CC, though this did not reach significance (Figure 4.3.27 D). As seen with CD31 (4.3.7.2), this is again suggestive of a *Cre* effect in this model.

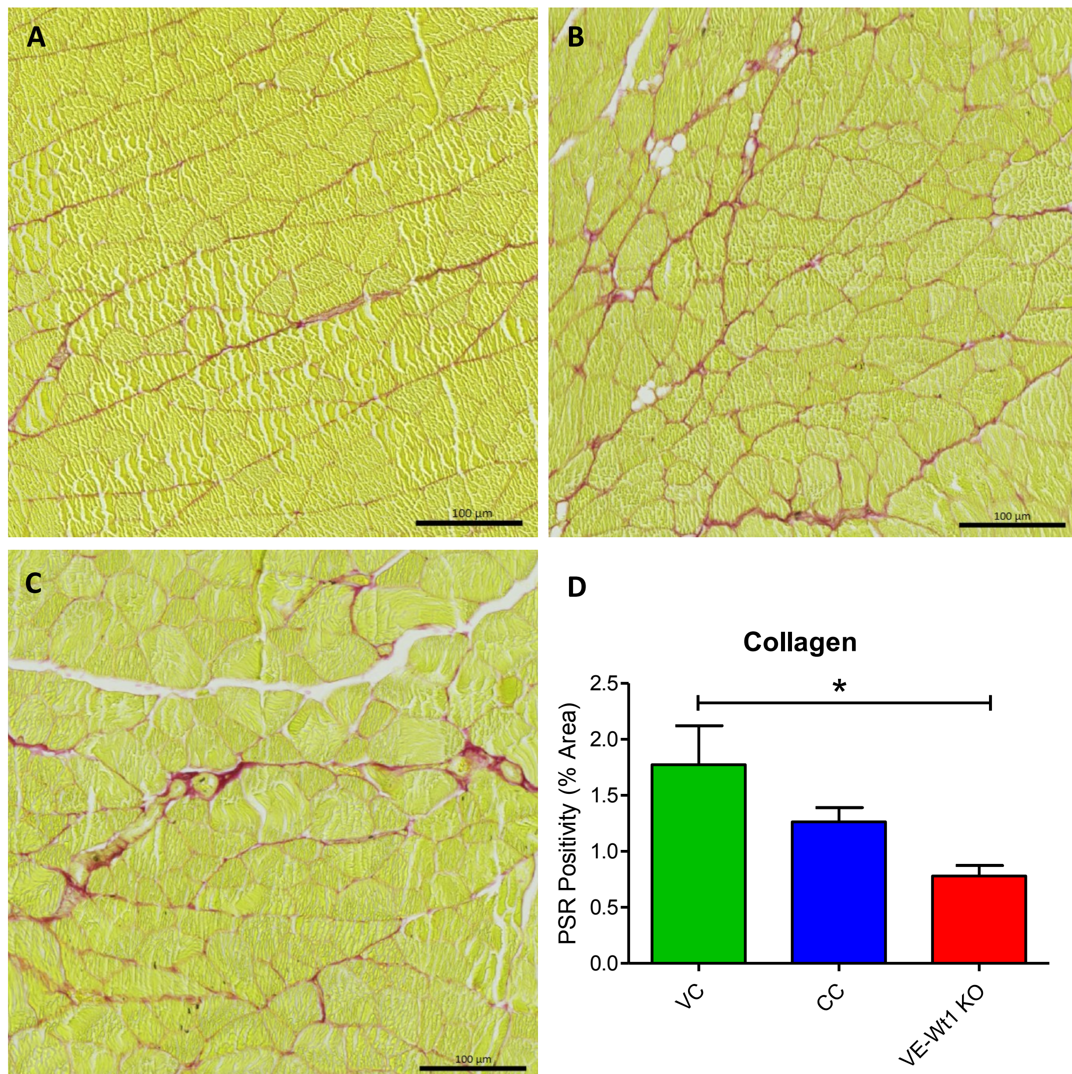

Figure 4.3.27: Vascular endothelial *Wt1* knock-out VE-Wt1 KO had no effect on collagen in HLI at day 28. Representative images of PSR staining in **A)** vehicle control (VC), **B)** *Cre* control (CC) and **C)** VE-Wt1 KO. **D)** Quantification of PSR staining (% area) in VC, CC and VE-Wt1 KO. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. *p<0.05, 1-way ANOVA with Bonferroni post-hoc tests. Scale bars = 100µm. n=8.

4.3.7.4 VE-Wt1 KO has no Effect on Macrophage Density in HLI

F4.80 staining in ischaemic gastrocnemius at HLI day 28 was carried out to visualise macrophages in the HLI model. There was very little F4.80 positivity in any samples, due to a very low density of macrophages at day 28 (Figure 4.3.28 A-C). There was no significant difference in macrophage density between groups, as quantified by percentage area of F4.80 staining (Figure 4.3.28 D).

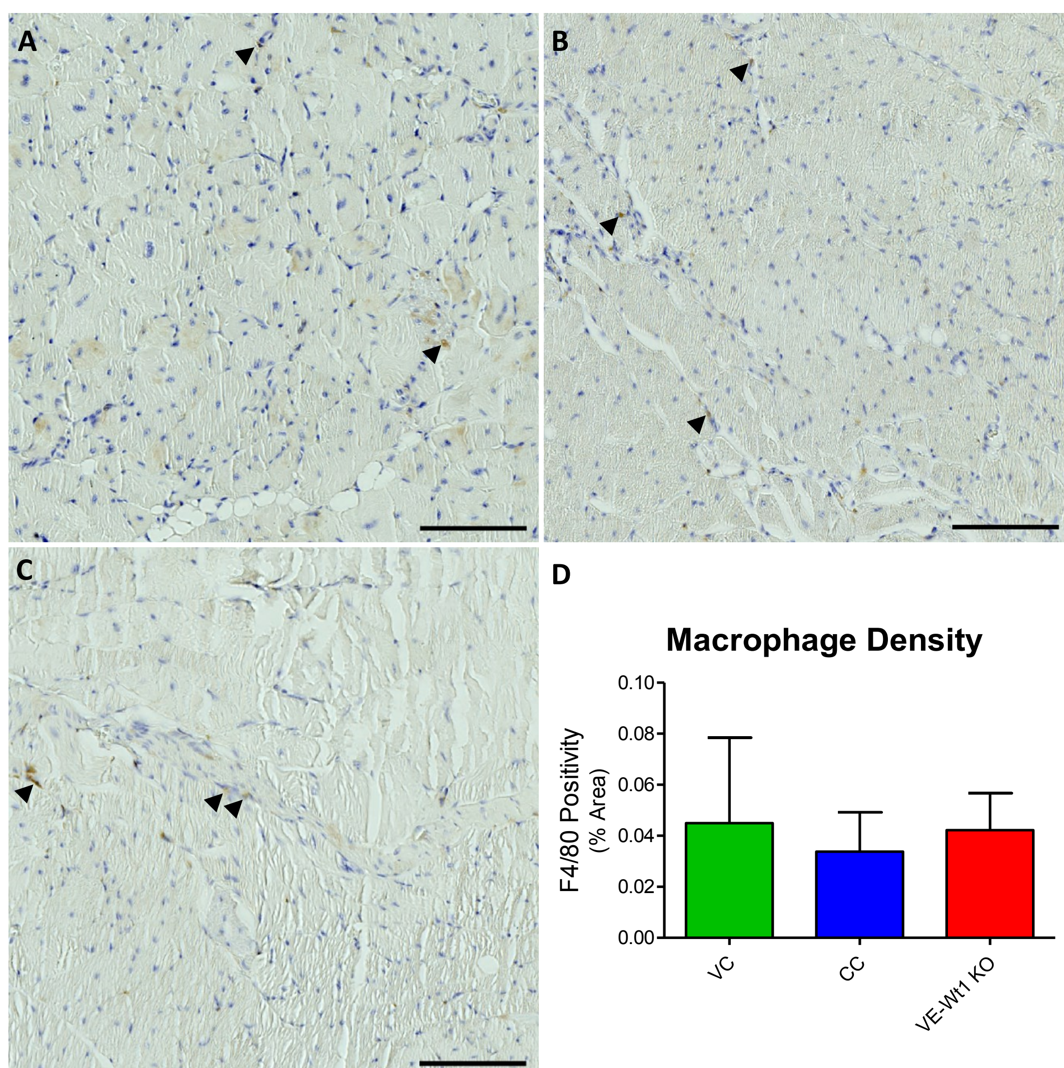

Figure 4.3.28: Vascular endothelial *Wt1* knock-out VE-Wt1 KO had no effect on macrophage density in HLI at day 28. Representative images of F4.80 staining in **A)** vehivle control (VC), **B)** *Cre* control (CC) and **C)** VE-Wt1 KO. **D)** Quantification of F4.80 staining (% area) in VC, CC and VE-Wt1 KO. Representative F4.80^{+} macrophages indicated with arrows. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. P>0.05, 1-way ANOVA with Bonferroni post-hoc tests. Scale bars = 100µm. n=6.

4.3.7.5 VE-Wt1 KO has no Effect on Muscle Damage in HLI

While no significant difference was found between VE-Wt1 KO and CC in any parameters assessed, it is possible that VE-Wt1 KO had a detrimental effect on the response to damage in HLI and repair. To quantify this, general muscle structure was visualised by H&E and assessed. As seen in C57Bl/6 muscle in HLI (Section 4.3.6.2), there were visible areas of damaged muscle. Random regions of interest were selected, the muscle damage in those areas was scored on a semi-quantitative scale (Figure 4.3.29A) and the mean cross-sectional myofibre area was measured (Figure 4.3.29B). Neither of these parameters were different between groups.

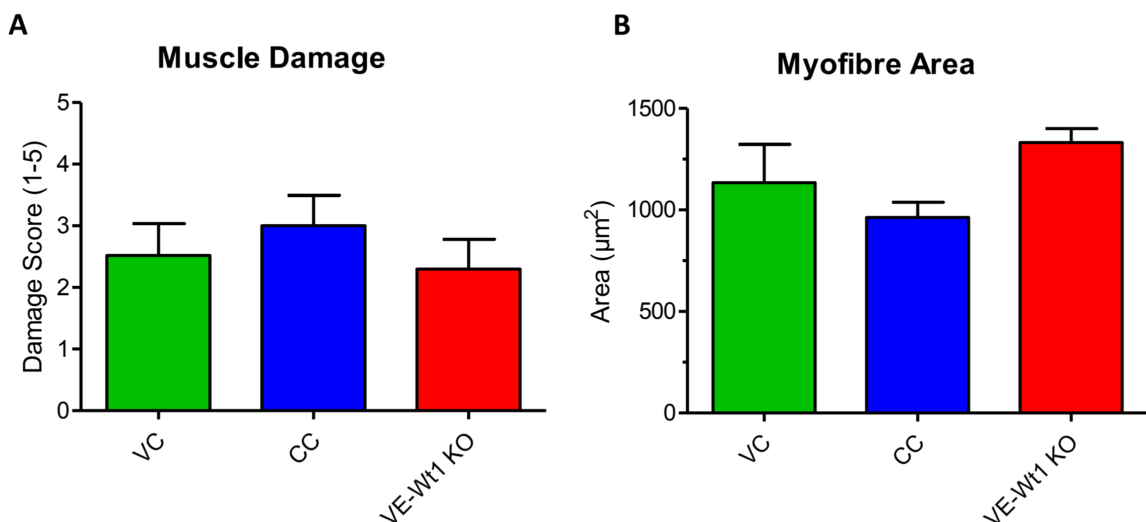

Figure 4.3.29: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) had no effect on skeletal muscle damage in HLI at day 28. Muscle damage was quantified in the ischaemic gastrocnemius from VE-Wt1 KO, vehicle control (VC) and *Cre* control (CC) mice, 28 days after femoral artery ligation. **A)** H&E images were quantified on a semi-quantitative scale for muscle damage. **B)** The area of myofibres was quantified. There was no difference in either of these parameters between groups. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. All data means +/- s.e. mean. P>0.05, 1-way ANOVA with Bonferroni post-hoc tests. n=8.

4.3.7.6 **VE-Wt1 KO Has No Effect on Vascular, Angiogenic or EMT Gene Expression in HLI**

To assess potential changes in gene expression following VE-Wt1 KO, ischaemic muscle tissue was extracted and gene expression was quantified by RT-qPCR for selected vascular cell markers (CD31, αSMA), genes associated with angiogenesis (HIF-1a, VEGF-A) and genes associated with EMT (Snail, Slug). First, 'housekeeper' genes were selected, to which the expression of all other genes were normalised. β-Actin and GAPDH were selected, as their expression did not differ significantly between groups and there was a significant correlation between their expression values ($p<0.0001$, linear regression) (Figure 4.3.30 A-D).

After normalising to β-Actin and GAPDH, expression of CD31, αSMA, HIF-1a, VEGF-A, Snail and Slug did not significantly differ between VE-Wt1 KO, VC and CC tissue (Figure 4.3.30 E). RT-qPCR was carried out for VE-Cadherin and PDGFR-β, but for unknown reasons, unlike in sponges (Section 4.3.4.4 & Figure 4.3.14), it was not possible to effectively detect expression levels of these genes.

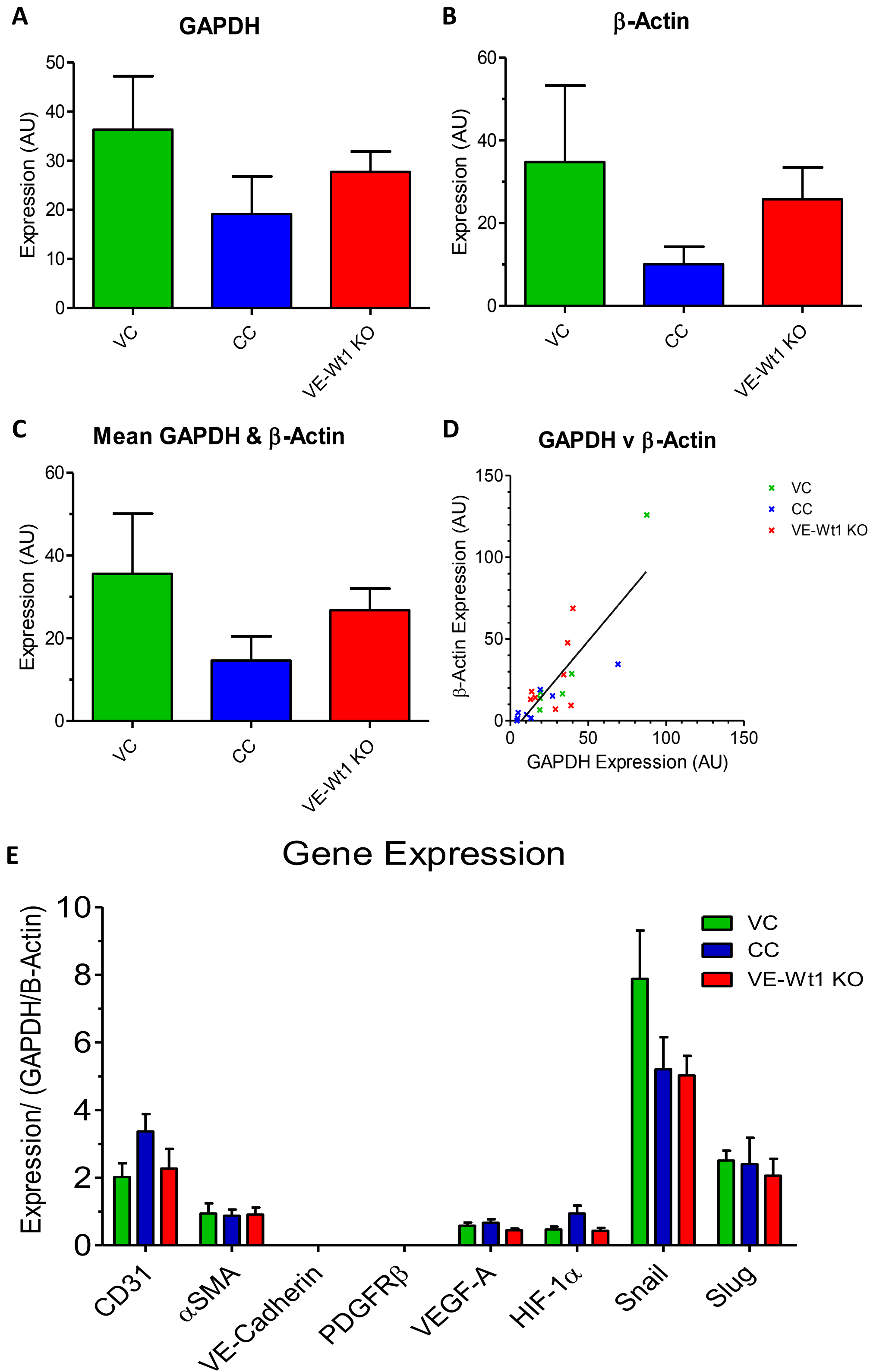

Figure 4.3.30: Vascular endothelial *Wt1* knock-out (VE-Wt1 KO) does not affect global gene expression of vascular, angiogenic or EMT genes in HLI. Global gene expression was assessed by RT-qPCR in extracted ischaemic gastrocnemius tissue at day 28. β-Actin and GAPDH were chosen as housekeeper genes as **A, B, C)** expression of all genes did not differ significantly between groups and **D)** there was a good correlation between their expression (non-linear regression ($p<0.05$)). All other genes were normalised to β-Actin and GAPDH expression. **E)** Expression of

CD31, αSMA, VE-Cadherin, PDGFRβ, HIF-1a, VEGF-A, Snail and Slug in VE-Wt1 KO, CC and VC muscle tissue as assessed by RT-qPCR. There was no difference between groups in expression of these. VC = vehicle control, CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. n=8.

4.4 Discussion

The experiments in this chapter were designed to investigate the role of WT1, specifically in the vascular endothelium, in angiogenesis *in vivo*. This was addressed using the subcutaneous sponge implantation model, a model of *in vivo* angiogenesis, and hindlimb ischaemia, a murine model of critical limb ischaemia, which stimulates pathophysiological ischaemic angiogenesis. This is the first time WT1 has been investigated in the context of critical limb ischaemia. Staining for WT1 in these models confirmed that WT1 was activated in endothelial cells and VSMCs of the vasculature, suggesting WT1 may be involved in angiogenesis. However, KO of WT1 selectively in the vascular endothelium had no effect on angiogenesis *in vivo*. Despite this, endothelial cell-selective WT1 KO did have some unexpected effects on macrophage infiltration (and general cell infiltration) into sponges. Furthermore, considerable non-vascular expression of WT1 was identified in ischaemic tissues, demonstrating that WT1 has a wider function than just in the vascular endothelium during tissue repair.

The original hypothesis for this chapter was that WT1 regulates angiogenesis in endothelial cells and hence, VE-Wt1 KO will impair *in vivo* angiogenesis. These experiments show VE-Wt1 KO does not significantly impair angiogenesis *in vivo*, though they do provide further novel and intriguing insights into the role of WT1 in angiogenesis and beyond.

4.4.1 Endothelial cell-selective *Wt1* KO does not impair Angiogenesis *In Vivo*

The potential for WT1 as an important factor in cardiovascular disease is a relatively new concept and is not completely understood. It is clear that WT1 is key to tumour angiogenesis, and in cardiovascular disease it has been shown that WT1 is expressed in ischaemic angiogenic tissue following induction of MI (K. Wagner *et al.*, 2002; Duim *et al.*, 2015). In the previous chapter it was demonstrated that vascular endothelial cell-selective *Wt1* KO impairs *ex vivo* angiogenesis; however, in this chapter, there was no evidence that endothelial cell-selective *Wt1* KO significantly affects angiogenesis *in vivo*. In the subcutaneous sponge implantation model, endothelial cell *Wt1* KO

had no effect on vessel infiltration into the sponges in samples provided by our collaborators in France, or in sampLes generated from experiments conducted in Edinburgh. In the hindlimb ischaemia model, endothelial cell *Wt1* KO had no effect on reperfusion of ischaemic tissue (as measured by laser Doppler) or on vessel density, with no difference in tissue damage. This suggests that either WT1 is not important in *in vivo* angiogenesis, or that endothelial cell *Wt1* KO was not sufficient to produce a physiologically relevant inhibition of angiogenesis.

It is worth noting that there was a significant reduction in CD31 expression in the HLI model in comparison to the vehicle control, but not the *Cre* control which had an intermediate level of expression. This is indicative of a potential *Cre* effect *in vivo*. *Cre* is known to have potentially toxic effects, even when it is inducible; as in the *VE-Cadherin* $CreER^{T2}$ used here (Loonstra *et al.*, 2001; Hameyer *et al.*, 2007; Naiche and Papaioannou, 2007). However, as these experiments have a *Cre* and a vehicle control this should not adversely impact the interpretability of the results. The fact that there is a potential small difference in CD31 staining, but not in vessel density, could be due to endothelial cell *Wt1* KO reducing the number of endothelial cells, rather than fully-formed vessels; for example by affecting non-vascular endothelial cells. In fact, Wt1 has been shown to regulate endothelial cell apoptosis (Morrison, English and Licht, 2005; Ito *et al.*, 2006; Lv *et al.*, 2015) and proliferation *in vitro* and *in vivo* (Wagner *et al.*, 2008; Duim *et al.*, 2015), disruption of which could reduce endothelial cell number. Concurrently, as has been previously demonstrated in murine MI, Wt1 co-expressed with PCNA in a proportion of $Wt1^+$ cells in HLI (K. Wagner *et al.*, 2002).

The reduction in CD31 expression with endothelial cell *Wt1* KO described above was not observed when *CD31* gene expression was quantified by qPCR. In fact, there was no difference in any of the markers (*CD31*, *VE-Cadherin*, *Snail*, *Slug*, *VEGF-A* and *HIF1α)* measured by qPCR in either *in vivo* model. If WT1 was playing a role in angiogenesis, it might have been expected that expression of CD31 and VE-Cadherin would have reduced in the whole tissue; however, no difference in *in vivo* angiogenesis was observed, so this is understandable. Given previous work showing that *Wt1* has a role in

regulating, markers of EMT *Snail* and *Slug* (Martínez-estrada *et al.*, 2010; Braitsch *et al.*, 2013; Takeichi *et al.*, 2013) and markers of angiogenesis *VEGF* and *HIF-1* (Wagner *et al.*, 2003; Graham *et al.*, 2006), it would be expected that expression of these markers would drop in endothelial cells following *Wt1* expression. However, as endothelial cells represent a relatively small proportion of the total cells in the sponges or gastrocnemius muscle, it is not surprising that no overall drop in expression of these markers was observed. This could be confirmed by FACs sorting for endothelial cells and then assessing gene expression in these *Wt1* KO endothelial cells to determine which genes and, hence, which processes are affected by endothelial cell *Wt1* KO.

Quantification of blood vessel density in these models was performed a relatively long time (21 days in sponges and 28 days in HLI) after induction of the initial surgery. Furthermore, reperfusion in the HLI model is attributable to both arteriogenesis and angiogenesis and cannot be considered a direct measurement of angiogenesis. Initial reperfusion is primarily attributable to arteriogenesis, with angiogenesis then contributing to reperfusion measurable by laser Doppler (Wu *et al*. unpublished data). Endothelial cell *Wt1* KO in the aortic ring model, presented in the previous chapter, showed that deletion of WT1 from the endothelium had a relatively acute effect on angiogenesis *ex vivo*. Therefore, it is possible that, similarly, endothelial cell *Wt1* KO acutely impairs angiogenesis but that this acts only to delay new vessel formation, or has a relatively minor effect on overall angiogenesis. Angiogenesis could be quantified in these models at earlier time points; however, it is not practical to quantify vessel density immediately following sponge implantation or femoral artery ligation due to the presence of very few quantifiable, competent vessels. It would perhaps be more effective, if attempting to confirm a role for WT1 in angiogenesis to use a broader vascular *Wt1* KO in endothelial cells, mural cells and vascular-associated inflammatory cells simultaneously to determine if this results in a measurable inhibition of *in vivo* angiogenesis. It would be particularly interesting to determine if complete vascular *Wt1* KO completely inhibits angiogenesis or not, hence revealing whether Wt1 is essential to angiogenesis.

Previous work, in cancer in particular, has shown that endothelial WT1 is intrinsic to angiogenesis. Endothelial cell-selective *Wt1* deletion was sufficient to impair tumour angiogenesis, considerably reducing vascular density in tumours and, hence, reduce their growth, or even inducing regression (Wagner *et al.*, 2014). Preliminary work with sponges from the endothelial cell *Wt1* KO mouse from our collaborators in France showed a roughly 8 fold reduction in vessel density in these sponges in the endothelial *Wt1* KO mouse (McGregor *et al.* 2014). However, this was not replicated by analysis of these samples in Edinburgh, or by experiments with endothelial cell *Wt1* KO mouse (which should, theoretically be genetically identical) performed in Edinburgh. The reason for the discrepancy between these results and previous work is unclear, however, it does question the extent to which endothelial cells are the main players in *Wt1*-regulated angiogenesis. This was evident in that the effect of *Wt1* KO on tumour angiogenesis was dramatically greater when it was affected under the control of *Tie2 Cre*, which is also expressed in some haematopoietic and immune cells, as opposed to the more endothelial cell-selective *VE-Cadherin Cre*, which is also used in the experiments in this chapter. The role of WT1 in endothelial and non-endothelial cells was also revealed by visualisation of WT1 in these models by histology.

4.4.2 **WT1 Does Have a Role in Angiogenic Vasculature**

WT1 expression was only assessed in sponges 21 days post-implantation, whereas in ischaemic gastrocnemius it was assessed at 3, 7 and 28 days post-induction. Therefore, the majority of observations relate to the role of WT1 in the more pathophysiologically-relevant HLI model. Analysis of WT1 expression in both the sponge and hindlimb ischaemia models revealed that WT1 was expressed in the vasculature. This is of particular relevance as previous work from our group has shown that WT1 is not expressed in healthy adult tissue in the vasculature or other cells, except for specific cell populations (McGregor *et al.* 2014). Therefore, WT1 expression must have been reactivated in vascular cells in these contexts and associated with a specific vascular response. Indeed, in the case of the sponge model, all vessels present in the sponges after implantation must have arisen by angiogenesis. The majority of endothelial and vascular WT1 observed was expressed acutely

after induction of ischaemia in HLI, with vascular WT1 decreasing over time. It would not be unreasonable to assume that WT1 is acutely activated in angiogenic vessels to bring about cellular changes that facilitate initiation or progression of angiogenesis.

The concept of WT1 regulating initiation of angiogenesis is supported by the fact that, in some vessels with endothelial WT1 expression, there was clear disruption of the endothelial and VSMC layers, with what appeared to be endothelial cells migrating outwards. This is consistent with the cellular processes occurring early in angiogenesis as an endothelial vessel sprout is established (Carmeliet and Jain, 2011). It has been proposed that a partial EndMT is required in endothelial cells during angiogenesis to increase their migratory potential (Welch-Reardon *et al.*, 2014; Welch-Reardon, Wu and Hughes, 2015). Given the known role of WT1 in EMT (Miller-hodges and Hohenstein, 2012), it could well be that at least one function of WT1 in endothelial cells is to activate EndMT to enable initiation of angiogenesis. Supporting this hypothesis, endothelial cells expressing WT1 had an unusual columnar morphology, more characteristic of mesenchymal than endothelial cells (Mendez, Kojima and Goldman, 2010) and Wt1 was co-expressed with Snail in ischaemic gastrocnemius.

Within the vasculature, WT1 was expressed in endothelial cells, but also in VSMCs/pericytes to much the same extent. In a few cases WT1 was present in what appeared to be vessel-associated immune cells. It should be confirmed whether Wt1 is expressed in vessel-associated immune cells with the cell type determined by co-staining with different cell markers. At no time point observed were all vessels or all vascular endothelial cells WT1^{+}; however, in vessels which had some WT1 expression, Wt1 was often identified in numerous cells proximal to each other. This implies that WT1 is discretely activated by some local cues and that the activation of *Wt1* in one cell may bring about transcriptional changes that result in release of molecular signals from one cell which correspondingly activate *Wt1* in other proximal cells. The nature of the signals which may activate *Wt1* in this context are unknown, though in hypoxia HIF-1 is known to transcriptionally regulate *Wt1* (Wagner *et al.*, 2003).

4.4.3 The Pathophysiological Function of WT1 is Not Restricted to the Vasculature

While endothelial cell *Wt1* KO did not significantly impair angiogenesis, it did have some unexpected effects in other cell types. These effects were all in comparison to vehicle controls, but not to *Cre* controls, suggestive of a *Cre* effect. In sponges from France, endothelial cell *Wt1* KO resulted in an increased depth of cell infiltration into sponges, though this was not evident in sponges implanted in endothelial cell *Wt1* KO mice in Edinburgh. WT1 is known to influence cell migration, in endothelial and non-endothelial cells (Wagner *et al.*, 2008; Brett, Pandey and Fraizer, 2013b), though it is interesting that endothelial cell *Wt1* KO seemingly increased infiltration of non-endothelial cells as well, with increased depth of collagen and macrophage infiltration into sponges. As there was no increase in the density of collagen or macrophages, this is most likely attributable to increased cell infiltration rather than to fibrosis or inflammation.

Endothelial *Wt1* KO did, however, increase macrophage density in sponges from experiments performed in Edinburgh and reduced collagen density in the ischaemic gastrocnemius. The idea of Wt1 regulating fibrosis and inflammation is not an entirely new concept; for one thing, *Wt1* is activated in cells responsible for inflammation and fibrosis in a number of diseases (McGregor *et al.* 2014, Hastie 2017). WT1 is expressed in areas of fibrosis post-MI (Braitsch *et al.*, 2013), and EMT, which WT1 is known to regulate, often contributes to fibrosis in disease (Zeisberg *et al.*, 2007; Liu, 2010). In the context of inflammation, WT1 directly regulates a number of inflammatory factors (reviewed by Toska & Roberts 2014). Furthermore, staining revealed WT1 expression in inflammatory cells in the sponge and HLI models. The highest level of WT1 expression occurred three days after induction of ischaemia in gastrocnemius, which is in accord with an acute, substantial influx of inflammatory immune cells. Altered endothelial cell function and endo-MT can increase pathogenic fibrosis and inflammation in disease (Goumans, van Zonneveld and ten Dijke, 2008; Goligorsky, 2015), which may in part explain the changes as a result of endothelial *Wt1* KO. These findings are particularly novel and exciting as they suggest endothelial *Wt1* KO is sufficient to induce

changes in associated cells and that, therefore, WT1^{+} endothelial cells may ordinarily interact with mesenchymal cells and immune cells. In order to investigate this further, there are a number of fibrotic and inflammatory murine disease models which could be applied to the endothelial *WT* KO mouse model used herein.

Finally, and perhaps most-excitingly, in the HLI model we discovered an entirely new potential role for WT1 in cardiovascular diseases, which demands further investigation. Unexpectedly, most of the WT1 expression in the ischaemic gastrocnemius over time was in the nuclei of skeletal myocytes. Due to the induction of ischaemia by femoral artery ligation and consequent arteriogenesis, certain areas of gastrocnemius muscle experience a greater extent of ischaemia than others. Simultaneously, and presumably as a result of this heterogeneity, there appears to be more muscle damage in some areas than in others; as identifiable by smaller myocytes with central nuclei. In these areas of increased muscle damage, WT1 was strongly expressed in these myocytes, in comparison to relatively uninjured areas where expression was lower. Moreover, this expression persisted throughout recovery, suggesting the activation of *Wt1* in skeletal muscle is not just as a result of hypoxia, but is reflective of a role for WT1 in muscle repair following damage.

As this is a novel observation, there is little to give context to this finding; however, WT1 has recently been found to be strongly expressed in developing human skeletal muscle tissue (Parenti *et al.*, 2013; Magro *et al.*, 2015). This implicates WT1 in myogenesis; a recapitulation of which may well be occurring in skeletal muscle tissue as seen here. In the heart, a predominantly muscular organ, WT1 expression is maintained in the epicardium, which post-MI generates a small population of transiently WT1^{+} cardiomyocytes (van Wijk *et al.*, 2012; Braitsch *et al.*, 2013). Mirroring this situation, skeletal muscle also has its own resident stem cell population, the satellite cell, which along with signals from macrophages and fibroblasts, is able to induce much more extensive muscle repair than induced in the myocardium (reviewed by Wang & Rudnicki 2011). These stem cells are dispersed at low density throughout the myocardium. Intriguingly then, not only was WT1 expressed in muscle in ischaemia, but at very low density in uninjured tissue consistent with WT1

being expressed physiologically in satellite cells. Preliminary staining for the satellite cell marker Pax7 seemed to show that WT1 co-expressed with Pax7 in muscle in non-ischaemic and ischaemic tissue, though this needs to be optimised further. This leads to generation of a working hypothesis that WT1 is expressed in the skeletal muscle satellite cells and these are activated in damaged tissue, in which they contribute to WT1-regulated regenerative myogenesis.

This needs to be investigated further, initially by FACs on ischaemic and non-ischaemic muscle tissue to isolate satellite cells and to assess whether or not they express WT1. The role of WT1 in muscle repair could then be investigated *in vitro* with skeletal muscle cells and satellite cells, using a number of functional assays, then inhibiting *Wt1*. These findings, if promising, could then be moved into *in vivo* models of muscle injury. If WT1 is found to have a role, in muscle repair, it could be applied therapeutically in cardiovascular diseases, as well as in myopathies such as muscular dystrophy and myositis.

4.4.4 Conclusions

In conclusion, these investigations demonstrated that WT1 is induced during tissue repair *in vivo*, but that endothelial *Wt1* KO was not sufficient to significantly impair new blood vessel formation. In concordance with the results from examining Wt1 *in vitro*, WT1 was largely expressed acutely in vasculature and also had a role in non-endothelial vascular cells. All of this may explain why endothelial WT1 has no effect on angiogenesis and WT1-expressing non-endothelial cells may increase WT1 expression to compensate, or even transition into endothelial cells themselves. WT1 also appeared to have a role in cell migration, cell transition, fibrosis and inflammation *in vivo*; much of which could be explained if WT1 is regulating endMT. Intriguingly, WT1 appeared to be involved in regenerative myogenesis; a novel role for this transcription factor. WT1 appears to be important in angiogenesis though certainly, WT1 has a broader scope of influence than previously anticipated.

CHAPTER 5:

The Gene Expression Profile of Wt1^{+} Cells *In Vivo*

5.1 Introduction

Determining the function of any gene or protein is a complicated process, requiring determination of the molecular pathways involved and how these in turn effect cellular processes. In the case of transcription factors, there is an added level of complexity, as their influence can be both direct and indirect through transcriptional activation of other genes. WT1 is an unusual transcription factor in that it is capable of transcriptionally activating and repressing factors on a DNA and RNA level. While WT1 has been studied now for more than twenty five years (Call *et al.*, 1990; Gessler *et al.*, 1990) and its diverse functions have slowly been elucidated, the molecular mechanisms of this multi-functional protein are still not well understood. This chapter will investigate the implications of WT1 expression in cells and provide some further insight into the molecular genetics of WT1 *in vivo* using next-generation sequencing and transcriptomic gene expression analysis.

WT1 is a relatively typical zinc-finger transcription factor containing four C-terminus C2H2 Krüppel-type zinc-fingers with a high specificity for GC-rich regions (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992). It is capable of activating and repressing gene expression with both transcriptional repression and activation domains (Madden, Cook and Rauscher, 1993; Wang *et al.*, 1993). Two isoforms of WT1, named +KTS and –KTS, are of particular importance, as it has been hypothesised that -KTS isoforms are mainly involved in transcriptional regulation of DNA, while +KTS isoforms have been implicated more in a post-transcriptional role in RNA processing (Larsson *et al.*, 1995). However, the situation is more complicated as both isoforms probably function in transcription and post-transcriptionally processes (Hastie, 2017). A recent, comprehensive study confirmed that WT1 binds to the 3' untranslated regions (UTRs) of target mRNAs involved in developmental processes and regulates their turnover (Bharathavikru *et al.*, 2017). As an added level of complexity, binding of WT1 to a number of co-factors alters transcriptional regulation by these WT1 complexes (Wang *et al.*, 2001, 2015; Reynolds *et al.*, 2003; Green *et al.*, 2009; Essafi *et al.*, 2011). Given the

complexity of transcriptional regulation by WT1, it is understandable that it has such a complex and elusive mechanism of action.

Chromatin immune-precipitation sequencing (ChIP-seq) has proved a powerful tool for validating direct targets of WT1 (Kim *et al.*, 2007, 2009; Sunny Hartwig *et al.*, 2010; Motamedi *et al.*, 2014; Kann *et al.*, 2015). These identified target genes are involved in a range of processes including; growth and development, cell differentiation, EMT/MET, cell adhesion and cytoskeletal organisation, cell proliferation, cell migration and apoptosis. Many of the targets of WT1 are also transcription factors. From *in vitro* DNA binding assays and ChIP-seq, a number of WT1-binding motifs have been discovered (Rauscher *et al.*, 1990; Wang *et al.*, 1993; Hamilton, Barilla and Romaniuk, 1995; Nakagama, Heinrich and Pelletier, 1995; Little *et al.*, 1996). However, to understand the functional role of WT1, it is not enough to just understand the genes WT1 is capable of regulating, as its transcriptional regulation is context dependent and so, this knowledge must be combined with an understanding of the role of WT1 expressing cells *in vivo*; not always a simple task.

In recent years, high-throughput sequencing has revolutionised analysis of gene expression. Unlike qPCR, high-throughput sequencing does not require selection of specific genes to be assessed and hence can provide an unbiased analysis of the entire gene expression profile of specific cells and tissues. As WT1 is a transcriptional regulator, identification of single proteins or genes associated with WT1 expression is of limited use; ideally one would build a map of the diverse role of WT1 in a particular context and this is the strength of sequencing technology. These mechanisms have begun to be applied to the study of WT1, revealing a number of new DNA and RNA targets and the processes with which WT1 and its targets are associated (Velecela *et al.*, 2013; Dong *et al.*, 2015; Bharathavikru *et al.*, 2017). Analysis of the gene expression profile associated with WT1 in a number of different cell types and contexts will build up a picture of the effect of WT1 on gene expression profile. This will build up a more complete understanding of the function of WT1, reveal new potential areas of interest and ultimately reveal the therapeutic consequences of targeting *Wt1*.

5.1.2 **Hypothesis**

This chapter aims to address the hypothesis that:

> "*Wt1* expression in endothelial cells is associated with angiogenesis and induces a more pro-angiogenic gene expression profile."

5.1.3 **Aims**

1. Determine the cell types in which WT1 is expressed in an *in vivo* model of angiogenesis.
2. Identify the gene expression profile of WT1 expressing cells.

WT1 expressing cells will be isolated from the subcutaneous sponge implantation model of *in vivo* angiogenesis by FACs. The identity of these WT1 expressing cells will then be determined. Using an RNA microarray, the gene expression profile of these cells will be characterised and the molecular, cellular and physiological processes with which this gene expression profile is associated will be determined. This will hopefully provide new insights into the function of WT1 *in vivo* and the effect of WT1 expression on cellular function.

5.2 Materials and Methods

Sponge implantation and FACs sorting in this section were carried out by Richard McGregor and You-Ying Chau (University of Edinburgh, IGMM). Data extraction and normalisation was carried out by Graeme Grimes at the University of Edinburgh Bioinformatics Analysis Core and analysis of transcriptomic data was carried out with technical help from Graeme Grimes.

5.2.1 WT1-GFP Mouse

WT1-GFP reporter mice (WT1GFP/+) generated by Hosen *et al.* (2007) have knock-in GFP under the control of the *Wt1* promoter. This enables detection of WT1 expression by generation of GFP. All mice used in this section were male and 12-16 weeks of age.

5.2.2 Fluorescence Activated Cell Sorting (FACs)

Sponges were implanted into 10 $Wt1^{GFP/+}$ mice, and 4 $Wt1^{+/+}$ (GFP^-) controls as in Section 2.7. Five $Wt1^{GFP/+}$ mice and 2 GFP^- controls were culled at day 7 and at day 21 post-implantation.

5.2.2.1 Isolation of GFP^+ Cells

Single cell suspensions from the sponges and kidneys of WT1-GFP mice were processed for FACS analysis. Sponges were removed and immediately placed Leibovitz's L-15 Medium (Invitrogen, L5520). In sterile conditions, sponges were disrupted using forceps and a scalpel. This homogenate was resuspended in 10 mL PBS plus BSA (4mg/mL, Sigma-Aldrich, A2058) with 1mg/mL Collagenase B (Roche, COLLB-RO). Samples were incubated for 45 minutes at 37°C before digestion was stopped by addition of 15ml Leibovitz's L-15 Medium +10% FCS. Digested sponge tissue was strained through a 100µm sterile cell strainer (Scientific Laboratory Supplies, 431752), then a 70µm sterile cell strainer (Scientific Laboratory Supplies, 431751). Sieved cells were washed by centrifugation, (1200rpm, 5 minutes) (Beckman Coulter, Allegra X-22R). Supernatant was discarded and pellet resuspended in 1mL Red Blood Cell lysis buffer (Biolegend, 420301) for 3mins at R.T. 15mL PBS

was added and suspension was centrifuged at 1200rpm for 5mins. Supernatant was discarded and pellet re-suspended in 1mL PBS containing 2% FCS. The sample was filtered through a 40µm sterile cell strainer (Scientific Laboratory Supplies, 431750). Cell suspensions of PBS +5% PBS were prepared for sorting.

5.2.2.2 Fluorescence Activated Cell Sorting on Sponges from WT1-GFP Mice

FACS was performed by Elisabeth Freyer (University of Edinburgh, MRC) using the BD FACSAriTM II System (BD Biosciences). Cell suspensions were sorted for GFP^+ and GFP^- cells by FACs. Cells were incubated at 4°C for 15 minutes with the antibodies in Table 5.2.1, washing with PBS between antibodies. Cell suspensions were then sorted for the expression of these antibodies in GFP^+ and GFP^- populations. Sorting gates were generated from sponge and kidney cells from GFP- mice. Isotype control antibodies and OneComp eBeads (eBioscience, 01-1111) were used as negative controls for background staining. And GFP^+ kidneys were used as positive controls. FlowJo Software Version 7.6.5 (TreeStar) was used for analysis.

Antibody	Conc.	Company
CD140b (Pdgf-β) Anti-Mouse, PE	1/100	Biolegend (136005)
CD31 Anti-Mouse, APC	1/80	eBioscience (17 0311-82)
Rat IgG2a Kappa Isotype, PE	1/500	Biolegend (400507)
Rat IgG 2a Kappa Isotype, APC	1/80	eBiosience (17 4321-41)

Table 5.2.1: Antibodies used for fluorescence activated cell sorting. Antibody description/name, concentration used, provider and catalogue number are all provided.

5.2.3 Whole Genome RNA Micro-Array

Samples sorted by FACs (Section 5.2.2.2) were analysed using the MouseWG-6 or MouseRef-8 v2 Expression BeadChip with Illumina iScan by the Illumina Whole Genome Expression Profiling Service (Qiagen) (Section

2.13.2). This was carried out on 3 WT1-GFP $WT1^{GFP/+}$ mice per time point (n=3).

Data from this was processed by Graeme Grimes and the IGMM Bioinformatics Core (University of Edinburgh). Raw microarray data (experimental probes and control probe files) was read into R using the bioconductor package limma using the function read.ilmn(). Microarray data was normalised using limma's neqc() function. This performs background correction using negative control probes followed by quantile normalization using both negative and positive control probes. Control probes were then removed from further analysis. Additionally, probes not detected on any array (Illlumina p.value of detection greater than 0.01) were also removed from further analysis. Differential expression of genes was then performed by a fitting a linear model to each probe. The empirical Bayes moderation was applied to the linear model fit to compute moderated t-statistics, moderated F-statistic, and log-odds of differential expression. The Benjamini & Hochberg (alias FDR) method was used to correct p-values for multiple testing. This generated p-values which were used in all further analysis.

5.2.4 Transcriptomic Analysis

Two different programs were used for analysis of processed data from 5.2.3; DAVID (Huang, Sherman and Lempicki, 2008, 2009) and GOrilla (Eden *et al.*, 2007, 2009), with ReviGO (Supek *et al.*, 2011) used for representation of some of the results of these. Data was input into these programs based on significance determined from data processing (Section 5.2.3) and log^{10} fold change in expression of genes between GFP^+ and GFP^- groups.

For DAVID, data is entered in a non-sorted manner with a list of genes of interest and a background list. In this case, the list of interest was significantly differentially expressed genes and the background list was all the genes listed in the array. For GOrilla, data is entered in a ranked list, which in this case was ranked on log^{10} fold change in expression between GFP^+ and GFP^- groups. GOrilla was generally used for identification of processes of interest and

DAVID was used when it was not possible to rank genes, or to identify pathways and cell types.

Potential direct targets of WT1 in the entire genome and from this list of genes identified by micro-array were determined using validated WT1-binding motifs and the programs BLASTN (Ensembl) (Aken *et al.*, 2016) and RSAT (Medina-Rivera *et al.*, 2015). See Section 2.13.4 for full details.

As it was not possible to rank genes generated from this analysis, DAVID was used for analysis of the generated data, with either the entire mouse genome or all genes detected by micro-array as the background, depending on the data used for analysis.

5.2.5 **Statistical Analysis**

Except in the case of the analysis of FACs data, all statistical analysis in this chapter is either from the initial data processing (Section 5.2.3) or p-values from the programs' (DAVID, GOrilla, RSAT, Ensembl, ReviGO) own statistical analysis. All data presented are means +/- s.e.m mean.

Results

5.3.1 A Small Proportion of WT1$^+$ Cells from WT1-GFP FACS in Sponges express CD31

Sponges from WT1-GFP mice were FACS sorted for the expression of GFP and hence, WT1, at day 7 and day 21 in order to identify the cell type of WT1$^+$ cells *in vivo*. Implantation of sponges and subsequent FACS analysis was carried out Richard McGregor and You-Ying Chau (University of Edinburgh).

WT1-GFP mice had a distinct population of GFP$^+$ cells in kidney, day 7 sponge and day 21 sponge tissue, in comparison to WT1-GFP$^-$ controls which had negligible GFP$^+$ cells in any of these tissues (Figure 5.3.1 A-E). There were significantly more GFP$^+$ cells in sponges at day 7 than at day 21 ($p<0.0001$) (Figure 5.3.1 F). GFP$^-$ and GFP$^+$ cells from sponges at both time points were FACS sorted for expression of CD31 and PDGF-β (Figure 5.3.2). CD31$^+$ (PDGF-β$^+$ and PDGF-β$^-$) cells made up a small minority of total GFP$^+$ cells (day 7 = 2.49%, day 21 = 1.07%). Notably, the largest proportion of GFP$^+$ cells were CD31$^-$/PDGF-β$^-$ and therefore of unidentified cell type.

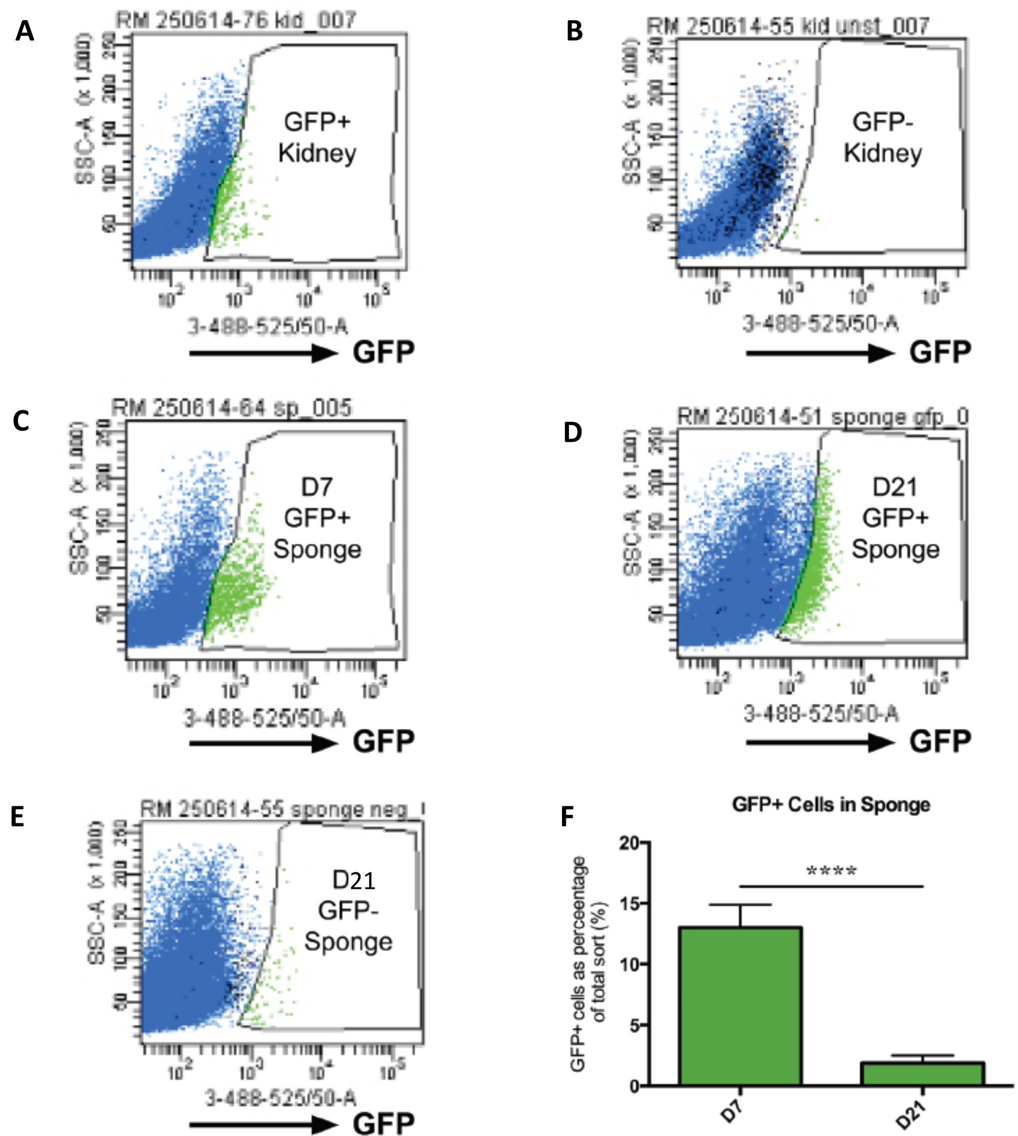

Figure 5.3.1: There are significantly fewer GFP$^+$ (WT1$^+$) cells in sponges at day 21 v day 7. Fluorescence activated cell sorting (FACS) scatter plots of side scatter (cell size) (y) against GFP fluorescence (x) in **A)** GFP$^+$ kidney, **B)** GFP$^-$ kidney, **C)** day 7 GFP$^+$ sponge, **D)** day 21 GFP$^+$ sponge, **E)** day 21 GFP$^-$ sponge. **F)** Quantification of cell counts from FACS sorting reveals significantly fewer GFP$^+$ cells at day 21 v day 7. **** $p<0.0001$, Student's t-test. D7 = day 7, D21 = day 21. Boxes in A-E represent gated GFP+ cell populations within scatter plot. GFP$^-$: n=2. GFP$^+$: n=5.

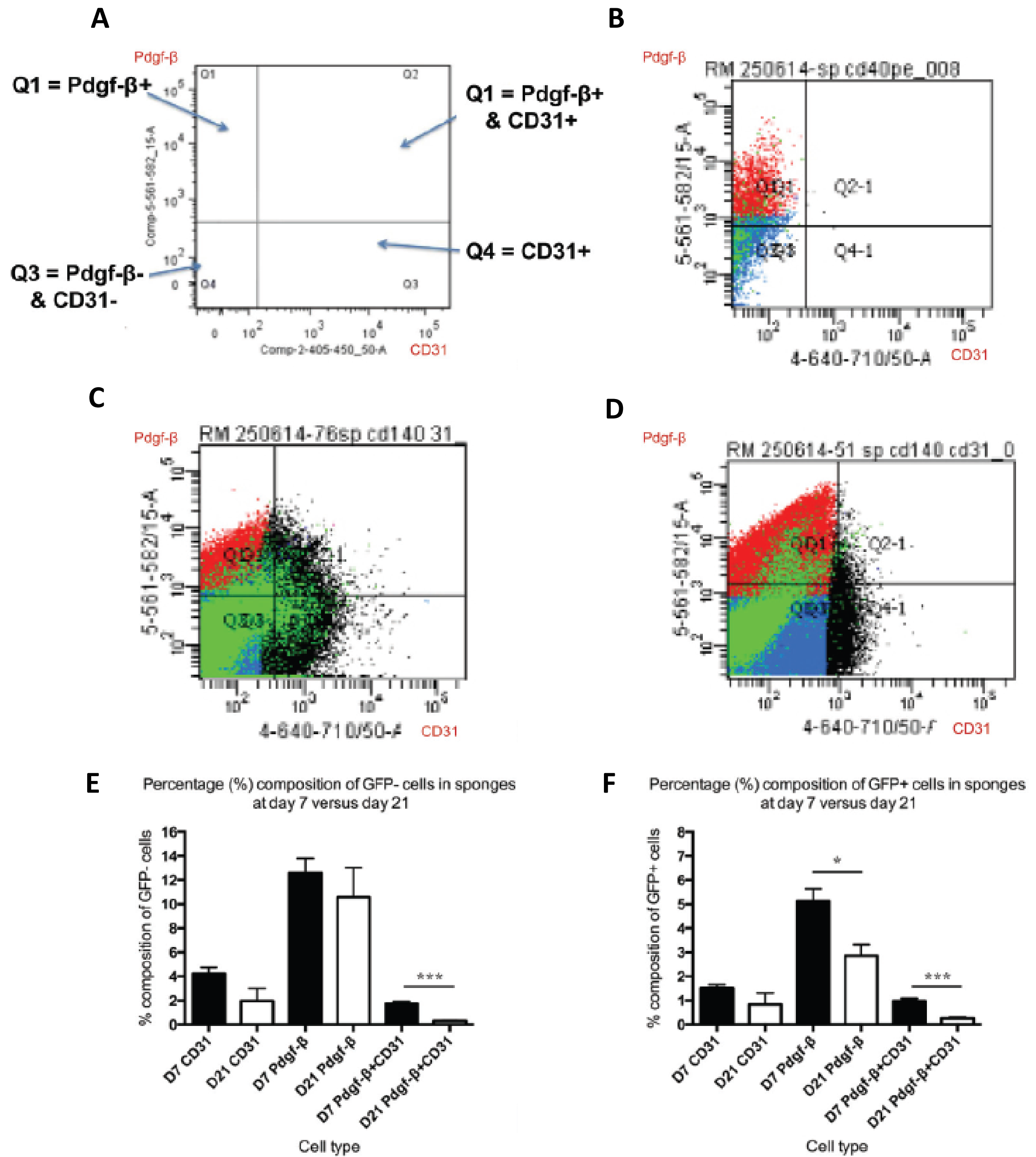

Figure 5.3.2: The majority of GFP^+ ($WT1^+$) cells from SSI sponges are $CD31^-$ and PDGF-β^-. Fluorescence activated cell sorting (FACS) scatter plots of PDGF-β fluorescence (y) against CD31 fluorescence (x) in SSI sponges. **A)** Key showing defined quartiles for CD31 and PDGF-β positivity and negativity. Scatter plot of FACs in WT1-GFP sponges **B)** with CD31 and PDGF-β isotype control antibodies, **C)** at day7 and **D)** at day 21. Quantification of cell counts from FACs sorting showing % of sorted **E)** GFP^+ and **F)** GFP^- cells at day 7 and 21 which also express CD31 and PDGF-β. * $p<0.05$, *** $p<0.005$, Student's t-test. GFP^+ cells = green, Pdgf-β^+ cells = red, $CD31^+$ cells = black, $CD31^+$/Pdgf-β^+ cells = black, $CD31^-$/Pdgf-β- cells = blue.

5.3.2 **RNA-Sequencing and Transcriptomic Analysis of WT1-GFP Sponges**

Sorted GFP^+ and GFP^- cells from WT1-GFP sponges in 5.3.1 were sent for microarray analysis for gene expression. This was carried out primarily in order to identify the cell type of the $WT1^+$ cells which are $CD31^-$/PDGF-β^- and determine the involvement of $WT1^+$ cells in angiogenesis. This returned the gene expression levels of 12,964 genes, 17,958 transcripts (due to multiple transcripts/gene) in GFP^+ (herein referred to as $WT1^+$) and GFP^- (herein referred to as $WT1^-$) cells at day 7 and day 21 by assessing mRNA abundance. This accounts for roughly 56% of the total mouse genome (~25,000 genes (Guénet, 2005)) 45.4% of these genes were not expressed at significantly different levels in $WT1^+$ v $WT1^-$ cells at either time point. 54.6% of genes were expressed at significantly different levels in $WT1^+$ v $WT1^-$ cells at day 7 or day 21 (Figure 5.3.3). Only 12.4% of genes were higher at both day 7 and 21 (~6.4% of the mouse genome) and 11.2% lower at both (~5.8% of the mouse genome).

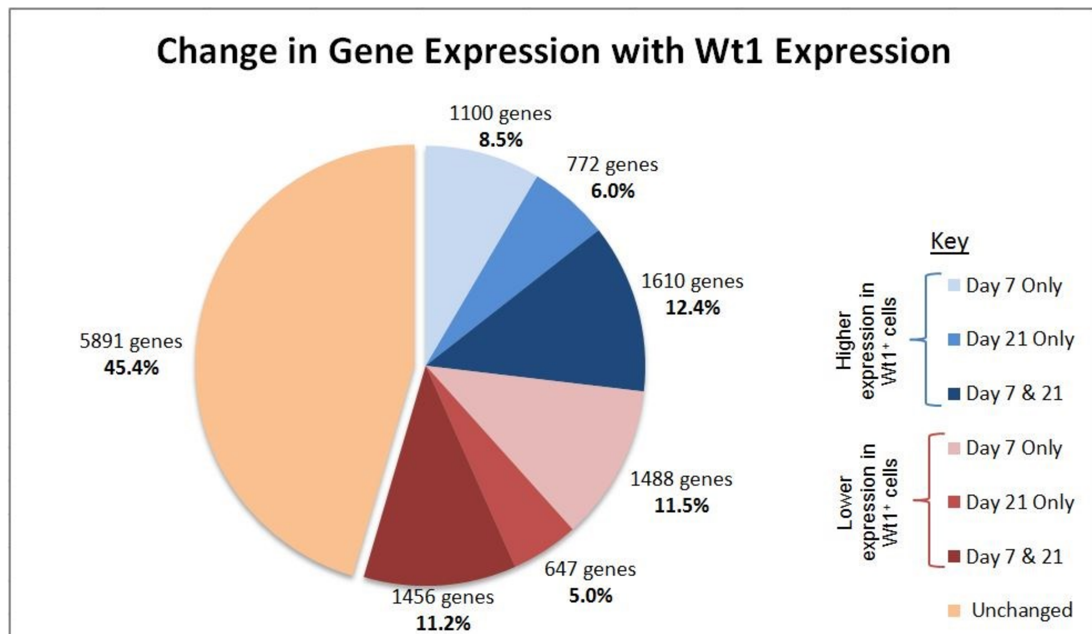

Figure 5.3.3: $WT1^+$ and $WT1^-$ cells have different gene expression profiles. $WT1^+$ (GFP^+) and $WT1^-$ cells were sorted from WT1-GFP SSI sponges at day 7 and 21 and gene expression was assessed by mRNA abundance from microarray analysis. Shown is a pie-chart of cells which have significantly higher (red) or lower (blue) gene expression in $WT1^+$ cells v $WT1^-$ cells, or unchanged (orange) expression between the two at day 7, day 21 and both day 7 & day 21.

5.3.2.1 WT1$^+$ Cells are Primarily Immune Cells

DAVID (Huang, Sherman and Lempicki, 2008, 2009) was used to determine the identity of WT1$^+$ cells from their entire gene expression profile. In order to reduce false positives and/or allow changes in cell type over time to affect results, only genes expressed at a significantly higher level in WT1$^+$ cells v WT1$^-$ cells at both day 7 and day 21 were selected and analysed to identify the cell types. Only the 12,964 genes detected in our micro-array were used as the background for analysis, to avoid any context-dependent bias. DAVID revealed 7 cell types which the WT1$^+$ gene expression profile significantly matched (Table 5.3.1). By far the most significant match was for macrophages, identified by 200 matching genes (p=3.41E-15). Four other immune cell types were also significant matches (mast cells, dendritic cells, peritoneal macrophages and pre-B cells), as well as fibroblasts, mesenchymal stem cells and endothelial cells. The genes which significantly match with the endothelial cell profile were VCAN, CD93, B9D1 and VCAM1. It is worth noting that these results are unable to reveal the relative abundance of these different types of WT1$^+$ cells.

Cell Type	P-Value	Gene no.	%
Macrophage	3.41E-15	200	6.14
Mast Cell	1.20E-05	84	2.47
Dendritic Cell	3.33E-05	48	1.74
Fibroblast	8.06E-05	38	1.05
Peritoneal Macrophage	2.27E-02	7	0.23
Pre-B Cell	3.03E-02	9	0.23
Mesenchymal Stem Cell	4.76E-02	13	0.46
Endothelial Cell	4.83E-02	4	0.18

Table 5.3.1: The identity of WT1$^+$ cells. DAVID (Huang, Sherman and Lempicki, 2008, 2009) was used to identify the cell types with which the gene expression profile of WT1$^+$ significantly matched, using genes that were elevated in WT1$^+$ v WT1$^-$ cells at both day 7 and day 21. The p-value for identifying each cell type is shown, along

with the number of elevated genes which match with this cell type (Gene no.) and the % of the total elevated genes this equates to (%). n=6.

5.3.2.2 **$WT1^+$ Cells are Associated with Angiogenesis**

Using GOrilla (Eden *et al.*, 2007, 2009), genes were ranked by log^{10} fold change ($WT1^+$ v $WT1^-$) from highest to lowest to carry out transcriptomic analysis of the gene expression profile of $WT1^+$ cells. This identified the gene ontology terms (GOterms) Angiogenesis, Regulation of Angiogenesis, Positive Regulation of Angiogenesis and Negative Regulation of Angiogenesis as being significantly associated with the gene expression profile of $WT1^+$ cells (Table 5.3.2)

	P-value	
Process	**Day 7**	**Day 21**
Angiogenesis	3.11E-08	1.07E-09
Regulation of Angiogenesis	4.02E-12	1.86E-04
Positive Regulation of Angiogenesis	4.60E-07	2.58E-05
Negative Regulation of Angiogenesis	1.51E-04	1.86E-04

Table 5.3.2: $WT1^+$ cells are involved in angiogenesis. GOterms identified by GOrilla (Eden *et al.*, 2007, 2009) as being associated with the gene expression profile of $WT1^+$ cells at day 7 and day 21, along with the identified p-value.

GOrilla also provided the names of genes associated with these terms which were significantly elevated in $WT1^+$ cells at day 7 and day 21 (Figure 5.3.4 & 5.3.5, respectively). Some of these, were expressed at higher levels at day 7 and day 21, such as Hif1a, Thbs1 and Ets1, while others were only higher at one time point.

5.3.2.2 **$WT1^+$ Cells have higher gene expression of a number of collagens, MMPs and EMT genes**

Given what is known about WT1 function from previous studies, the expression of a number of collagen genes, matrix metalloproteinases (MMPs)

and genes involved in EMT/MET were examined. The gene expression of a number of collagens (Figure 5.3.6 A,B) and MMPs was significantly elevated in WT1$^+$ cells at day 7 and day 21(Figure 5.3.6 C,D). Col1a2, Col3a1, Col5a1, Col5a2, Col5a3, Col6a1, Col6a2, Col6a3, Col8a1, Col9a3, MMP3, MMP14 and MMP23 were all significantly higher at both time points in WT1$^+$ cells. WT1$^+$ cells also had a significantly different gene expression profile for genes involved in EMT/MET at day 7 and day 21 (Figure 5.3.6 E,F), with Snai1, Slug, Twist1, Twist2, ETS-1 and ZEB1 all higher in WT1$^+$ cells at both time points and ETS-2 lower at day 7 and day 21.

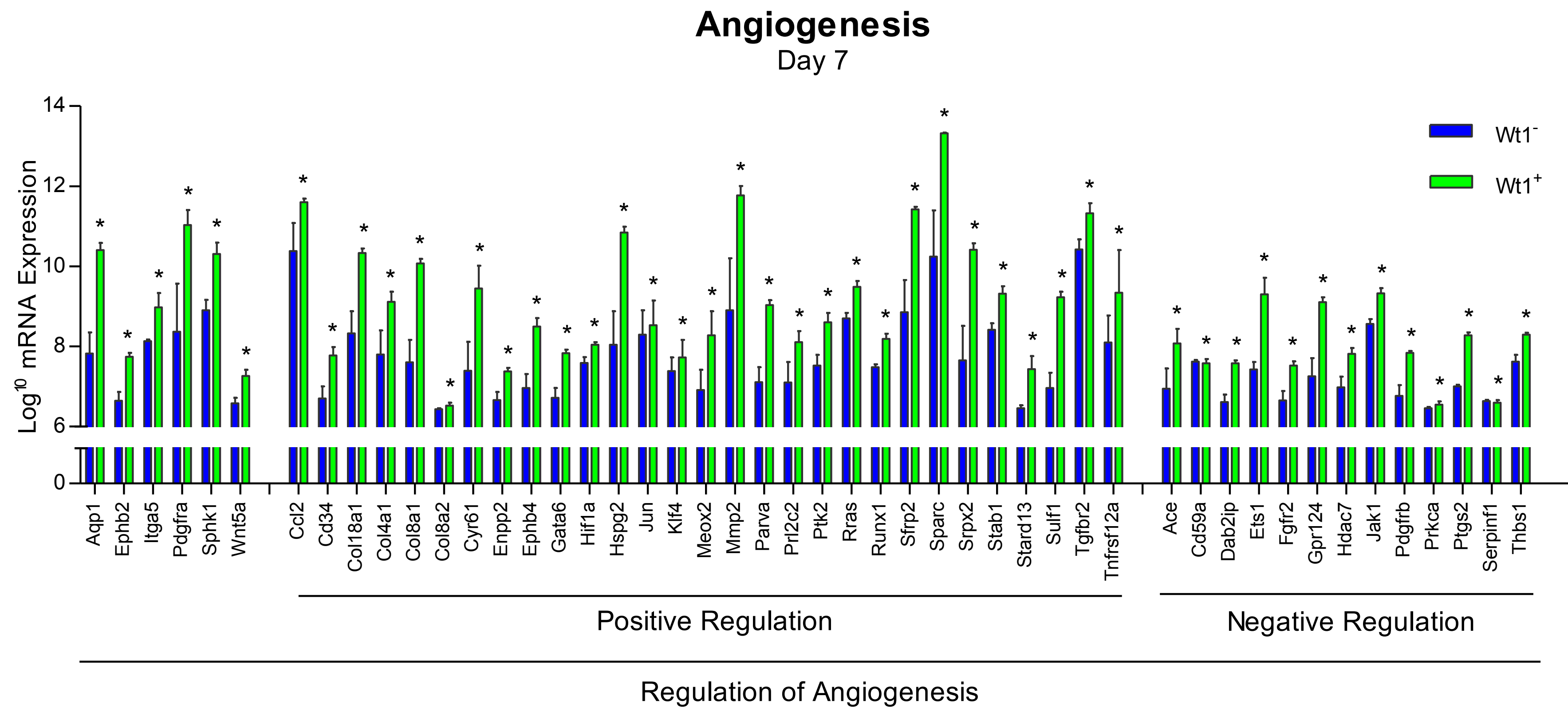

Figure 5.3.4: WT1$^+$ cells at day 7 have higher expression of a number of genes involved in angiogenesis. Graph shows log^{10} mRNA expression in WT1$^+$ and WT1$^-$ cells of various genes identified by GOrilla (Eden *et al.*, 2007, 2009) as being associated with angiogenesis (regulation, positive regulation and negative regulation, denoted below graph). Cells were FACS sorted from SSI sponges from WT1-GFP mice at day 7. Gene names are the official gene symbols. *fdr $q<0.05$, limma R bioconductor package. WT1$^-$ v WT1$^+$. n=3.

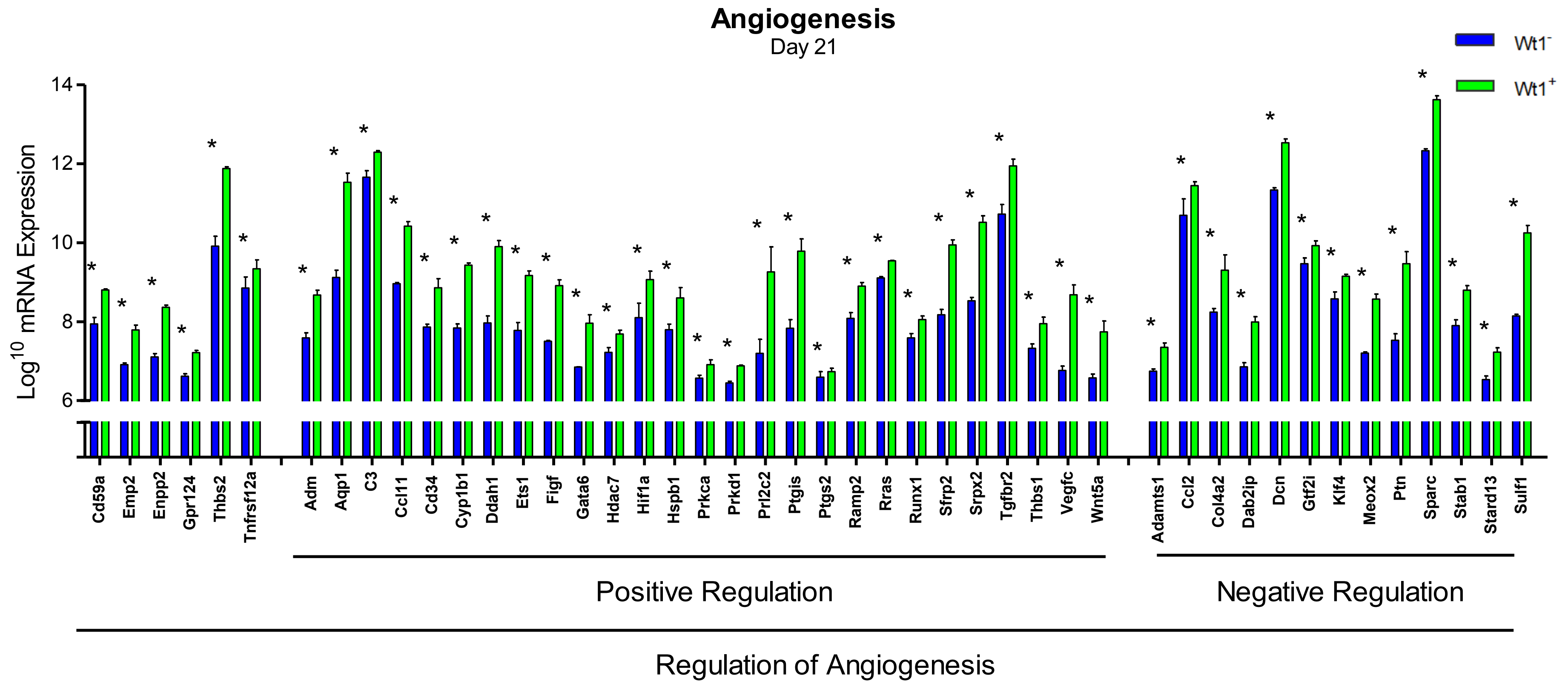

Figure 5.3.5: WT1$^+$ cells at day 21 have higher expression of a number of genes involved in angiogenesis. Graph shows log^{10} mRNA expression in WT1$^+$ and WT1$^-$ cells of various genes identified by GOrilla (Eden *et al.*, 2007, 2009) as being associated with angiogenesis (regulation, positive regulation and negative regulation, denoted below graph). Cells were FACS sorted from SSI sponges from WT1-GFP mice at day 21. Gene names are the official gene symbols. *fdr q<0.05, limma R bioconductor package, WT1$^-$ v WT1$^+$. n=3.

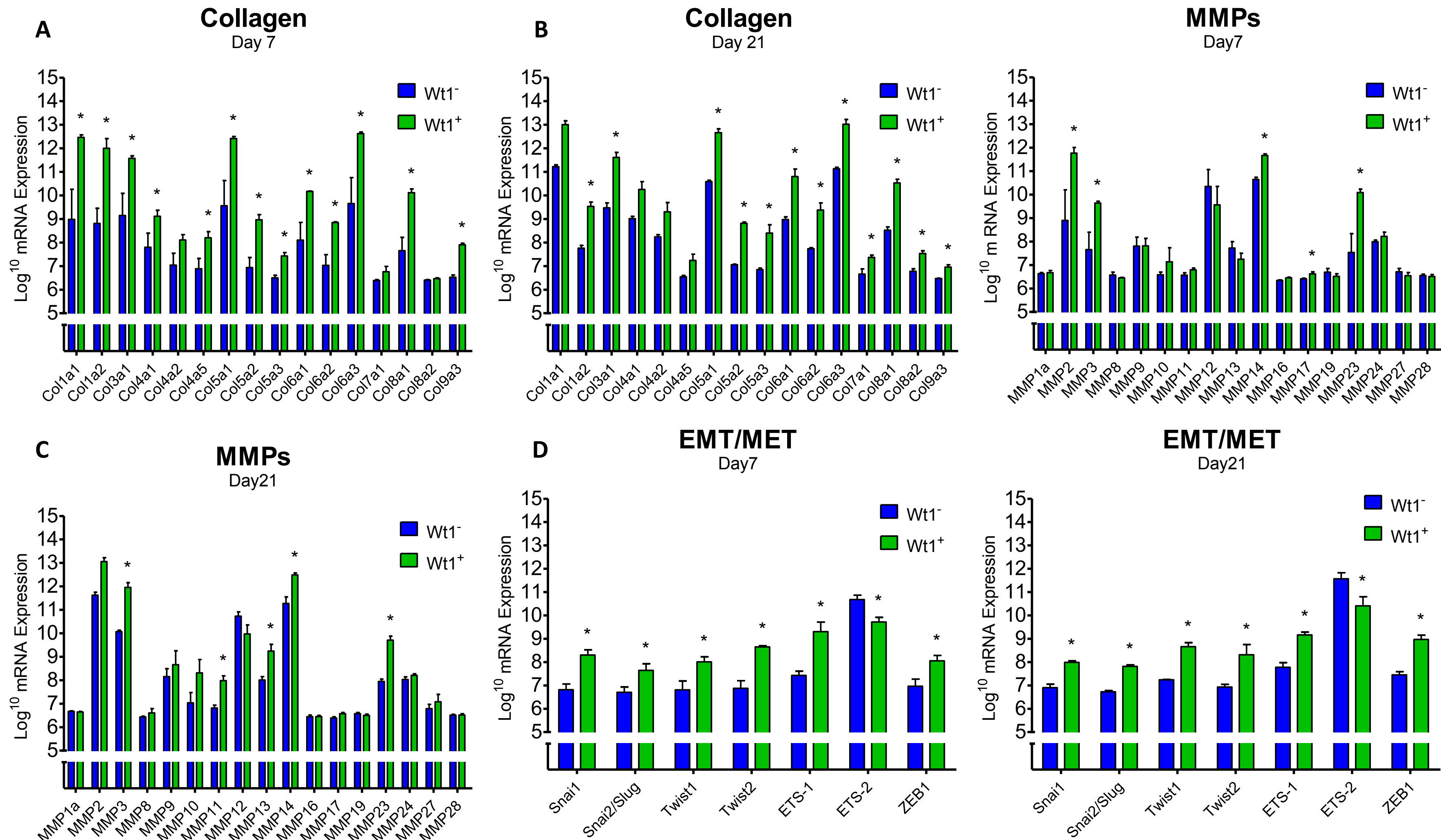

Figure 5.3.6: $WT1^+$ and $WT1^-$ cells have higher expression of a number of collagens, MMPs and genes involved in cell transition. Graph shows log^{10} mRNA expression in $WT1^+$ and $WT1^-$ cells of **A, B)** collagen genes at day 7 and 21, respectively, **C, D)** matrix metalloproteinases (MMPs) at day 7 and 21, respectively and **E, F)** genes known to be regulators of epithelial-to-mesenchymal transition (EMT)/ mesenchymal-to-

epithelial transition (MET) at day 7 and 21, respectively. Cells were FACS sorted from SSI sponges from WT1-GFP mice at day 7 and day 21. Gene names are the official gene symbols. *fdr $q<0.05$, limma R bioconductor package, $WT1^-$ v $WT1^+$. n=

5.3.2.4 Differentially Expressed Genes in WT1$^+$ Cells are Involved in a Number of Processes

Using GOrilla (Eden *et al.*, 2007, 2009), genes were ranked by log^{10} fold change between WT1$^+$ and WT1$^-$ cells to determine the cellular locations at which WT1 was inducing genetic changes (Figure 5.3.7). The molecular functions (Figure 5.3.8) and biological processes (Figure 5.3.9) associated with WT1-expressing cells were also determined. This was all carried out at day 7 and day 21 separately and it was found that the WT1$^+$ gene expression profile at day 7 and day 21 was similar enough that much the same GOterms were significant at both. GOterms were presented using ReviGO (Supek *et al.*, 2011), which reduces down long lists of GOterms by removing redundant terms and spatially distributes terms based on biological similarity.

WT1$^+$ cells primarily had different gene expression to WT1$^-$ cells in genes associated with the membrane and extracellular space of cells (Figure 5.3.7). Genes encoded proteins involved with the function of the plasma membrane, extracellular vesicles, extracellular matrix and collagens. There were also genes associated with the actin filament bundle, actomyosin and stress fibres, as well as blood microparticles and platelet alpha granules.

As well as molecular functions relating to RNA polymerase II, with which WT1 is known to interact, the genes expressed at higher levels in WT1$^+$ cells were associated with a number of molecular activities (Figure 5.3.8). One group of biologically related molecular functions related to binding of proteins including Laminin-1, Integrins, Fibronectin, Actin and other growth factors. Another group of related functions concerned the activity of a number of different peptidases. Other functions include, metal ion binding, calcium binding and heparin binding.

In terms of biological processes (Figure 5.3.9), while angiogenesis was a process significantly associated with WT1$^+$ cells, the WT1$^+$ gene expression profile also significantly correlated with general cellular processes, including cell growth, proliferation, locomotion, adhesion and death. WT1$^+$ cells were also involved in a general response to hypoxia, oxygen levels and wounding

and wound healing. A number of processes related to extracellular matrix and collagen were also significant.

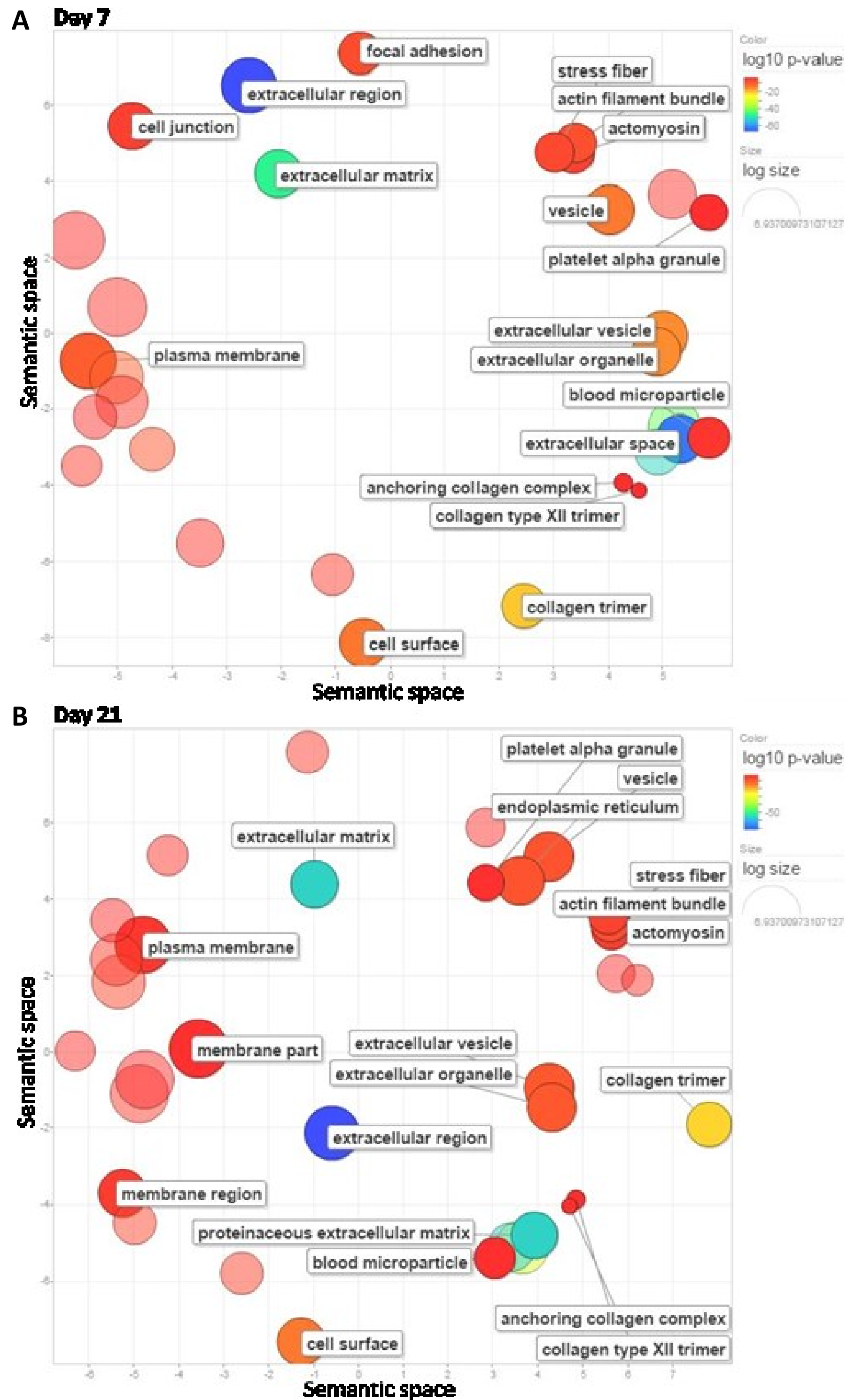

Figure 5.3.7: The cellular location of genes differentially expressed in WT1$^+$ cells. The gene expression profile of WT1$^+$ cells was analysed by GOrilla, in comparison to that of WT1$^-$ cells to determine significant GOterms associated with the expression profile. This was sorted for redundancy and visualised by ReviGO (Supek *et al.*, 2011) for **A)** day 7 and **B)** day 21. Each circle is a GOterm. Size of circle indicates log size of changes in expression and colour represents log^{10} p-value for significance of each term. X and Y axis are semantic space, such that GOterms that are biologically similar are closer together. n=3.

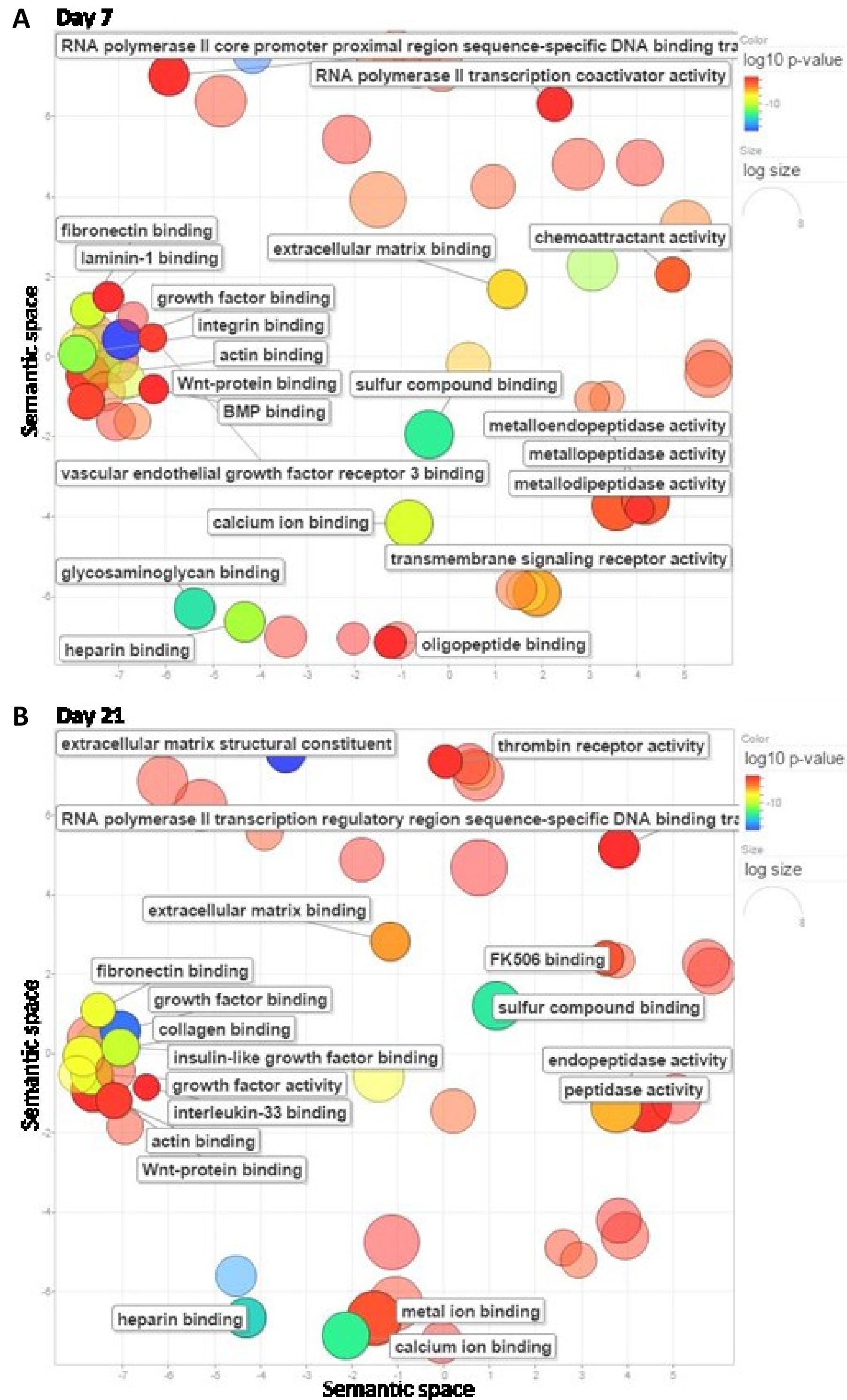

Figure 5.3.8: Molecular functions associated with the gene expression profile of WT1⁺ cells. The gene expression profile of WT1⁺ cells was analysed by GOrilla, in comparison to that of WT1⁻ cells to determine significant GOterms associated with the expression profile. This was sorted for redundancy and visualised by ReviGO (Supek *et al.*, 2011) for **A)** day 7 and **B)** day 21. Each circle is a GOterm. Size of circle indicates log size of changes in expression and colour represents $\log^{10}$ p-value for significance of each term. X and Y axis are semantic space, such that GOterms that are biologically similar are closer together. n=3.

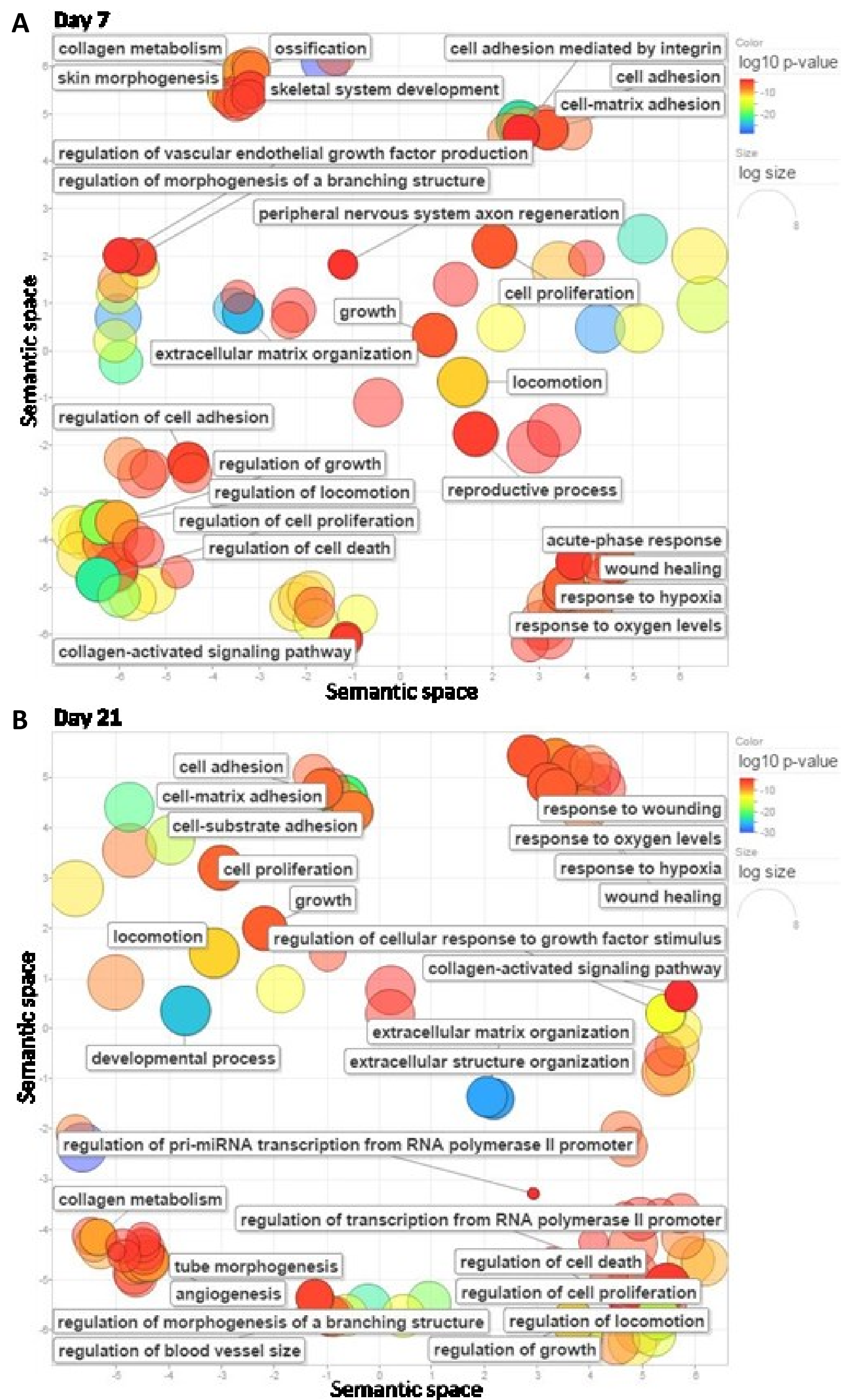

Figure 5.3.9: Biological processes associated with the gene expression profile of WT1⁺ cells. The gene expression profile of WT1⁺ cells was analysed by GOrilla, in comparison to that of WT1⁻ cells to determine significant GOterms associated with the expression profile. This was sorted for redundancy and visualised by ReviGO (Supek *et al.*, 2011) for **A)** day 7 and **B)** day 21. Each circle is a GOterm. Size of circle indicates log size of changes in expression and colour represents $\log^{10}$ p-value for

significance of each term. X and Y axis are semantic space, such that GOterms that are biologically similar are closer together. n=3.

5.3.2.5 Pathway Analysis of WT1$^+$ Cells

DAVID (Huang, Sherman and Lempicki, 2008, 2009) was used, as previously described (5.3.2.1) to identify pathways from the KEGG pathway database with which the gene expression profile of WT1$^+$ cells was significantly associated. Again, only genes expressed at a significantly higher level in WT1$^+$ cells v WT1$^-$ cells at both day 7 and day 21 were selected and analysed. 29 KEGG pathways were significantly associated with WT1$^+$ cells (Table 5.3.3), suggesting these pathways are upregulated in WT1$^+$ cells. The pathway with the most associated genes (PI3K-Akt signalling pathway) had 67 genes from the pathway elevated in WT1$^+$ cells.

Pathway	Genes	P-Value
Lysosome	54	2.54E-08
Phagosome	50	1.32E-06
Rheumatoid arthritis	32	4.18E-05
Amoebiasis	36	1.02E-04
Platelet activation	36	2.77E-04
Protein digestion and absorption	21	2.79E-04
ECM-receptor interaction	24	5.04E-04
Focal adhesion	51	0.0023
Rap1 signalling pathway	49	0.0035
Intestinal immune network for IgA production	14	0.0045
Calcium signalling pathway	35	0.0051
Jak-STAT signalling pathway	34	0.0051
Transcriptional misregulation in cancer	41	0.0071
Regulation of actin cytoskeleton	48	0.0072
Steroid hormone biosynthesis	9	0.0096

Proteoglycans in cancer	51	0.0108
Leukocyte trans-endothelial migration	28	0.0127
Collecting duct acid secretion	10	0.0130
Synaptic vesicle cycle	17	0.0137
Malaria	17	0.0137
Mineral absorption	13	0.0140
Bacterial invasion of epithelial cells	23	0.0160
Cytokine-cytokine receptor interaction	43	0.0161
Cell adhesion molecules (CAMs)	31	0.0184
Adherens junction	21	0.0202
PI3K-Akt signalling pathway	67	0.0227
Ras signalling pathway	45	0.0272
Hematopoietic cell lineage	21	0.0322
Endocytosis	60	0.0369

Table 5.3.3: KEGG pathways upregulated in $WT1^+$ cells. 29 pathways were identified as being associated with genes elevated in $WT1^+$ cells using DAVID (Huang, Sherman and Lempicki, 2008, 2009) and the KEGG pathway database. Only genes higher in $WT1^+$ cells at both day 7 and day 21 were analysed. Shown are the number of genes in that pathway elevated in $WT1^+$ cells and the p-value for identifying that pathway. n=6.

5.4 Discussion

The experiments in this chapter were intended to determine what cell types were expressing WT1 *in* vivo, in the context of angiogenesis, using the gene expression profile of these cells. Additionally, some insights into the role of these WT1 expressing cells were deduced from their gene expression. Characterisation of the effect of WT1 on a molecular level is essential to understanding the implications of WT1 expression and manipulation of this expression, on a cellular, tissue and organismal level. Furthermore, unbiased gene-sequencing as carried out here has the potential to reveal new areas of importance in relation to WT1, which would not otherwise have been envisioned; effectively allowing a ‘bottom-up’ approach to interrogation of WT1 and its function.

This chapter investigated the gene expression profile of WT1 in a number of different cell types in a model of *in vivo* angiogenesis, working on the hypothesis that WT1 would be expressed in endothelial cells and that this expression would induce a more pro-angiogenic gene expression profile. Cell sorting and analysis of the gene expression profile of these cells revealed that the vast majority of WT1 expressing cells were non-endothelial and that while WT1 positive cells were involved in angiogenesis to some extent, they were also implicated in a number of other cellular and physiological processes.

5.4.1 Issues to Consider in Transcriptomic Analysis

It is important to note here a few caveats regarding this analysis. Firstly, changes in gene expression between WT1 positive and WT1 negative cells simply reflect genes which are expressed to a greater or lesser extent in WT1 expressing cells. Hence, while changes in the expression of particular genes may be correlated with WT1 expression, this does not prove that they are regulated by WT1 directly or indirectly. Instead, the gene expression of these different cells can provide insights into the processes WT1 expressing cells are involved in. For example, it is more than possible that a specific factor could transcriptionally activate *Wt1* and another gene, simultaneously increasing expression of both genes (and protein levels), or that *Wt1* and other genes in question could be activated by the specific process a cell is undergoing or the location of this cell within the tissue.

As all cells were sorted from sponges for WT1 expression and the total RNA levels of all these cells then quantified, the gene expression profiles will be from a number of different cell types which can complicate analysis. For example, if more inflammatory immune cells express WT1 than do not express WT1, the expression of genes associated with these cells will, necessarily, be elevated in the WT1 positive cell population; this could appear as if WT1 is associated with activation of inflammatory genes, when, in fact, it may be involved in entirely different processes in these cells. However, this also does not preclude a role for WT1 in inflammation and certainly, the fact that WT1 is expressed to a greater degree in these cell types would be of relevance. While this broad transcriptomic analysis was necessary to enable further identification of different cell types, it would be interesting in the future to carry out sequencing comparing WT1 positive and WT1 negative cells of a particular cell type, such as endothelial cells or macrophages.

There are a number of things that this whole-genome sequencing cannot tell us about the molecular genetic environment associated with WT1. Where there are changes in gene expression and identification of different processes associated with WT1 expression, it is not possible to tell, for example, whether this is the result of a large change in a subpopulation of WT1 expressing cells, or a small change in all WT1 expressing cells. Different WT1 protein isoforms (particularly +/- KTS) have been shown to have different effects on cellular function, but this sequencing does not provide any information on WT1 isoform expression. Moving forward, it would be very interesting to sort and compare the expression profile of +KTS WT1 and –KTS WT1 to provide more insight into the role of these different isoforms. The sequencing carried out in this chapter is based on RNA expression, while in practice protein function depends on a number of post-transcriptional modifications such as phosphorylation, methylation and acetylation. Therefore, increases in gene expression do not always correlate with increases in protein function, though in the majority of cases and for the purpose of this investigation, it is fair to assume that an increase in gene expression effectively means an increase in protein function.

5.4.2 **WT1 is Expressed in a Number of Non-Endothelial Cell Types**

In the context of angiogenesis, the focus has primarily been on WT1 in endothelial cells (Wagner *et al.*, 2014). In previous chapters it was shown that WT1 is expressed

in endothelial cells, but also in a number of other cell types, including vascular mural cells and non-vascular cells. Cell sorting revealed that a greater proportion of cells are WT1 positive in sponges 7 days post-implantation that 21 days post-implantation, supporting the idea that WT1 has a role relatively acutely after injury. Identification of cell types from the sponges revealed that a very small proportion (less than 2%) of WT1 positive cells were endothelial. A larger proportion of WT1 positive cells expressed the pericyte marker PDGFR-β, but this still accounted for less than 6% of all WT1 positive cells. Interestingly, a proportion of endothelial cells expressed endothelial and pericyte markers, especially at day 7 where this accounted for the majority of endothelial cells. This is interesting as it supports the possibility that WT1 positive endothelial cells often sit in a transitionary state, as has been seen *in vivo* and *in vitro* in the previous chapters.

Although endothelial cells made up a very small proportion of WT1 expressing cells, it is understandable, given that vessels comprise a small proportion of the total cells in sponges post-implantation. However, the proportion of WT1 positive cells which were endothelial was about half the proportion of WT1 negative cells which were endothelial. While many genes are ubiquitously expressed, certain genes are only expressed in certain cell types, and so from gene-sequencing, it is possible to determine from genes that are more highly expressed in WT1 positive cells than WT1 negative cells, what cell types are expressing WT1. From this it was determined that a proportion of WT1 expressing cells were endothelial cells.

Utilising the gene sequencing, a fascinating array of cell types were found to comprise the WT1 positive cell population. The majority of WT1 positive cell types identified were of an immune cell lineage; macrophages, mast cells, dendritic cells, peritoneal macrophages and pre-B cells. Two mesenchymal cell types were also identified; fibroblasts and mesenchymal stem cells. While it is not possible to determine which cells most abundantly express WT1, it is worth noting that 200 genes (6.14% of all genes assessed) matched with the macrophage cell identity while 84, 48 and 38 genes matched with the mast cell, dendritic cell and fibroblast cell identities, respectively. The approach used here (sequencing to identify the potential cell types associated with a gene, protein, or process followed by FACs sorting for markers of these cells) would appear to be effective in contexts where there is little prior information, especially as sequencing technology becomes cheaper and more accessible. Now that it has been

confirmed that WT1 is expressed in these cell types, further FACs should be carried out in sponges to identify the relative proportions of these cells that express WT1 and how much of total WT1 expression this comprises. This would confirm the robustness of this method of analysis for identifying specific cell types and could then be applied to other contexts in which WT1 is of interest, such as different types of cancer (avoiding arduous and imprecise histological analysis for every cell type). The first context in which this should be applied is the hindlimb ischaemia model, where our work suggests WT1 may additionally be expressed in myocytes and satellite cells.

That WT1 is expressed in a range of immune cells, is an exciting discovery, with far reaching implications. There has been little specific focus on WT1 in immune cells to date, despite previous evidence suggesting WT1 may regulate immunity. WT1 expression has been previously noted in macrophages, where it regulates Il-10 and macrophage differentiation (Smith *et al.*, 1998, 2000; Sciesielski *et al.*, 2010) and B-lymphocytes, which are generated by pre-B cells (Spinsanti *et al.*, 2000; Marcet-Palacios, Davoine, *et al.*, 2007), but not (to the best of this author's knowledge) in pre-B cells themselves, mast cells, or dendritic cells; this should be assessed by histology *in vivo*, both in the context of cardiovascular diseases, where inflammation is a key component and other inflammatory diseases. WT1 is involved in haematopoiesis and haematopoietic stem cell (HSC) growth, proliferation and differentiation (Leif W Ellisen *et al.*, 2001; Alberta *et al.*, 2003; Cunningham *et al.*, 2013). Macrophages (including peritoneal macrophages), mast cells, dendritic cells and pre-B cells all arise from HSCs during haematopoiesis, suggesting that all HSC-derived cells could have the potential to express WT1. Furthermore, all of these cell types are involved in angiogenesis, raising questions about the interactions between WT1 expressing endothelial cells and immune cells during angiogenesis.

It is well established that WT1 is expressed in fibrotic tissue and fibroblasts in a number of contexts. Following myocardial infarction, WT1 is expressed in areas of fibrosis (Braitsch *et al.*, 2013) and fibroblasts are derived from WT1 expressing epicardium (Zhou and Pu, 2012). Detection of fibroblasts from gene sequencing is in concordance with WT1 transcriptionally regulating fibroblast genes and fibrosis. Mesenchymal stem cells (MSCs) are a fascinating pluripotent cell type with the capacity to generate fibroblasts, endothelial cells, adipocytes, chondrocytes, osteoblasts and all forms of muscle cells (Uccelli, Moretta and Pistoia, 2008). They also have immunomodulatory

function (Aggarwal and Pittenger, 2009) and form a niche in the bone marrow, along with HSCs, from which they both mobilise (Méndez-Ferrer *et al.*, 2010). In this respect, MSCs seem to be a crucial link between a number of the functions of WT1 and the cell types detected here. Surprisingly, however, the role of WT1 in MSCs is largely unknown. Supporting this link between WT1 and MSCs, FACs and gene-sequencing of cells isolated from Wilms' tumours with *Wt1* mutations had gene expression characteristic of MSCs (Royer-Pokora *et al.*, 2010). In a more cardiovascular context, a population of MSCs was discovered in the heart which was derived from WT1 positive proepicardium (Chong *et al.*, 2011). The importance of WT1 in MSCs needs to be determined *in vivo*, as they are not only sources of a number of important cell types, but have been shown to be beneficial in promoting angiogenesis when implanted into ischaemic hindlimbs (Al-Khaldi *et al.*, 2003; Li *et al.*, 2011).

5.4.3 **The Gene Expression Profile is Consistent with a Role for WT1 in Angiogenesis**

As well as identifying WT1 in endothelial cells and other cell types involved in angiogenesis, the gene expression profile of WT1 expressing cells was consistent with these cells having a role in angiogenesis. At both day 7 and day 21 post-implantation of sponges, it was determined that WT1 expressing cells were involved in positive and negative regulation of angiogenesis, through increased expression of pro-angiogenic and anti-angiogenic factors. Some of these factors are known to be regulated by WT1, such as *Ets1* (Wagner *et al.*, 2008), *Col4a1* (S.-R. Chen *et al.*, 2013) and *Thbs1* (Dejong *et al.*, 1999), while *Hifa* is known to regulate *Wt1* (Wagner *et al.*, 2003). However, the majority of these genes have not previously been implicated with WT1. *Hif1a* encodes the HIF-1α protein, a major regulator of response to hypoxia. Both HIF-1α and WT1, transcriptionally activate *Vegf*. Concordantly, *VegfC* expression was increased at day 21 in WT1 positive cells, while a number of other genes in the VEGF-signalling pathway were changed with WT1 expression. This supports a role for WT1 in VEGF-signalling as a possible mechanism through which WT1 influences angiogenesis (Cash *et al.*, 2007; Hanson *et al.*, 2007; Wagner *et al.*, 2008; McCarty, Awad and Loeb, 2011); though it is worth considering WT1 and VEGF interact not only in angiogenesis, but haematopoiesis too (Cunningham *et al.*, 2013). In line with the

role of WT1 as a transcriptional regulator, WT1 likely has a regulatory role in angiogenesis, rather than being pro-angiogenic or anti-angiogenic.

It is important to note however, that correlation does not mean causation and it is possible that WT1 is expressed in cells undergoing angiogenesis for unrelated reasons. It is also not clear whether this association between WT1 expression and other gene expression occurs in a particular subset of WT1 positive cells (for example only in endothelial cells) or in numerous cell types expressing WT1. Regardless of whether WT1 is directly regulating angiogenesis, it is a robust conclusion that *Wt1* is transcriptionally activated in cells undergoing angiogenesis, or that initiation of angiogenesis results in transcriptional activation of *Wt1*.

It is interesting to note that the majority of genes which change in expression with WT1 expression encode proteins located at the cell surface, suggesting WT1 not only induces changes in the cell in which it is expressed, but also affect cell-cell and cell-matrix interactions. This is further supported by association between WT1 expression and signalling via cellular vesicles. Perhaps expression of WT1 in clusters of proximal cells within the vasculature (observed *in vivo* in the HLI and sponge models), where WT1 could activate WT1 expression in proximal cells by cell-cell interactions or through factors released in vesicles. It would be interesting to assess the difference in the composition of vesicles released from WT1 positive and negative cells *in vitro*. Furthermore, endothelial or immune cells expressing WT1 may be releasing circulating signals into the vasculature, such as cytokines and chemokines (Velecela *et al.*, 2013).

5.4.4 WT1 Expressing Cells have a Number of Non-Angiogenic Functions

Alongside a clear role for WT1 in cells undergoing angiogenesis, the transcriptomic analysis carried out here reveals a role for WT1 in a number of processes *in vivo* in the sponge model, including a general response to hypoxia, wounding and wound healing. As previously mentioned, certain processes could solely be correlated with WT1 expression, due to the cell types in which WT1 is expressed. However, WT1 affected cellular processes not specific to any particular lineage including proliferation, growth, locomotion, adhesion and death, as has been previously described

(Maheswaran *et al.*, 1995; Roberts, 2008; Wagner *et al.*, 2008; Shandilya and Roberts, 2015). One of the key roles of WT1 is regulation of EMT (Hastie, 2017). Concordantly, the key EMT regulators *Snail*, *Slug*, *Twist1*, *Twist2*, *Ets1* and *Zeb1* are all expressed at higher levels in WT1 positive cells, suggesting that at least a proportion of these cells are undergoing a transition event. It was also evident that WT1 expressing cells were undergoing cytoskeletal rearrangements. These are necessary for changes in cellular function, to increase cell motility and in transitions such as EMT (reviewed by Yilmaz & Christofori 2009). Given the regulatory role WT1 plays in cellular processes including EMT and cell migration, it would not be surprising if WT1-induced modification of the cytoskeleton was a part of this and in fact, changes in cytoskeletal structure have been previously shown in relation to WT1 in a number of contexts (Hosono *et al.*, 2000; Barbolina *et al.*, 2008; Scholz, Wagner and Wagner, 2009; Brett, Pandey and Fraizer, 2013b).

Numerous collagen and MMP genes were highly expressed in WT1 positive cells, consistent with WT1 expression in fibroblasts and immune cells and a role in collagen and extracellular matrix turnover (Goetzl, Bando and Leppert, 1996; Distler *et al.*, 2005). While this could be solely due to the expression of WT1 in cell types involved in collagen deposition, such as fibroblasts, all cells interact with the extracellular matrix (ECM) and basement membrane to some extent. The focal adhesion pathway was associated with WT1 as were numerous processes relating to interaction of cells with collagen and ECM, which potentially implicates WT1 in regulation of cellular interaction with ECM components. In agreement with this, in the testis, WT1 directly regulates *Col4a1* and *Col4a2* (S.-R. Chen *et al.*, 2013), both of which were more highly expressed in WT1 positive cells, here. Modulation of cell-ECM interaction is essential in angiogenesis, regeneration, cell proliferation, migration and differentiation (Newby, 2006; Guilak *et al.*, 2009; Schultz and Wysocki, 2009).

5.4.5 **Conclusions**

This chapter set out to address the hypothesis that WT1 is expressed in endothelial cells and that WT1 expression induces a more pro-angiogenic gene expression profile in these cells. Through cell sorting and transcriptomic analysis, it was confirmed that WT1 was expressed in endothelial cells, but that this only comprised a very small proportion of WT1 expression. WT1 was expressed in immune cells (macrophages,

peritoneal macrophages, dendritic cells and mast cells), fibroblasts and mesenchymal stem cells. However, all of these cells are also known to influence angiogenesis and indeed, from transcriptomic analysis, WT1 positive cells were clearly involved in angiogenesis. The gene expression analysis carried out also attempted to identify the gene expression profile of WT1 expressing cells to help draw functional conclusions about the implications of WT1 expression *in vivo*. This confirmed that WT1 was also involved in a more broad regenerative response with roles in previously discovered processes such as migration, proliferation, apoptosis and cell transition, including EMT.

Ultimately, the work in this chapter provided a wealth of stimulating molecular genetic data, which needs to be corroborated by *in vivo* and *in vitro* work. It proved the efficacy of this kind of transcriptomic approach in novel contexts where little prior information is available. In the context of WT1 and angiogenesis, it was shown that WT1 was involved in cells undergoing angiogenesis, as shown previously *in vivo*, but could not confirm that this was the direct result of WT1 expression. Importantly, endothelial cells do not seem to be the only cell in which WT1 plays a role in angiogenesis and future investigation should also focus on immune cells, mesenchymal cells and progenitor populations. It seems likely, given the roles of WT1 in cellular function and differentiation that WT1 is required to induce cellular changes necessary to facilitate angiogenesis, though there are no doubt functions of WT1 in angiogenesis and regeneration that are yet to be discovered.

CHAPTER 6:

Discussion & Conclusions

Cardiovascular disease is the leading cause of mortality worldwide, accounting for 4 million deaths a year across Europe, 45% of all mortality (Townsend *et al.*, 2016). While the treatment of cardiovascular complications, such as myocardial infarction, is improving, patients are still left with detrimental functional impairment; for sufferers of peripheral arterial disease, this may mean limb amputation and for those unsuitable for amputation there is an even more severe and potentially fatal prognosis. Furthermore, the increasingly sedentary lifestyles and unhealthy diets of many people are increasing the prevalence of a number of cardiovascular diseases (Go *et al.*, 2014). This all means the need has never been greater to develop regenerative strategies for deployment during and after cardiovascular events, for example, by increasing angiogenesis which is capable of reperfusing ischaemic tissue, hence augmenting repair processes (Khurana *et al.*, 2005). However, trials of pro-angiogenic therapeutics, primarily focusing on growth factors such as VEGFs and FGFs, have to date, been ineffective in clinical trials (Bouïs *et al.*, 2006; Brenner *et al.*, 2016); this suggests the need for a new approach to therapeutically targeting angiogenesis in cardiovascular disease.

One such approach could be targeting transcriptional regulation of a number of different factors simultaneously. In this respect, WT1 came to light as a potential factor of interest, due to its known regulation of a range of cellular and physiological processes (reviewed by Hastie 2017) and its implication in angiogenesis in cancer. (Timár *et al.*, 2005; Dohi *et al.*, 2010; Wagner *et al.*, 2014). WT1 has been further implicated in angiogenesis by *in vitro* experiments showing that inhibition of WT1 impairs angiogenesis (Wagner *et al.*, 2008, 2014; Duim *et al.*, 2015) and that *in vivo*, post-MI, WT1 is expressed in the ischaemic (and potentially angiogenic) vasculature (K. Wagner *et al.*, 2002; Duim *et al.*, 2015). In fact, among the numerous transcriptional targets of WT1 are factors such as VEGFs (Cash *et al.*, 2007; Hanson *et al.*, 2007; McCarty, Awad and Loeb, 2011) and FGFs (Bra *et al.*, 2014) and pro-angiogenic drugs against these targets have previously gone to clinical trials. All of this suggests WT1 could be a powerful therapeutic target, manipulation of which could have therapeutic benefits on outcomes from cardiovascular diseases.

Whilst previous work has strongly implicated WT1 in angiogenesis, this has not been sufficient to elucidate the role which WT1 is playing in ischaemic vasculature in cardiovascular disease. The work contained in this thesis was designed to clarify the

role of WT1 in angiogenesis and tissue repair in the context of cardiovascular disease. The work utilised a number of experimental approaches to provide a fuller picture of the role of WT1 in ischaemic, regenerative angiogenesis and hence reveal whether it is a promising target for further investigation.

6.1 **WT1 in Angiogenesis**

WT1 is expressed in the cardiac vasculature throughout development (Duim *et al.*, 2016), but not in the majority of quiescent, adult vasculature; the blood vessels of the female endometrium and mammary being examples of exceptions to this, due to the physiological remodelling that occurs in these tissues (McGregor et al. 2014). However, WT1 is reactivated in the ischaemic cardiac vasculature post-MI (K. Wagner *et al.*, 2002; Duim *et al.*, 2015) and is also expressed in tumour vasculature (Timár *et al.*, 2005; Dohi *et al.*, 2010), two contexts in which angiogenesis is known to occur. Inhibition or deletion of WT1 has been shown to reduce angiogenesis in the *in vitro* 2D tube formation and *ex vivo* aortic ring assays (Wagner *et al.*, 2008, 2014; Duim *et al.*, 2015); while inhibition of WT1 specifically in the vascular endothelium impaired angiogenesis *ex vivo* as well as *in vivo* in the context of tumour angiogenesis (Wagner *et al.*, 2014). The experiments detailed in this thesis support these findings, while to some extent questioning the previous assertions on the role of WT1 in angiogenesis and adding new information to the field.

Visualisation of WT1 expression during angiogenesis, *ex vivo* in the aortic ring assay and *in vivo* in the subcutaneous sponge implantation and hindlimb ischaemia models, confirmed that WT1 was expressed in the vascular endothelium of a proportion of blood vessels, showing that WT1 is activated during angiogenesis and ischaemia. It is unclear whether this expression is due to activation of WT1 during hypoxia, as has been previously demonstrated (Wagner *et al.*, 2003), or due specifically to angiogenesis. However, as the *ex vivo* aortic ring assay was carried out in normoxic conditions, it seems likely the expression of WT1 is related to angiogenesis. This was confirmed by cell sorting of sponges which showed endothelial cells and pericytes comprised an identifiable, though relatively small proportion of, WT1-expressing cells. Transcriptomic analysis confirmed the presence of WT1-expressing endothelial cells and showed that WT1-expressing cells were involved in angiogenesis. Which occurs first, activation of WT1, or initiation of angiogenesis is undetermined. In the HLI model,

WT1-expressing vessels were more evident acutely after induction of injury, with considerably less vascular WT1 later on, suggesting WT1 is playing a role in the induction or early stages of angiogenesis. This is promising for WT1 in a therapeutic context, where manipulation of angiogenesis would be most beneficial if it could be promoted immediately after injury.

However, WT1 was not expressed solely in the endothelial component of the vasculature in angiogenic tissues. There was also considerable expression of WT1 in other mural cells (pericytes and VSMCs) of the vasculature, often concurrent with endothelial WT1 expression within the vessel. This was confirmed by cell sorting which identified a population of WT1 positive pericytes, *in vivo*. There were also what appeared to be immune cells in close proximity to the vasculature and in one instance, what could be a haematopoietic cell (both WT1 positive) in contact with WT1 positive vascular endothelium. Transcriptomic analysis confirmed that WT1 is expressed in a number of different immune cells; macrophages, including peritoneal macrophages, dendritic cells, mast cells and pre-B cells. All of these cells arise by haematopoiesis, in which WT1 is involved (Ariyaratana and Loeb, 2007) and have a role in angiogenesis. Of these cell types, WT1 has only previously been shown to be expressed in macrophages and never in the context of angiogenesis (Smith *et al.*, 1998, 2000; Sciesielski *et al.*, 2010). This has exciting implications in a number of contexts, but in angiogenesis, it is very feasible that activated, WT1 positive immune cells migrate to the site of other WT1 positive cells and promote angiogenesis. Following *in vivo* confirmation that WT1 is important in these cell types, these findings may open many novel areas of investigation. Finally, WT1 was also expressed in two other cell types from transcriptomic analysis; fibroblasts and MSCs. Both of these have been shown to express WT1 (Chau *et al.*, 2011; Braitsch *et al.*, 2013) and influence angiogenesis (Newman *et al.*, 2011; Ubil *et al.*, 2014; Gong *et al.*, 2017). Clearly then, endothelial cells are not the only angiogenesis-associated cells in which WT1 has a role in tissue repair.

Interestingly, there were often clusters of WT1 expressing cells proximal to each other within the vasculature, in endothelial cells and mural cells and on some occasions there was clear focal disruption of the basement membrane, with endothelial cells migrating outwards. This is consistent with the processes which occur at the early stages of angiogenesis, in which the basement membrane is degraded and endothelial

and mural cells migrate outwards to form the initial angiogenic vascular outgrowth (Carmeliet and Jain, 2011). Endothelial cells expressing WT1 had a more mesenchymal-like shape and mural cells expressing WT1 also expressed endothelial and mesenchymal markers simultaneously. WT1 is known to have a role in EMT (Miller-hodges and Hohenstein, 2012), though it has not been investigated in the context of the related process EndMT. These observations could be explained by WT1 inducing complete or partial EndMT and in the future, the role of WT1 in EndMT should be investigated. Indeed, it has been hypothesised that partial EndMT is an essential step in angiogenesis (Welch-Reardon *et al.*, 2014; Welch-Reardon, Wu and Hughes, 2015). A sensible first step would be using an *in vitro* EndMT assay (Zeisberg *et al.*, 2007) to assess WT1 expression during EndMT and the effect of WT1 inhibition on EndMT.

6.2 The Angiogenic Implications of Endothelial WT1 KO

WT1 was knocked-out selectively in the vascular endothelium in an inducible manner (VE-Wt1 KO). The mice generated in this way had a significant reduction in endothelial WT1 expression (in the uterus) following KO of WT1 in the endothelium. Consistent with the findings described above, endothelial WT1 KO significantly impaired angiogenesis in the *ex vivo* aortic ring assay, delaying the formation of angiogenic vessel outgrowths. This suggests that WT1 does have an endothelial role in angiogenesis. When VEGF-A was added to the media, though, angiogenesis was not impaired. This could be due to VEGF-A providing an improved angiogenic environment, but could also reflect a role for WT1 in activating VEGF, supporting previous findings that WT1 directly regulates *VEGF* (Cash *et al.*, 2007; Hanson *et al.*, 2007; McCarty, Awad and Loeb, 2011). Transcriptomic analysis also supported this, by showing increased *VEGF-C* expression in WT1 positive cells and a potential role for WT1 in the VEGF signalling pathway. WT1 expression also correlated with regulation of a number of other cellular processes including proliferation, cell cycle, migration, survival and apoptosis (Aksamitiene, Kiyatkin and Kholodenko, 2012).

In the endothelial WT1 KO mouse, however, WT1 had no detectable effect on *in vivo* angiogenesis in sponges or in muscles from the ischaemic hindlimb, despite endothelial WT1 expression in these models. WT1 has never previously been investigated in the HLI model, but previous work on the sponge model using a mouse

effectively identical to our endothelial WT1 KO (Université de Nice), found an eight fold reduction in vessel density following endothelial WT1 KO (McGregor et al. 2014). When we analysed these samples there was no difference found in any measures of angiogenesis. The reason for this discrepancy is unclear. The *in vivo* and *ex vivo* results initially appear contradictory, but there are a number of possible explanations for the differing outcomes; this is also a nice demonstration of the importance of using *ex vivo* and *in vivo* approaches to tackle the same question.

First, it is possible that the reduction in WT1 induced in our endothelial WT1 KO mouse is insufficient to impair angiogenesis *in vivo*, as the knockout of WT1 in the endothelium is incomplete. A complete endothelial WT1 KO may impair angiogenesis *in vivo* and completely inhibit angiogenesis *ex vivo*. Furthermore, as WT1 is a transcriptional regulator, a 50% reduction in *Wt1* expression would not lead to complete activation or inactivation of target genes. However, an incomplete WT1 KO *in vivo* was previously shown to achieve dramatic effects in adult tissues (Chau *et al.*, 2011).

Another explanation is that as WT1 is expressed in a number of different cell types, not only endothelial cells. It is possible that expression in these other cell types is sufficient to compensate for the reduction in endothelial WT1 expression. Factors released by non-endothelial WT1 cells could also be sufficient to elicit the same effect in WT1 KO endothelial cells. Given the potential of a number of other cell types (including pericytes, fibroblasts and macrophages) to transition into endothelial cells, it is also possible these cells transition into endothelial cells in the KO to generate endothelial cells capable of WT1 expression. While the mechanisms behind this could be interrogated in further detail, this could first be confirmed by determining whether a ubiquitous tamoxifen-inducible WT1 KO (siRNA or *Cre-lox* (Chau *et al.*, 2011)), impaired angiogenesis to a greater extent in the *ex vivo* aortic ring assay than an endothelial WT1 KO alone. Then, an inducible endothelial WT1 KO combined with an inducible mural cell and/or macrophage WT1 KO could be used in the sponge and HLI models to determine if there was any inhibition of angiogenesis. Previous work in the aortic ring assay and in murine tumours found that KO of WT1 impaired angiogenesis and tumour growth to a much greater extent under the control of *Tie2 CreER*^{T2}, which is also expressed in haematopoietic cells including monocytes and HSCs (Kisanuki *et al.*, 2001; Arai *et al.*, 2004), than when under the control of *VE-Cadherin CreER*^{T2}

(Wagner *et al.*, 2014). Therefore, it would be logical to assume that Tie2 positive non-endothelial cells have an effect on angiogenesis and in the HLI model it was shown that delivery of Tie2 expressing monocytes and macrophages dramatically increases *in vivo* angiogenesis and improves outcomes (Patel *et al.*, 2013).

Finally, the difference in timing between the *ex vivo* and *in vivo* models is likely to contribute, in part, to impaired of angiogenesis having been observed *ex vivo*, but not *in vivo*; that is to say that angiogenesis could be measured much more acutely *ex vivo* than *in vivo*, due to the nature of the models themselves. The evidence from this work suggests that WT1 is having an acute role in angiogenesis, which may be missed at the later time points at which angiogenesis could be quantified *in vivo*. As previously mentioned endothelial WT1 KO only delayed angiogenesis in the aortic ring model, with an early, transient reduction in vessel density that recovered by one week after start of angiogenesis. In the HLI model, WT1 was more widely expressed acutely after injury than later on. The implication is that in the *in vivo* models, an acute impairment of angiogenesis may also occur, but be unquantifiable and function is recovered by compensatory mechanisms by the time neovascularisation is quantified. The murine model of mouse menstruation developed by Brasted *et al.* (2003) and adapted by Cousins *et al.* (2014) could be a model of *in vivo* angiogenesis in which the effect of endothelial WT1 KO could be quantified at earlier time points.

6.3 Physiological Roles of Vascular WT1

WT1 has been shown herein to have a role in regenerative angiogenesis; however, putative evidence suggests a broader function of WT1 within the vasculature. As WT1 is not known to be expressed in healthy adult vasculature, especially in males used in these experiments (Chau *et al.* 2011, McGregor et al. 2014), it was not envisioned that WT1 would affect normal vascular endothelial function in non-pathological circumstances. In agreement with this, endothelial WT1 KO had no effect on the morphology of a number of tissues. However, it was observed that the male gonad was visibly enlarged after sustained (<4 weeks) endothelial WT1 KO, while conversely, the testis was reduced in size. This suggests endothelial WT1 plays a normal physiological role in the testicular vasculature, which is disrupted following disruption of WT1 function. In line with this hypothesis, WT1 was, for the first time, found to be expressed in endothelial cells of a number of testicular blood vessels. This

adds to the very few known contexts in which WT1 is expressed in the adult vasculature and shows that along with the female uterine tract and mammary, WT1 is expressed in the vascular component of a number of reproductive tissues (McGregor et al. 2014). WT1 expression should also be assessed in the seminal vesicles and epididymal fat to determine whether WT1 is also expressed in the vasculature here, which would confirm that WT1 is expressed in the vasculature of all reproductive tissues. However, the weight of the seminal vesicles and epididymal fat was unchanged in endothelial KO mice, so it is likely WT1 is expressed in the vasculature to a lesser extent in these tissues.

As the testis continues to develop and grow throughout early adulthood (Sharpe *et al.*, 2003), the simplest explanation for the reduced testis size, given the function of WT1 in angiogenesis, is that impaired angiogenesis in the endothelial WT1 KO results in impaired testicular growth. It is also possible, however, that atrophy of the testis, , is contributing to the reduced testis weight observed. Atrophy was also observed in the heart, spleen and pancreas of mice ubiquitously deleted for WT1 (Chau *et al.*, 2011). In this same study (Chau *et al.*, 2011), a small, though non-significant reduction in testis weight was seen, though endothelial WT1 KO was maintained for a longer period of time. The mechanism responsible for reduced testis size could be identified by observing endothelial WT1 expression in older adult mice in which testes are fully developed and then inducing endothelial WT1 KO in these older mice to see whether it results in a reduction in testicular size. Regardless, WT1 in the adult testicular vasculature is a completely novel area which demands further investigation.

Given the reduced testis size and unchanged epididymal and seminal vesicle size, the simplest explanation for the enlarged male gonad is that endothelial WT1 KO is resulting in increased vascular permeability, leading to fluid accumulation and oedema. Generalised oedema was also observed following ubiquitous WT1 KO in adult mice (Chau *et al.*, 2011), for which the deletion of WT1 in the endothelium may have been responsible. Whether oedema is localised to the gonad or more widespread following endothelial WT1 KO needs to be determined. WT1 regulates VEGF in the endothelium (Wagner *et al.*, 2003) which regulates angiogenesis, but also vascular permeability (Nagy, Dvorak and Dvorak, 2007). This all leads to the hypothesis that WT1 regulates physiological vascular permeability in endothelial cells. This should be

explored further, looking at the vascular permeability in endothelial WT1 KO mice using an *in vivo* permeability assay (Chen *et al.*, 2009).

Myography was used to determine if endothelial WT1 KO impaired normal endothelial function. Surprisingly, endothelial WT1 KO aortae exhibited increased contractile responses to two vasoconstrictor agonists, 5HT (serotonin) and NA (noradrenaline) (though not potassium), with vasodilation unaffected. Therefore, in all likelihood WT1 has a role in serotonin and noradrenaline signalling in endothelial cells and VSMCs which is disrupted by endothelial WT1 KO, though it is worth considering it could be an indirect effect. WT1 may prove to have a role in physiological regulation of vascular tone. In support of this hypothesis, hypertension is evident in some glomerulopathies resulting from *Wt1* mutation (Little *et al.*, 1993; Wagner *et al.*, 2008; Lipska *et al.*, 2014) and WT1 has been shown to regulate NO signalling (Johannesen *et al.*, 2003; Mazzei *et al.*, 2010). Intriguingly, serotonin and noradrenaline signalling are intertwined (Molderings *et al.*, 1990) and both are involved in angiogenesis, in some cases through factors associated with WT1 (either previously or in this work), such as Thrombosponin-1 (Qin *et al.*, 2013), Akt (Iwabayashi *et al.*, 2012), PI3K (Steinle *et al.*, 2003) and VEGF (Yang *et al.*, 2006).

Part of what makes these results surprising is that WT1 is not thought to be expressed in quiescent adult vasculature, such as the aorta. Two possible options are that WT1 is expressed at very low levels in the aorta, or is rapidly activated in response to these vasoconstrictors. The expression of WT1 in the adult aorta should be assessed by histology. The function of WT1 in regulation of vascular tone should first be examined by assessing the blood pressure of endothelial WT1 KO mice *in* vivo as a role for endothelial WT1 in regulation of vascular tone and blood pressure would be highly relevant in the context of cardiovascular disease, where hypertension is a significant risk factor. Angiogenesis, vascular permeability and vascular tone are all linked and the regulation of the latter two processes is required for regulation of angiogenesis. Furthermore, factors (including Akt, NO and VEGF) associated with WT1 (either here or previously) are involved in all three processes (Fukumura *et al.*, 2001; Shiojima and Walsh, 2002; Chen *et al.*, 2009).

6.4 Non-Vascular Functions of WT1

The work described in this thesis confirms that WT1 has a number of roles outside the vasculature. Transcriptomic analysis revealed that, alongside angiogenesis, WT1 expressing cells were involved in a number of cellular processes including cell proliferation, growth, locomotion, adhesion and death; all previously shown to be regulated by WT1 (Maheswaran *et al.*, 1995; Roberts, 2008; Wagner *et al.*, 2008; Shandilya and Roberts, 2015). These processes are all important to enable cells to participate in angiogenesis, but also reflect the role of WT1 as a broad regulator of cellular function and demonstrate that the role of WT1 is unlikely to be restricted exclusively to angiogenesis. This was confirmed by histology which showed WT1 positive cells were involved in EMT (by colocalisation with Snail) in HLI and a proportion of these cells were proliferative. While the *in vivo* models deployed herein are primarily used as models of angiogenesis, they also encompass other regenerative responses to damage (Andrade, Fan and Lewis, 1987; Limbourg *et al.*, 2009).

In the sponge and HLI models WT1 was expressed in cells which were morphologically consistent with fibroblasts and inflammatory immune cells. In ischaemic muscle following induction of HLI, there was acute infiltration of immune cells which were WT1 positive and the greatest expression of WT1 throughout this muscle tissue occurred acutely after injury. Transcriptomic analysis indicated that fibroblasts, macrophages, dendritic cells, mast cells and pre-B cells comprised a proportion of WT1 expressing cells. Furthermore, in the sponge and HLI models, there was putative evidence that endothelial WT1 KO affected macrophage infiltration and collagen deposition, respectively. WT1 has been previously shown to be involved in fibrosis (Braitsch *et al.*, 2013) and inflammation (Smith *et al.*, 1998, 2000; Sciesielski *et al.*, 2010); one of the interesting things about this result is that WT1-expressing endothelial cells may act cooperatively with fibroblasts and immune cells, as endothelial WT1 KO affects fibrosis and inflammation. Transcriptomic analysis further confirmed changes in collagens, MMPs and immune markers associated with WT1 expression, as well as involvement of WT1 expressing cells in ECM remodelling and immune processes. One such process was leukocyte transendothelial migration, a process in which endothelial cells increase vascular permeability to allow leukocytes

into specific tissues (Aghajanian *et al.*, 2008). This may represent one instance of the cross-talk between WT1 expressing endothelial and immune cells.

WT1 expressing cells were also involved in a general wound healing response, activated by hypoxia, as confirmed by transcriptomic analysis. One of the most striking and exciting observations in the HLI model of critical limb ischaemia was that skeletal muscle cells comprised a large proportion of WT1 expressing cells. This myocyte WT1 expression was particularly widespread and persistent in areas of muscle damage within the gastrocnemius, where there was also increased angiogenesis, implicating WT1 in skeletal muscle regeneration. This is a completely novel observation; however, WT1 has recently been found to be strongly expressed in developing human skeletal muscle tissue (Parenti *et al.*, 2013; Magro *et al.*, 2015) and deletion of *Wt1* from the kidney mesenchyme results in activation of myogenic markers and ectopic muscle formation (Berry *et al.*, 2015). Skeletal muscle has its own resident stem cell population, satellite cells, which when activated in part by macrophages and fibroblasts, induces extensive muscle regeneration (reviewed by Wang & Rudnicki 2011). Preliminary histological analysis suggested that WT1 may be expressed in satellite cells in quiescent and damaged skeletal muscle. This needs to be investigated in more detail *in vitro* and *in vivo*, but a working hypothesis based on these results is that WT1 is expressed in satellite cells which contribute to a WT1-regulated regenerative myogenesis.

This is intriguing in isolation, but it is also easy to see the parallels between this situation and the maintained WT1 expression in the epicardium which has the potential to generate cardiomyocytes post-MI (van Wijk *et al.*, 2012; Braitsch *et al.*, 2013). This contributes to the known adult stem cell populations in which WT1 is essential, which along with epicardial cells comprises haematopoietic stem cells (Cunningham *et al.*, 2013) and adipose progenitor cells (Chau *et al.*, 2014), while transcriptomic analysis carried out here also contributes pre-B cells and MSCs to this list. It is tempting to propose that WT1 is expressed in a large proportion of adult tissue-resident stem cell populations and should therefore be investigated in other populations, including mesenchymal stem cells. One particular population of interest, given the role for WT1 demonstrated in this work could be endothelial progenitor cells. These cells of haematopoietic origin have high regenerative potential, contribute to adult

vasculogenesis (Zammaretti and Zisch, 2005) and undergo EndMT in certain circumstances (Díez *et al.*, 2010).

EMT, a process in which WT1 is known to be essential (Martínez-Estrada *et al.*, 2010), has been shown to induce stem cell-like properties in transitioning cells (Mani *et al.*, 2008), generating the following hypothesis; constant WT1 expression is required to maintain stem cell characteristics in adult stem cell niches, while transient WT1 expression is required to activate these stem cell-like characteristics in differentiated cells, such as when undergoing transition, before being inactivated to allow return to a quiescent adult cell type.

6.5 Future Work

Alongside interrogating the areas investigated herein in more detail and the future work already suggested within this chapter, there are some other experiments which were started during the course of this work, but have not yet yielded revealing data in relation to WT1.

6.5.1 Determining the Function of WT1 in Endothelial Cells *In Vitro*

In order to provide further insight into the functional implications of WT1 expression *in vitro*, endothelial cell culture was initiated. This would be useful to investigate the role of WT1, specifically in EndMT using an EndMT assay, but could also be applied to a 2D-tube formation assay to confirm endothelial WT1 KO does impair angiogenesis as seen in the aortic ring assay.

Endothelial cells were extracted from VE-Wt1 KO and CC mice and cultured for up to 7 days post-extraction (Figure 6.1). Initial small colonies of endothelial cells attached to the bottom of the plates as early as Day 3 and began to proliferate and migrate out to form a monolayer. By Day 7, confluence was achieved and cells were re-plated. Both VE-Wt1 KO and CC endothelial cells had the capacity to form a monolayer and reach confluence and there was no noticeable difference in speed or extent of cell proliferation or migration, though this was not quantified. This shows it is possible to isolate and culture VE-Wt1 KO endothelial cells for further experiments. However, alongside cells with a typical 'cobblestone' endothelial morphology, more spindle-shaped non-endothelial cells were noted, suggesting some mesenchymal cells had

also been isolated from aortae. This technique will need to be further optimised to reduce contamination from non-endothelial cell types.

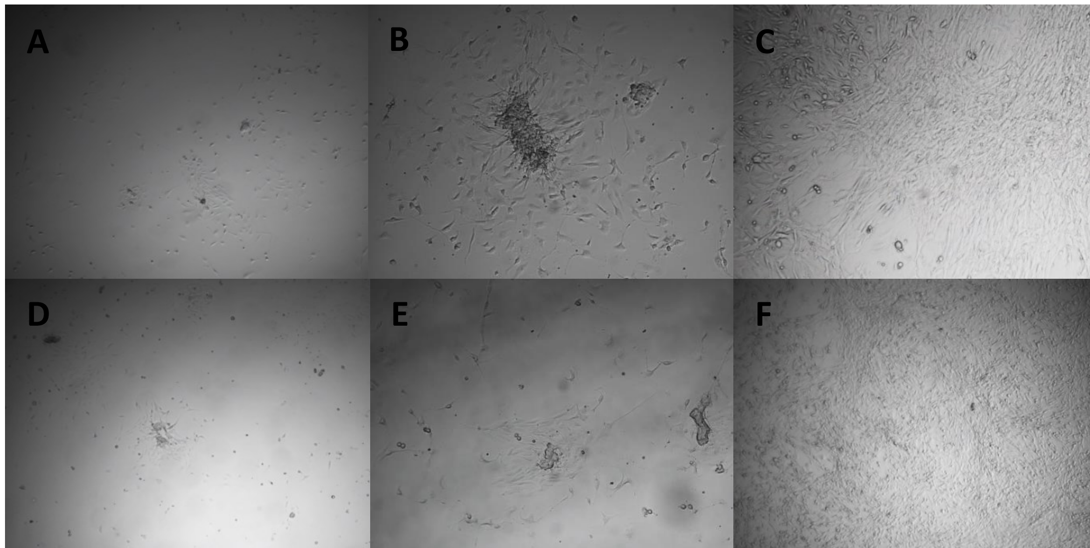

Figure 6.1 Cultured endothelial cells from CC & VE-Wt1 KO aortae *in vitro*. Endothelial cells were extracted from **A-C)** CC & **D-F)** VE-Wt1 KO aortae with collagenase and cultured *in vitro*. Endothelial cells attached, proliferated and migrated to form a monolayer, though other cell types were present. Images are from brightfield microscopy at Day 3, Day 5 & Day 7 (left to right). **A, B, D, E)** magnification 5x, **C, E)** magnification 10x. CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. Representative images from n=5.

6.5.2 Murine Menstruation as an Acute Model of Angiogenesis

The murine model of mouse menstruation developed by Brasted *et al.* (2003) and adapted by Cousins *et al.* (2014) could be a model of *in vivo* angiogenesis in which the effect of endothelial WT1 KO could be more effectively quantified. This model may also exhibit more dramatic effects as a result of endothelial WT1 KO, due to the previously observed WT1 expression in the vessels of the adult uterine tract.

Surgical work was carried out by Dr. Douglas Gibson and Prof. Philippa Saunders (Centre for Reproductive Health, University of Edinburgh) who kindly provided samples. Samples from this model of menstruation were provided at 8 hours, 12 hours, 24 hours and 48 hours after induction of decidualisation of the endometrial layer. WT1 was found to be expressed in the stromal cells of the endometrium and in a proportion of the endothelial cells of the uterine blood vessels.

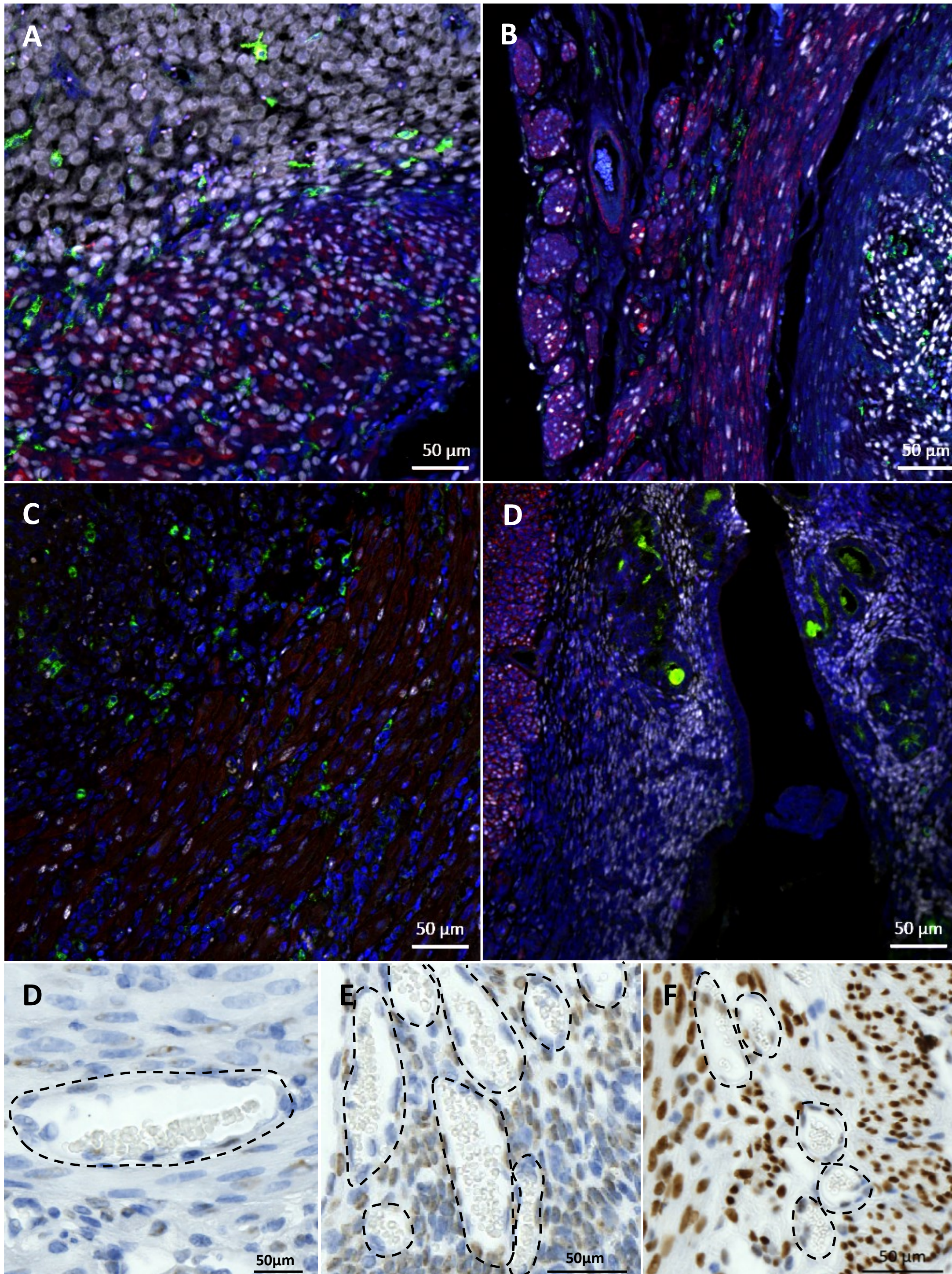

Figure 6.2 WT1 is expressed in the murine model of menstruation. The murine model of mouse menstruation (Cousins *et al.*, 2014) was assessed for WT1 expression by Isolectin-B4/αSMA/WT1/DAPI staining at **A)** 8 hours, **B)** 12 hours, **C)** 24 hours and **D)** 48 hours after induction of decidualisation. WT1 was also assessed by WT1 immunoprecipitation staining. **D)** 8 hours, **E)** 12 hours and **F)** 24 hours after decidualisation. WT1 was found to be expressed in the stromal cells and in the endothelium of vessels. Vessels indicated by dotted lines. Representative images from n=3.

6.5.3 **Wire Injury Model of Neointima Remodelling**

As WT1 was discovered to have a role, not only in angiogenesis, but in general vascular function, the wire injury model of neoinitimal remodelling was thought to be an effective model of the effect of WT1 on general vascular function. This model is of relevance to retinosis and atherosclerosis, in which endothelial proliferation and regeneration, fibrosis and inflammation are integral.

Intra-luminal injury was surgically induced in the left femoral artery using a wire injury model (Sata *et al.*, 2000). In C57Bl/6 mice, the role of WT1 should be assessed in this model by histology over the course of damage and regeneration, to determine when and where WT1 is expressed in vessels during damage, repair and remodelling. Neointimal remodelling was assessed in 3D by optical projection tomography (Kirkby *et al.*, 2011) at day 21 after induction of injury in VE-WT1 KO and CC mice. Quantification of lesion size could be carried out in the future from 3D images and by histology to determine if VE-WT1 KO mice have larger lesions than controls.

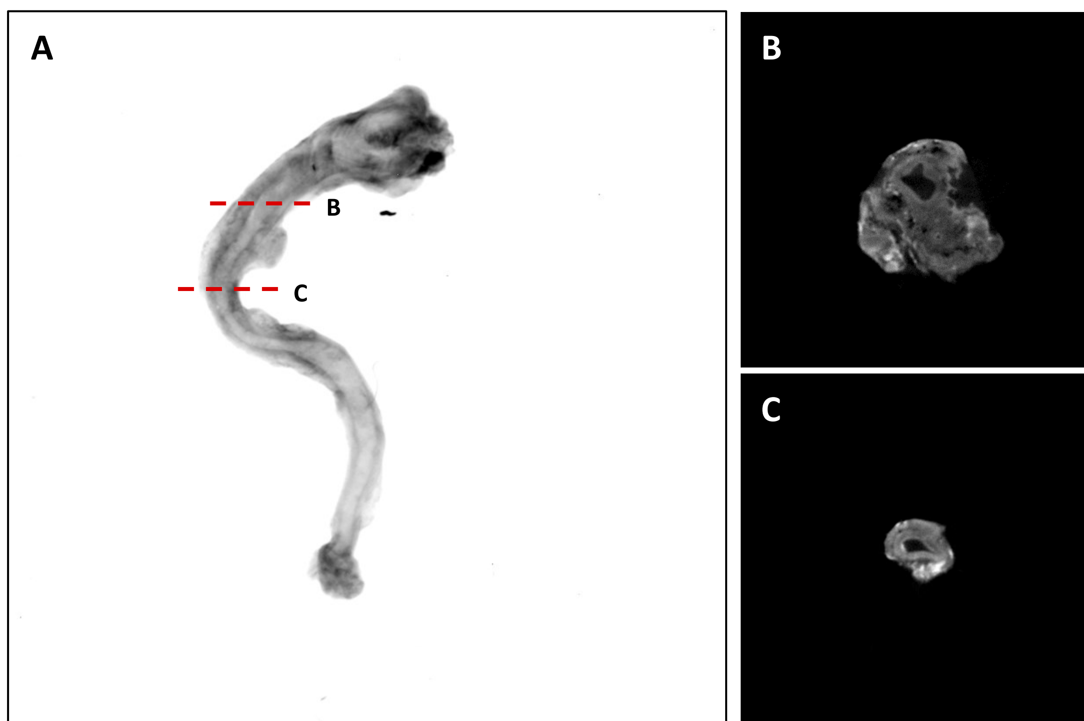

Figure 6.3 Neointima remodelling in the wire injury model. 3D Optical Projection images in CC mice from the wire injury model of neointima remodelling. **A)** A whole femoral artery at 21 days following wire injury. Wire injury was carried out at the top of the artery where remodelling is evident by increased tissue mass. Cross sections through the vessel shown in **B)** and **C)** are indicated by dotted red lines. CC = Cre control, VE-Wt1 KO = vascular endothelial WT1 KO. Representative images from n=5.

6.5.4 WT1 in Human Limb Ischaemia

To confirm the relevance of the findings from HLI in humans, clinical samples from limb amputation were obtained from patients at the Royal Infirmary of Edinburgh with limb ischaemia, as a result of diabetes or critical limb ischaemia. Samples were kindly provided with the help of Dr. Richard McGregor, Dr. Rachael Forsythe and Dr. Ross Jones (Royal Infirmary of Edinburgh). Samples were taken distally within diseased tissue and proximally in relatively healthy tissue. WT1 expression should be assessed in the vessels and muscle tissue in both these locations to determine the involvement of WT1 in ischaemia and ischaemic angiogenesis.

6.6 Conclusions

This thesis set out to determine the role of WT1 in cardiovascular disease, specifically in endothelial cells in ischaemic angiogenesis, utilising *in vivo* and *ex vivo* models of angiogenesis, alongside a murine endothelial WT1 KO and integrating comprehensive transcriptomic analysis. The hypothesis explored in this thesis was that WT1 is intrinsically involved in the response to ischaemic cardiovascular disease and hence, WT1 KO in the vascular endothelium would impair angiogenesis. Ultimately, this thesis adds to an exciting field, consolidating the role of WT1 in angiogenesis and in cardiovascular disease in general, while generating some completely novel insights relating to WT1 in areas which demand further investigation.

WT1 is an important factor in angiogenesis, though the focus on endothelial cells is too narrow to be physiologically relevant in cardiovascular disease. In agreement with this, endothelial WT1 KO impairs angiogenesis *ex vivo*, but not *in vivo*, with WT1 having a relatively early, transient role in angiogenesis. WT1 additionally acts as a transcription factor in mural cells, fibroblasts and immune cells both in the context of angiogenesis and beyond. Whether WT1 is directly regulating factors of importance to angiogenesis is unclear; it seems possible WT1 is enacting a functional shift in cells which increases their capacity to undergo angiogenesis through cellular processes such as migration and proliferation and potentially, through inducing a partial EndMT to improve their regenerative potential.

Certainly, WT1 has a broader scope of influence than previously anticipated. Within the vasculature, intriguing preliminary evidence suggests WT1 also regulates

vasculature tone and vascular permeability; both important in angiogenesis and cardiovascular disease in a broader sense. Outside of the vasculature, WT1 is also implicated in fibrosis and inflammation. One novel exciting observation is that WT1 may play a role in regenerative myogenesis in satellite cells and skeletal muscle myocytes. Along with identification of WT1 expressing MSCs and pre-B cells, this questions the broader implications of WT1 expression in adult stem cell populations; WT1 may be an essential regulator of adult stem cell function and differentiation, in part through EMT.

Angiogenesis and the other processes in which WT1 is implicated in cardiovascular disease *in vivo* could prove to be beneficial or detrimental depending on the nature of WT1 regulation; either WT1 may prove to be a silver bullet which can therapeutically target, angiogenesis, fibrosis, inflammation and muscle regeneration in one shot, or these may throw up unwanted side effects. This work questions the established consensus on WT1 as an important regulator of angiogenesis in endothelial cells alone and suggests WT1 may be regulating a number of cellular, vascular and regenerative processes of therapeutic relevance to targeting regeneration in cardiovascular disease, but also in a number of other pathological contexts. Finally, WT1 retains its status as a potentially beneficial therapeutic target in cardiovascular disease, but should be considered not only in endothelial cells in angiogenesis, where WT1 has scope to influence a number of processes, but in the vasculature in general and in the general pathophysiology and regeneration central to a number of cardiovascular diseases

5.3.3 **Identification of Potential WT1-Binding Sites in the Genome**

BLASTN by Ensembl (Aken *et al.*, 2016) is a tool for searching the genome for the location of short nucleotide sequences. Using three validated WT1-binding sequences the entire Mus musculus genome was screened for the location of these sequences within genes. In total, 2683 hits were found (Table 5.3.4), most of these were within introns.

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Appendix

A.i. **Identification of Potential WT1-Binding Sites in the Genome**

BLASTN by Ensembl (Aken *et al.*, 2016) is a tool for searching the genome for the location of short nucleotide sequences. Using three validated WT1-binding sequences the entire Mus musculus genome was screened for the location of these sequences within genes. In total, 2683 hits were found (Table A.i), most of these were within introns.

Sequence	BP	Source	Hits
5'- GCGTGGGAGT -3'	10	(Nakagama, Heinrich and Pelletier, 1995; Motamedi *et al.*, 2014; Kann *et al.*, 2015)	881
5'- GCG-TGG-GCG-(T/G)(G/A/T)(T/G) -3'	12	(Hamilton, Barilla and Romaniuk, 1995; Motamedi *et al.*, 2014; Kann *et al.*, 2015)	319
5'- GGAGAGGGAGGATC -3'	14	(Little *et al.*, 1996; Motamedi *et al.*, 2014; Dong *et al.*, 2015)	1483

Table A.i: BLASTN screening for potential WT1-binding sites within the mouse genome. Three validated WT1-binding sequences were used to identify potential WT1 binding within the Mus musculus genome. Shown are the sequences, number of base pairs (BP) in the sequence, the earliest source where this sequence was identified and the number of hits within the genome for each sequence.

The overlap between these genes and the gene expression data obtained from the *in vivo* microarray data was assessed, examining the number of genes identified from BLASTN that were also identified from the gene expression data (Table A.ii). However, between the two datasets, the number of genes which overlapped was not larger in genes whose expression was different between $WT1^+$ and $WT1^-$ than in genes which were completely unchanged. Further, 45.88% of all the genes identified from the microarray, whether changed or not between $WT1^+$ and $WT1^-$ cells, were also identified by BLASTN screening. While there will likely be direct targets of WT1 in this list of genes, the false hit rate is too high for meaningful analysis; these 12,964 genes comprise ~56% of the total mouse genome, which suggests the number of hits from BLASTN is not greater than would be expected by chance.

	Matches	Genes/category	Overlap
Increased at day 7 only	76	1,100	6.91%
Increased at day 21 only	70	772	9.07%
Increased at day 7 & 21	166	1,611	10.30%
Decreased at day 7 only	106	1,488	7.12%
Decreased at day 21 only	38	647	5.87%
Decreased at day 7 & 21	102	1,457	7.00%
Unchanged	539	7,520	7.17%
Total	1097	12,964	45.88%

Table A.ii:Overlap between genes identified by BLASTN and *in vivo* SSI microarray data. There is very little overlap between genes which are changed in $WT1^+$ v $WT1^-$ cells and those identifies by BLASTN screening for WT1-binding motifs. "Increased" and "Decreased" refers to genes which have higher and lower expression in $WT1^+$ cells, respectively. "Unchanged" are ones which weren't changed with WT1 expression and "Total" are all genes in the database. Number of matches are shown, genes/category in total are shown and the overlap as a % of these genes in each category.

A.ii. Potential WT1-Binding to Promoter Sequences

Genes with higher or lower expression at both day 7 and day 21 were analysed with RSAT (Medina-Rivera *et al.*, 2015) to extract the Mus musculus promoter sequences of all these genes and then screen promoters for the location of previously identified and validated WT1-binding motifs (Table A.iii). Three motifs elicited a number of hits. False hit rates were identified by generating a number of random nucleotide sequences of equal length to the actual motif and screening promoters for hits. The WT1-binding motif GGAGG(A/G) (Reynolds *et al.*, 2003) elicited 1786 hits, but with a 67.1% false hit rate due to its short length of 6bp, so was removed from further analysis. To reduce the number of false positive hits, no substitutions in sequence were permitted, though it is known WT1 will often bind to sequences with some substitutions.

Sequence	Source	False hits	# hits
GCGGGGGCG or CGCCCCCGC	(Rauscher *et al.*, 1990)	24.9%	128
TCCTCCTCCTCCTCTCC	(Wang *et al.*, 1993; Lefebvre *et al.*, 2015)	-	0
GCGTGGGAGT	(Nakagama, Heinrich and Pelletier, 1995; Motamedi *et al.*, 2014; Kann *et al.*, 2015)	-	3
GGAGG(A/G)	(Reynolds *et al.*, 2003; Motamedi *et al.*, 2014; Dong *et al.*, 2015)	67.1%	1786
GCGTGGGCG(T/G)(G/A/T)(T/G)	(Hamilton, Barilla and Romaniuk, 1995; Motamedi *et al.*, 2014; Kann *et al.*, 2015)	1.8%	22
GGAGAGGGAGGATC	(Little *et al.*, 1996; Motamedi *et al.*, 2014; Dong *et al.*, 2015)	-	1

Table A.iii: Number of potential WT1-binding sites in promoter sequences from RNA-seq genes. Genes changed at both day 7 and day 21 in $WT1^+$ v $WT1^-$ cells had their promoter sequences screened for the location of WT1-binding motifs using RSAT (Medina-Rivera *et al.*, 2015). The sequences, their source, the false hit rate (determined with randomly generated nucleotide sequences of the same length) and the number of hits in the extracted promoter regions is shown.

The 22 genes identified with the GCGTGGGCG(T/G)(G/A/T)(T/G) sequence (Hamilton, Barilla and Romaniuk, 1995) in their promoter and 128 genes with the GCGGGGGCG or CGCCCCCGC sequence (Rauscher *et al.*, 1990) in their promoter are shown in Figure A.i. The majority of these genes had one, or often multiple, WT1-binding motifs in the promoter within 200bp of the upstream start site of the promoter region. Some of these identified genes are already recognised as genes that are directly regulated by WT1, such as Thrombospondin-1 (Thbs1), while others need to be validated.

-3000
-2500
-2000
-1500
-1000
-500
0
002N15Rik
455G09Rik
068E07Rik
Abca3
Abhd8
Actn1
Adamts7
Akirin1
Akr1b8
Alg5
Anks1
Arpc5
Atmin
B3galnt2
B3gnt8
B4galt1
Bcl2l11
Cacna2d1
Camk2n2
Capn6
Cbl
Cds1
Clk3
Cmtm6
Col12a1
Col8a1
Coro1b
Cotl1
Crip2
Crlf3
Cstb
Ctnnbip1
Ctsb
Ctsl
Cxcr4
Dag1
Dctd
Ddhd1
Dlg5
Dmwd
Dok4
Dusp2
Efs
Elmo1
Evc
Evc2
Faim
Fam13c
Fam171a2
Fbxo21
Fbxw7
Fem1c
Fhl1
Frmd6
Fst
Galk1
Gldc
Gli2
Gltp
Gpr153
Hes1
Hic1
Hist2h3c1
Hoxb7
Hpcal1
Hspa9
Hspb8
Htatip2
Ifitm6
Il13ra1
Itpr3
Josd1
Kdelr2
Lamc1
Large
Lgi3
Lgmn
Lin54
Lysmd2
Mafk
Mapk8ip1
Meis1
Mmp14
Myl9
Nr2f6
Nradd
Nuak1
Nubp2
Olfml3
Pabpc1
Pard6g
Pccb
Pepd
Pip4k2a
Pja2
Pkn1
Plcb3
Plcl2
Plekhf1
Plekho2
Ppargc1b
Ptpn11
Ptpre
Rasgef1b
Rbbp7
Rel
Rgl1
Rhog
Rora
Rps24
Rps6ka1
Rqcd1
Rrbp1
Sccpdh
Sec24d
Sema5a
Shf
Slc13a3
Slc24a3
Slc25a28
Slc7a6
Slit2
Snx30
Snx8
Sox5
Ssfa2
Syf2
Tagap
Thbs1
Tmem135
Tmem86a
Tnip2
Tshz1
Tspan14
Ttc28

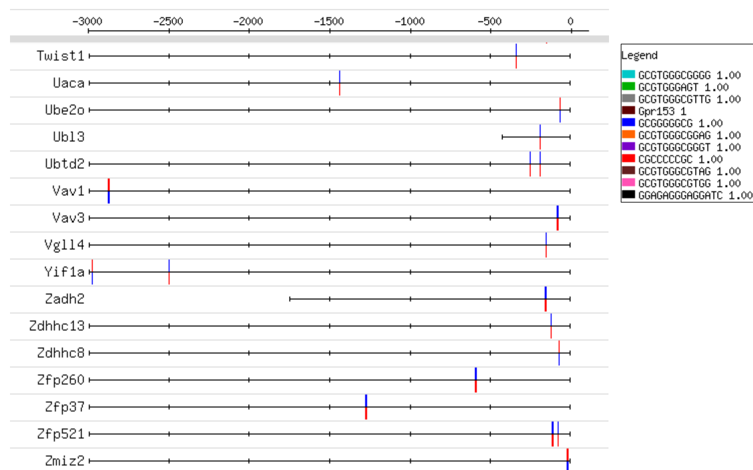


Figure A.i: WT1-binding sites in promoter sequences from RNA-seq genes are primarily at the 3' end of the promoter. Genes changed at both day 7 and day 21 in WT1^{+} v WT1^{-} cells had their promoter sequences screened for the location of WT1-binding motifs using RSAT (Medina-Rivera *et al.*, 2015). Sequences matching with known WT1-binding motifs are shown by coloured lines (above the line for 5'-3' and below for 3'-5'). Scale indicates number of base pairs from the 3' end of the promoter sequence.

A.iii. **Role of Potential WT1 Target Genes**

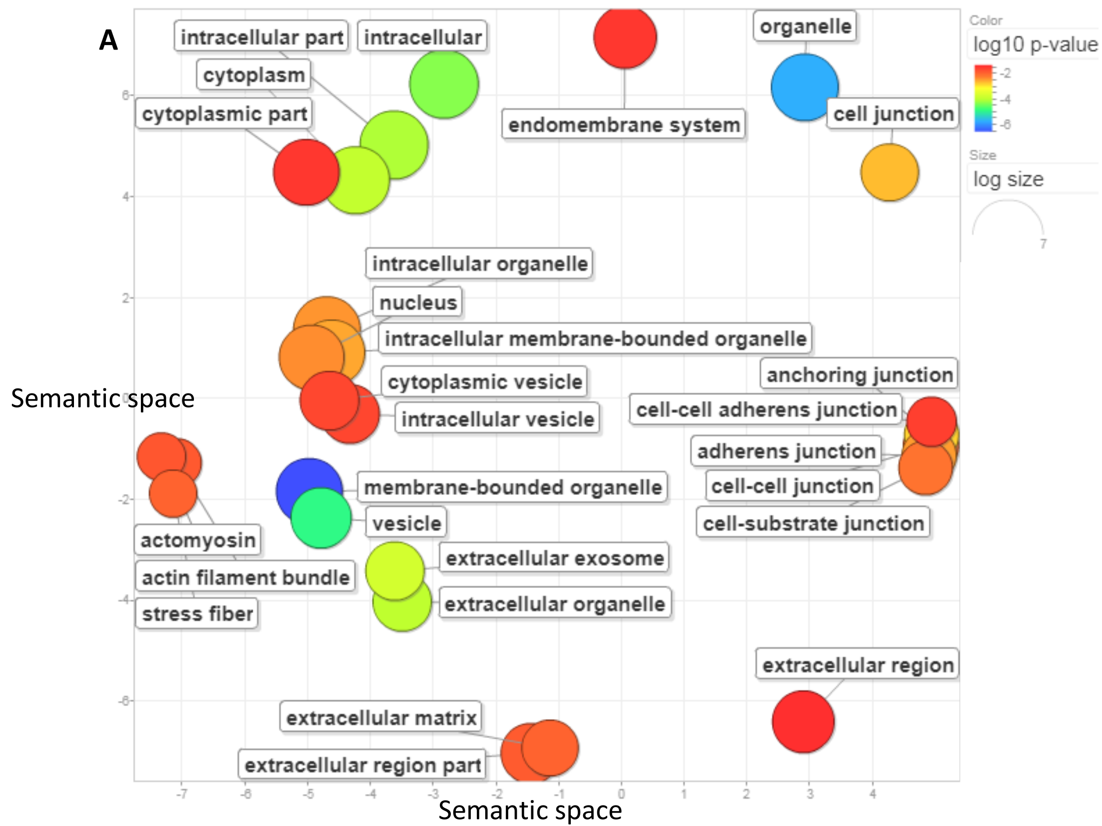
DAVID (Huang *et al.* 2008; Huang *et al.* 2009) was used to identify GOterms associated with the genes identified by RSAT (Medina-Rivera *et al.*, 2015). Only the 12,964 genes detected in our micro-array were used as the background for analysis, to avoid any context-dependent bias. GOterms were reduced by redundancy and presented using ReviGO (Supek *et al.*, 2011) to show the cellular locations (Figure A.ii A), molecular functions (Figure A.ii B) and biological processes (Figure A.ii C) with which these genes are associated. There were a degree of overlap between these GOterms and the GOterms identified with the WT1^{+}cell gene expression profile in section A.i.

In terms of cellular localisation (Figure A.ii A), as in 5.3.2.4, actin filament bundle, actomyosin and stress fibres were identified. There were also terms relating to extracellular space and extracellular matrix and different types of vesicles. However, there were more references to cytoplasmic terms, cell-cell junctions and the nucleus than in section A.i.

In terms of molecular functions (Figure A.ii B), the two main groups of GOterms overlapped to some extent with section A.i. One group related to the binding of various

metal ions, while the other related to binding of various proteins, including Laminin-1. There were also significant GOterms relating to the activity of various peptidases.

In terms of biological processes (Figure A.ii C), as in section A.i, angiogenesis was important. There were also processes relating to cell migration, proliferation, adhesion and death. Regulation of stem cell differentiation was significant, as was vesicle mediated transport, collagen catabolism and actin filament-based process. Some other processes related to DNA transcription, such as DNA-binding and RNA polymerase II activity.


A
intracellular part
intracellular
cytoplasm
cytoplasmic part
endomembrane system
organelle
cell junction
intracellular organelle
nucleus
intracellular membrane-bounded organelle
cytoplasmic vesicle
intracellular vesicle
anchoring junction
cell-cell adherens junction
adherens junction
cell-cell junction
cell-substrate junction
membrane-bounded organelle
vesicle
actomyosin
actin filament bundle
stress fiber
extracellular exosome
extracellular organelle
extracellular region
extracellular matrix
extracellular region part
Semantic space
Semantic space
Color
log10 p-value
Size
log size

B
calcium ion binding
zinc ion binding
transition metal ion binding
metal ion binding
cation binding
ion binding
laminin binding
cell adhesion molecule binding
proteoglycan binding
cytoskeletal protein binding
protein kinase binding
protein domain specific binding
laminin-1 binding
protein complex binding
kininogen binding
ligand-dependent nuclear receptor transcription coactivator activity
signaling adaptor activity
cysteine-type peptidase activity
cysteine-type endopeptidase activity
guanyl-nucleotide exchange factor activity
aminopeptidase activity
Semantic space
Semantic space
Color
log10 p-value
Size
log size

Figure A.ii: Cellular locations, molecular functions and biological processes associated with potential WT1 target genes. Genes detected as having potential WT1-binding sites within their promoter regions were analysed using DAVID (Huang, Sherman and Lempicki, 2008, 2009) to determine GOterms associated with these genes. Terms were sorted for redundancy and visualised by ReviGO (Supek *et al.*, 2011) for associated **A)** cellular locations, **B)** molecular functions and **C)** biological processes. Each circle is a GOterm. Size of circle indicates log size of changes in expression and colour represents $\log^{10}$ p-value for significance of each term. X and Y axis are semantic space, such that GOterms that are biologically similar are closer together.

A.iv. **Pathway Analysis of Potential WT1 Target Genes**

DAVID (Huang, Sherman and Lempicki, 2008, 2009) was used, to identify pathways from the KEGG pathway database with which potential WT1 target genes were significantly associated. 9 KEGG pathways were significantly associated with these genes (Table A.iv), suggesting these pathways may be directly regulated to some extent by WT1.

Pathway	Genes	P-Value
Leukocyte trans-endothelial migration	6	2.22E-03
Proteoglycans in cancer	6	1.90E-02
Focal adhesion	6	2.05E-02
Bacterial invasion of epithelial cells	4	2.15E-02
Regulation of actin cytoskeleton	6	2.33E-02
Galactose metabolism	3	2.46E-02
Phosphatidylinositol signalling system	4	3.77E-02
Chemokine signalling pathway	5	6.22E-02
Long-term potentiation	3	9.03E-02

Table A.iv: KEGG pathways associated with potential WT1 target genes. 9 pathways were identified as being associated with potential WT1 target genes using DAVID (Huang, Sherman and Lempicki, 2008, 2009) and the KEGG pathway database. Only genes higher in WT1^{+} cells at both day 7 and day 21 were analysed. Shown are the number of genes in that pathway elevated in WT1^{+} cells and the p-value for identifying that pathway.

A.x. **Integrative Analysis of WT1^{+} Gene Profile and Potential WT1 Binding**

In order to attempt to further refine the effect WT1 has on gene expression how this impacts the function of WT1^{+} cells, integrated analysis on the data from RNA-seq and RSAT was carried out. As previously mentioned, analysis of both the RNA-seq and RSAT data revealed WT1 as a significant process. Using data from RNA-seq, integrated with the RNA-seq expression data and genes selected by DAVID as being

associated with angiogenesis, the change in gene expression was quantified between WT1⁺ cells and WT1⁻ cells in the 11 genes associated with angiogenesis which are potentially directly regulated by WT1 (Figure A.iii). This revealed expression of 8 of these genes was higher in WT1⁺ cells at day 7 and day 21 and expression of 3 was lower.

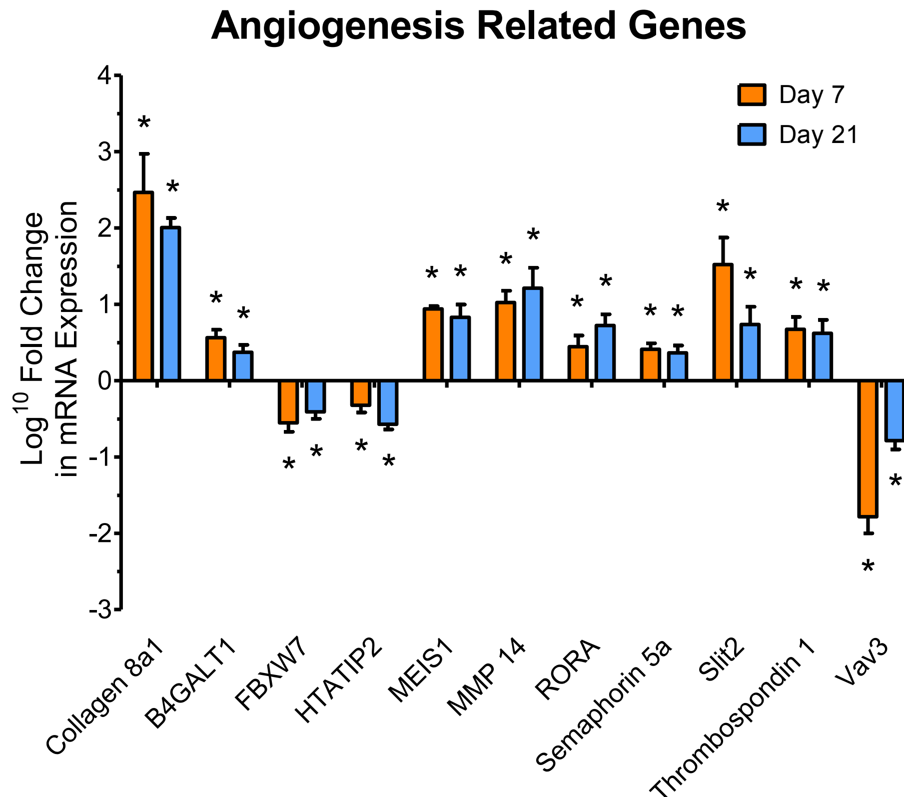


Figure A.iii: WT1 directly affects gene expression of 11 genes involved in angiogenesis. Graph shows log^{10} fold change in mRNA expression between WT1⁻ and WT1⁺ cells of various genes identified as being associated with angiogenesis and which also have potential WT1 binding sites in their promoters. Cells were FACS sorted from SSI sponges from WT1-GFP mice at day 7 and 21. RNA-seq was carried out to determine expression levels. Promoters of genes were screened for WT1-binding motifs using RSAT (Supek *et al.*, 2011) Gene names are the official gene symbols or full names. *fdr $q<0.05$, limma R bioconductor package. WT1⁻ v WT1⁺. n=3.

From previously identified KEGG pathways, 5 of the 9 pathways identified as being significant from analysis of RSAT WT1-binding genes (Section A.ii) overlapped with significant pathways from the pathways identified relating to the RNA-seq data (Section 5.3.2.5) (Table A.v). From these pathways, using KEGG, a number of

pathways were visualised, demonstrating thes stages in the pathways in which there were changes in gene expression between $WT1^+$ and $WT1^-$ cells and the stages in the pathway at which WT1 was potentially directly regulating intrinsic genes. VEGF-Signalling (Figure A.iv), Leukocyte Trans-Endothelial Migration (Figure A.v), Regulation of Actin Cytoskeleton (Figure A.vi) and Focal Adhesion (Figure A.vii) were visualised. These WT1 appears to having an effect at multiple stages in these pathways and these pathways interact with each other to some extent. From visualising these pathways, it became clear that Phosphoinositide 3-kinases (PI3Ks) played a role in all these pathways and number of PI3Ks were influenced by WT1. On interrogation of the PI3K-Akt pathway (Figure A.viii), it was evident that more than a third of all the genes in the pathway were significantly higher of lower in $WT1^+$ cells v $WT1^-$ cells at day 7 or day 21. WT1 potentially directly regulates 4 genes in this pathway; Pkn1, Bcl2l11, Lamc1 and Thbs1.

	Matches by Dataset	
Pathway	RNA-seq	WT1-binding
Bacterial invasion of epithelial cells		
Focal adhesion		
Leukocyte trans-endothelial migration		
Proteoglycans in cancer		
Regulation of actin cytoskeleton		

Table A.v: Biological pathways in which changes in WT1 expression and direct regulation by WT1 have an effect. KEGG pathways were identified by DAVID (Huang, Sherman and Lempicki, 2008, 2009) from RNA-seq data and RSAT Wt-binding data. Shown above are the 5 pathways which were identified as significant to both these datasets.

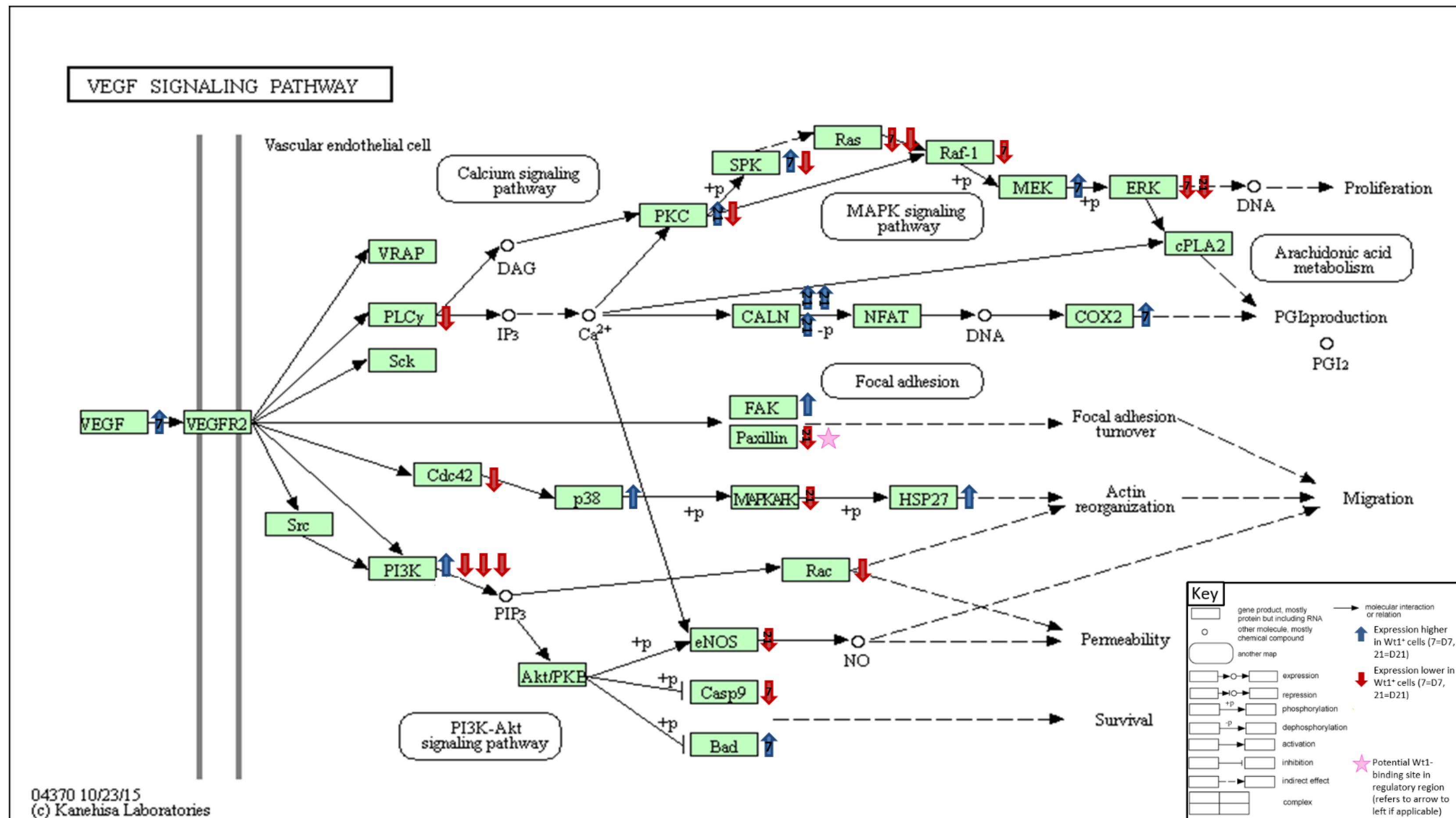


Figure A.iv: The effect of WT1 on VEGF signalling. The VEGF signalling pathway, as extract from KEGG. Annotated are the changes in the expression of genes at each step in the pathway, where up arrows indicate increased expression in WT1^{+} cells and down arrows indicate decreased expression in WT1^{+} cells from WT1-GFP RNA-seq data. If no annotation, day 7 & 21, 7 = day 7 only, 21 = day 21 only. Stars indicate genes identified from RSAT (Supek *et al.*, 2011) as being potential direct targets of WT1.

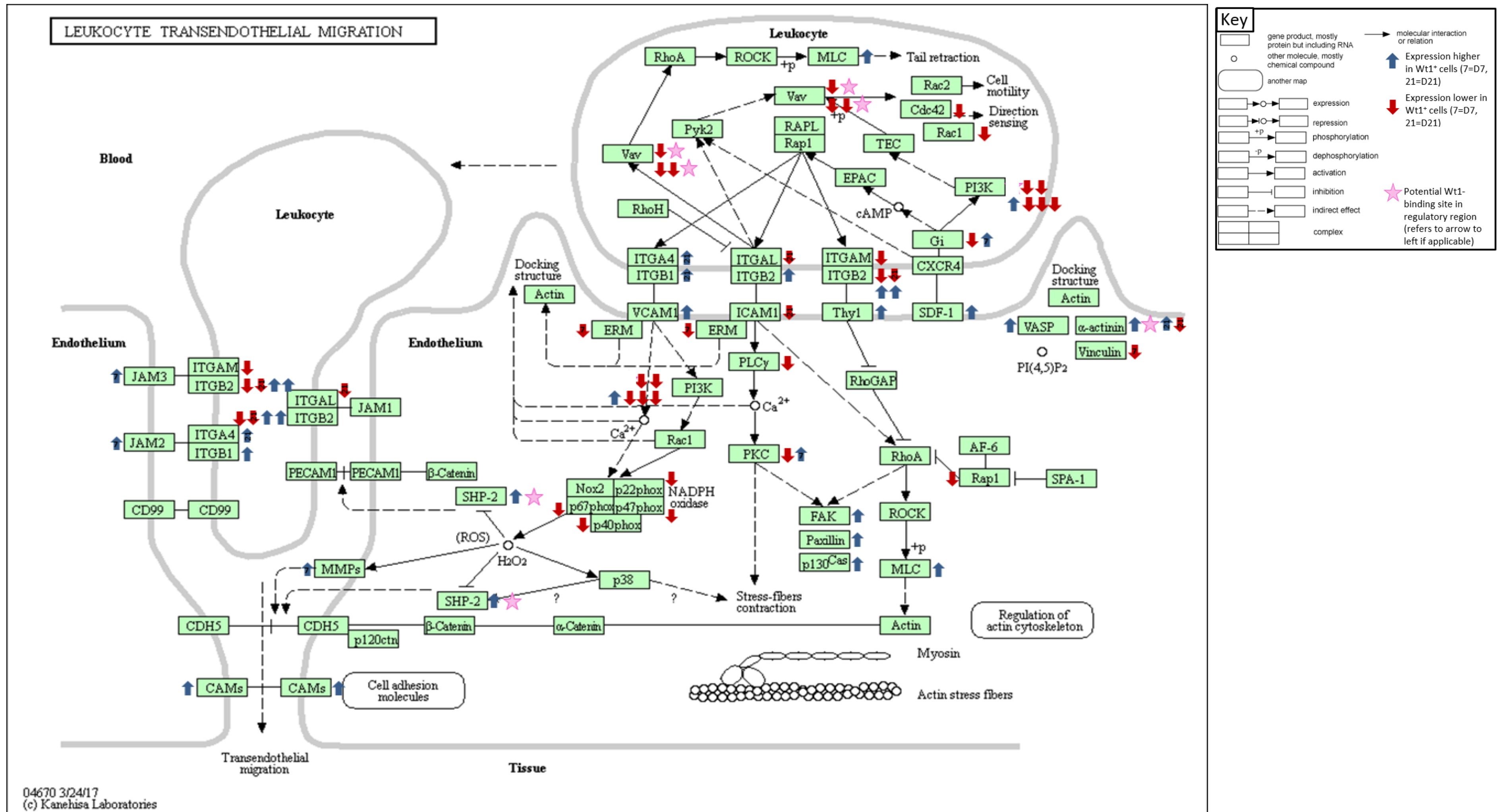


Figure A.v: The effect of WT1 on Leukocyte transendothelial migration. The leukocyte transendothelial pathway, as extract from KEGG. Annotated are the changes in the expression of genes at each step in the pathway, where up arrows indicate increased expression in WT1$^+$ cells and down arrows indicate decreased expression in WT1$^+$ cells from WT1-GFP RNA-seq data. If no annotation, day 7 & 21, 7 = day 7 only, 21 = day 21 only. Stars indicate genes identified from RSAT (Supek *et al.*, 2011) as being potential direct targets of WT1.

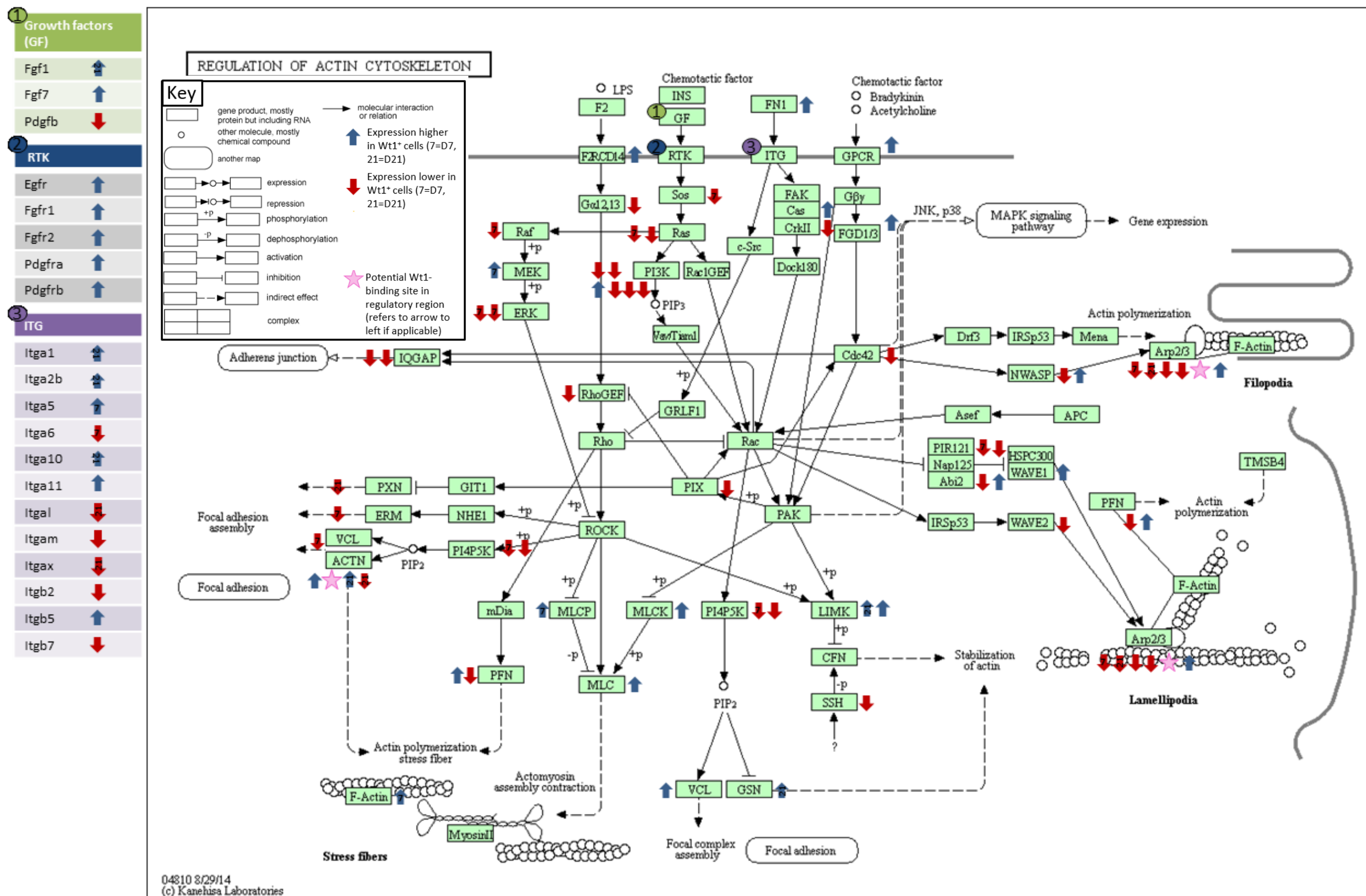


Figure A.vi: The effect of WT1 on regulation of actin cytoskeleton. Regulation of actin cytoskeleton, as extract from KEGG. Annotated are the changes in the expression of genes at each step in the pathway, where up arrows indicate increased expression in WT1$^+$ cells and down arrows indicate decreased expression in WT1$^+$ cells from WT1-GFP RNA-seq data. If no annotation, day 7 & 21, 7 = day 7 only, 21 = day 21 only. Stars indicate genes identified from RSAT (Supek *et al.*, 2011) as being potential direct targets of WT1.

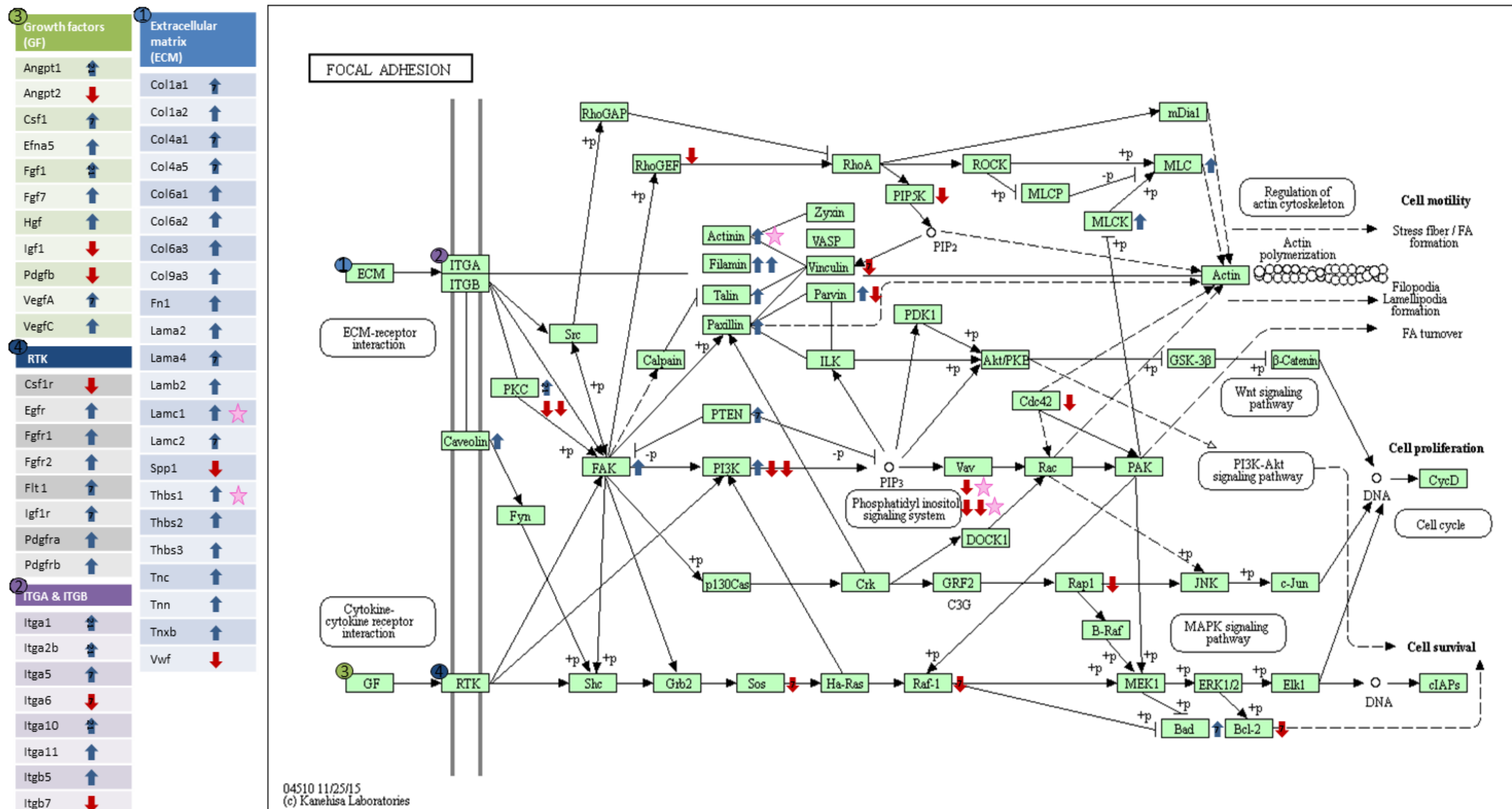


Figure A.vii: The effect of WT1 on focal adhesion. The focal adhesion pathway, as extract from KEGG. Annotated are the changes in the expression of genes at each step in the pathway, where up arrows indicate increased expression in WT1^{+} cells and down arrows indicate decreased expression in WT1^{+} cells from WT1-GFP RNA-seq data. If no annotation, day 7 & 21, 7 = day 7 only, 21 = day 21 only. Stars indicate genes identified from RSAT (Supek *et al.*, 2011) as being potential direct targets of WT1.

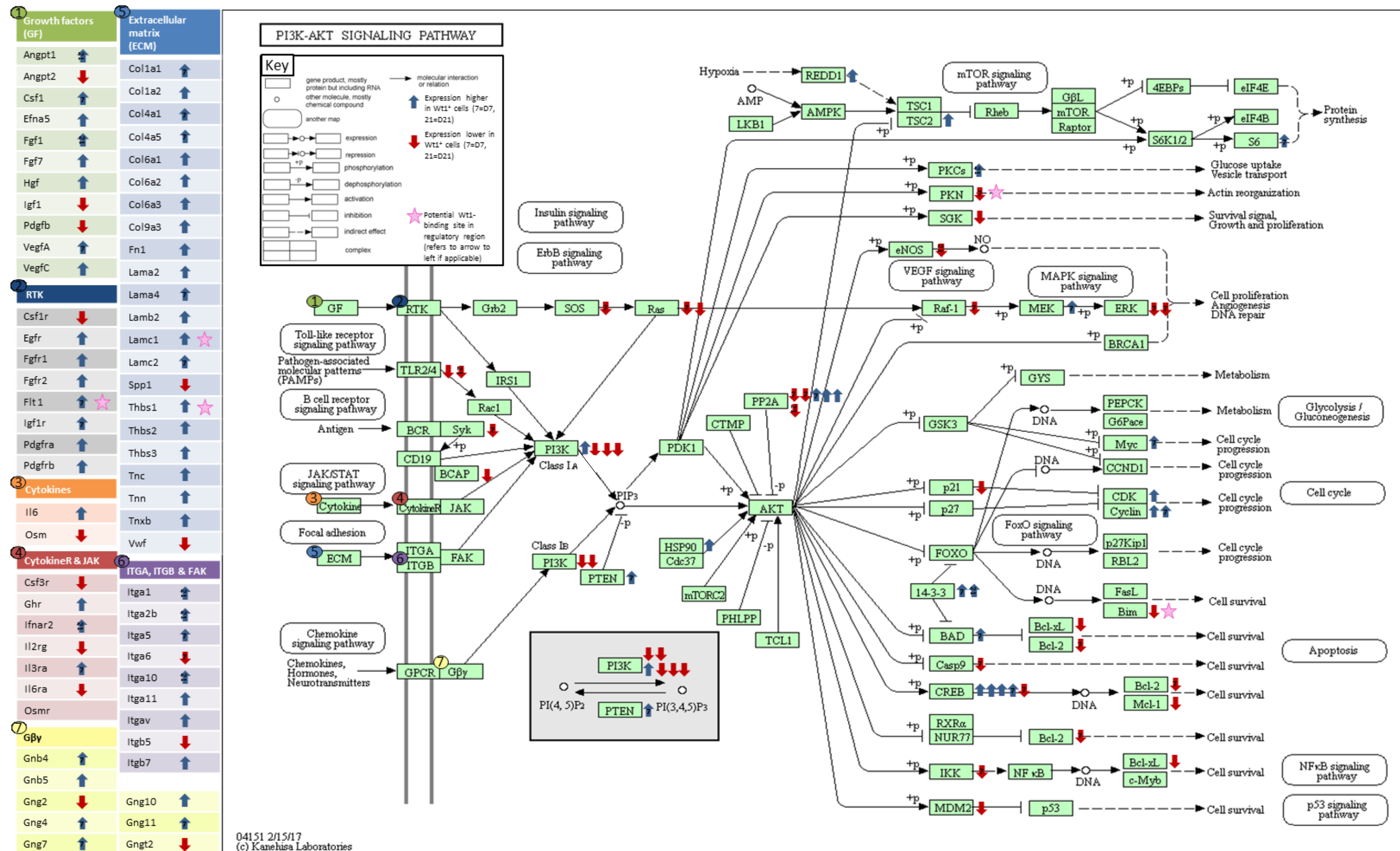


Figure A.viii: The effect of WT1 on the PI3K-Akt signalling pathway. The PI3K-Akt pathway, as extract from KEGG. Annotated are the changes in the expression of genes at each step in the pathway, where up arrows indicate increased expression in WT1^{+} cells and down arrows indicate decreased expression in WT1^{+} cells from WT1-GFP RNA-seq data. If no annotation, day 7 & 21, 7 = day 7 only, 21 = day 21 only. Stars indicate genes identified from RSAT (Supek *et al.*, 2011) as being potential direct targets of WT1.