UNIVERSITY OF LEICESTER

Studies on the development of pharmacological inhibitors of Angiopoietin-2

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

By

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Omeed,

Congratulations on your thesis.

It has been a pleasure supervising you for your PhD.

Good luck with your future research

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Abstract

Blocking Angiopoietin-2 pathogenic effects mediated through Tie2 and other receptor is the main goal of this study. Angiopoietin-2 is a vascular growth factor that binds to the Tie2 receptor and is expressed mainly in endothelial cells. Several studies demonstrate the destabilizing effects of Ang2 in the vascular system of the human body. On the other hand, Ang1 has a stabilizing effect necessary for maintaining the integrity of the vessels. Increased levels of Ang2 can block the binding of the Ang1 to the Tie2 receptor and stimulate pathogenic signalling through Tie2 and β-integrin pathways. These effects can promote vessel destabilization through the induction of apoptosis, inflammation, and leakage.

Therefore, the aim of this study was to improve an Ang2 ligand-trap as a potential inhibitor for its regressive effects, and to investigate the possibility of Ang2 signalling through a Tie2 independent pathway. In order to inhibit Ang2 effects, a ligand-trap previously described was improved by mutagenesis and by pentamerization. The mutagenesis resulted in increased relative affinity 2-4 fold, while pentamerization improved the relative affinity of the current ligand-trap (R3-Fc) up to 18-fold using pentameric R3 design (R3-COMP). In addition, in this study the possibility that Ang2 signalling through Tie2 independent pathway was investigated for VE-cadherin and Tie1 cleavage. This study found Ang2 as a novel activator of VE-cadherin and Tie1 cleavage through an integrin pathway and by activation of ADAM10 and ADAM17 respectively. Furthermore, the study found Ang2 is required for the pro-cleavage effects of TNF-α.

In conclusion this study produced a higher affinity Ang2 ligand-trap and showed that Ang2 signals through integrins and this pathway can be inhibited to suppress Ang2 effects on VE-cadherin and Tie1 cleavage.

Overall, these data provide a strategy for inhibition of Ang2 effects through Tie2, using a high affinity ligand-trap, as well as inhibition of integrin mediated effects by use of integrin blocking reagents.

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List of abbreviations

Ang1	Angiopoietin-1
Ang2	Angiopoietin-2
Bmax	Maximal binding
CHD	Coronary heart disease
CHF	Congestive heart failure
COMP	Cartilage oligomeric matrix protein
CRP	C-reactive protein
CVD	Cardiovascular disease
dsDNA	Double-stranded DNA.
E12.5	Embryonic age 12.5
EAHY	Immortalized human umbilical vein endothelial cells
ECs	Endothelial cells
EGF	Epidermal growth factor.
ESR,	Erythrocyte Sedimentation Rate
FBS	Fetal bovine serum
FCS	Fetal calf serum
HCMECs	Human cardiac microvascular endothelial cells
HEK cell	Human embryonic kidney cell
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
LPS	Lipopolysaccharide
LSGS	Low Serum Growth Supplement
MI	Myocardial infarction
PI3K	Phosphatidylinositol 3-kinase
siRNA	Small interfering RNA
SMC	Smooth muscle cell
Tek	Endothelial-specific receptor tyrosine kinase
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains.
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor A
VEGF-R	Vascular endothelial growth factor receptor

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Chapter 1: Introduction

The angiopoietins are a family of secreted ligands that act primarily through the receptor tyrosine kinase Tie2 (Fukuhara *et al*, 2010). In humans the angiopoietins expressed are Ang1, 2 and 4 (Brindle *et al*, 2006). The angiopoietin / Tie system has a critical role in regulating angiogenesis, maintaining vascular homeostasis, vascular maturation as well as destabilization and remodelling (Fiedler *et al*, 2003; Bogdanovic, 2009). Angiopoietin-2 (Ang2) is a competitive inhibitor of Angiopoietin-1 (Ang1) binding; it suppresses the protective effects of Ang1, which can affect the integrity of the vascular wall and induce leakage, oedema, and increases inflammation (Brindle *et al*, 2006). Ang2 contributes to the progression of some diseases by controlling the signalling pathways that are responsible for angiogenic remodelling (Reiss *et al*, 2007). Defects in the endothelial integrity of hypertensive and atherosclerotic patients have a great impact on the progression of certain pathologies such as myocardial infarction and stroke (Felmeden *et al*, 2003). This study focuses on the possibility of finding a drug that can block the pathological effects of the Ang2 acting through its primary receptor Tie2. The study will also investigate alternative pathways through which Ang2 can exert its pathogenic effects.

Enhancing vessel integrity and suppressing inflammation and endothelial permeability by blocking the antagonistic effect of Ang2 on Tie2 and other receptors can protect the microvascular system of the body (Chen *et al*, 2013). Therefore, identifying Ang2 signalling pathways and blocking Ang2 pathogenic effects will increase the survival of the cells and decrease damage to the vascular endothelial cells.

1.1 The Tie family of receptors

The Tie family are tyrosine kinases with immunoglobulin-like and EGF-like domains (Fox *et al*, 2012). This family play a crucial role in maintaining the integrity and organization of the vascular endothelial cell wall (Pfaff *et al*, 2006). Tie expression occurs primarily in endothelial cells of the inner lining wall of the blood vessels (Thurston, 2003; Ray *et al*, 2000). The family consists of two receptors, Tie1 and Tie2, which both share similar overall structures. The amino acid identity between Tie1 and Tie2 reaches 30% in the extracellular domains and approximately 76% in the intracellular domains (Sato *et al*, 1993).

The extracellular domains of Tie1 and 2 share the same basic structure (Nakashima *et al*, 2001) (Figure 1.1). Tie2 responds to angiopoietin ligands which can control the angiogenesis process including sprouting, proliferation, and maturation of vessels as well as regulating normal vascular maintenance (Chen and Stinnett, 2008; Chong and Lip, 2004; Brindle *et al*, 2006). These ligands control vascular leakage and inflammation (Ghosh *et al*, 2012; Parikh, 2013). All these actions indicate the important role of a Tie2/Ang system which can contribute to vessel protection as well as vessel regression (Chen *et al*, 2013; Garcia *et al*, 2012).

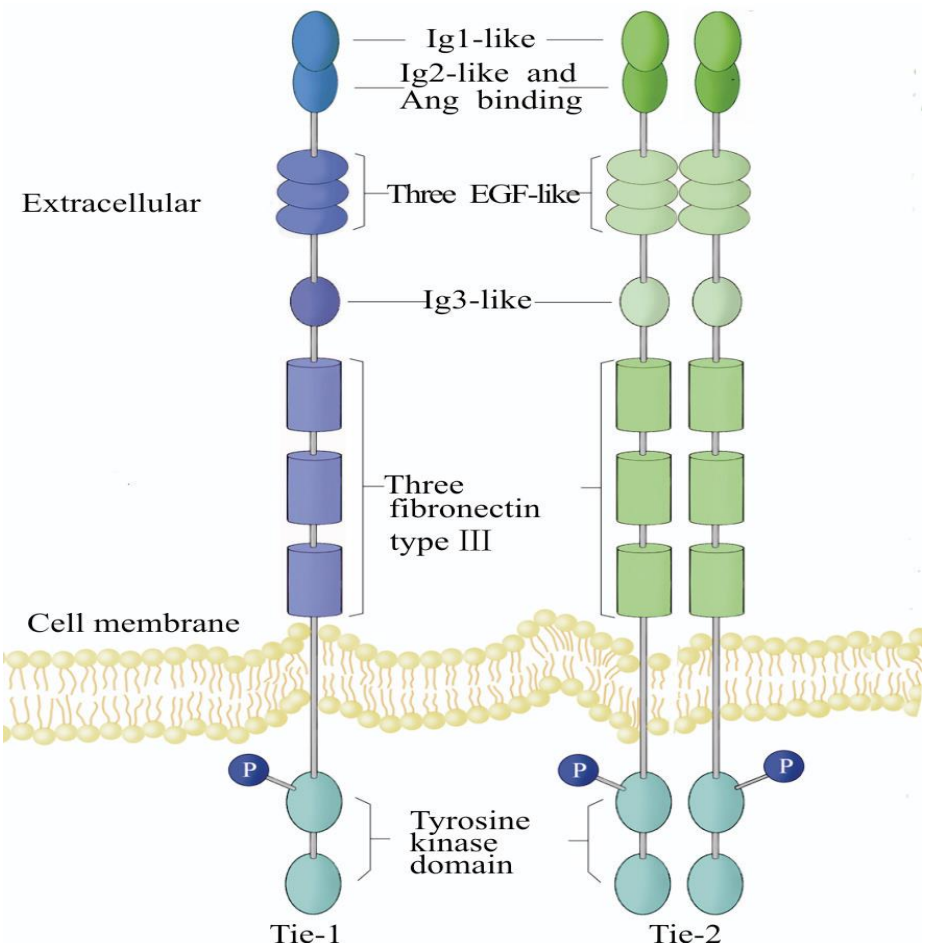


Figure 1.1: The structure of Tie receptor.

The Tie structure consists of three domains: extracellular, transmembrane, and the intracellular domain. The extracellular domain of Tie1 and 2 has a multi-domain form that contains three epidermal growth factor (EGF) homology domains set between two immunoglobulin-like loops followed by three-fibronectin type III. The image was taken from (Yang *et al*, 2015).

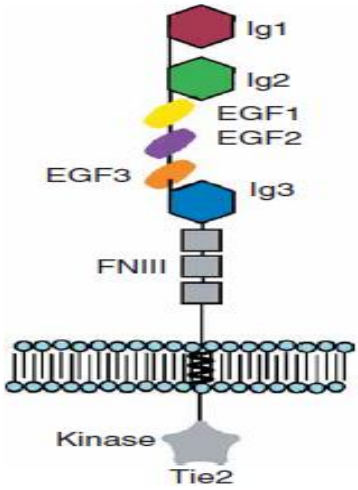
1.1.1 Tie2 receptor

Tie2 (also known as TEK) is essential for vascular remodelling and maintaining the integrity of the blood vessels (Augustin *et al*, 2009; Gjini *et al*, 2011). It is expressed mainly by endothelial cells (ECs), specifically on cells that cover the interior walls of the blood and lymphatic vessels (Chung, 2014). It has a critical role in the development of the cardiovascular system.

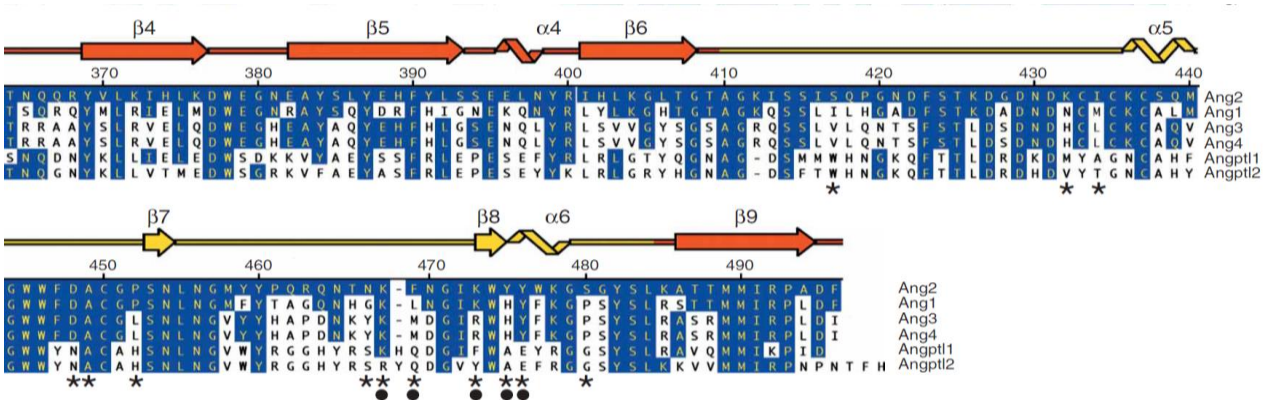
The Tie2 receptor consists of three main domains: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Barton *et al*, 2006). The extracellular ligand-binding domain contains two amino-terminal immunoglobulin (Ig)-like domains plus another Ig-homology, three epidermal growth factors (EGF) and the fibronectin type III domains, that are located close to the transmembrane domain (Barton *et al*, 2006). Both Ig domains together with the EGF domains cause the structure of the Tie2 to appear like an arrowhead (Barton *et al*, 2006). Immunoglobulin-2 (Ig2) is the extracellular segment that is responsible for binding to the angiopoietins (Fiedler *et al*, 2003) (Figure 1.2). The two-immunoglobulin domains (Ig1and Ig2) consist of 97 amino acids for Ig-1 (residues 23-120), 87 amino acids for Ig-2 (residues 122-209) plus another 94 amino acids for the third immunoglobulin (Ig3) (residues 348–442). Structural studies suggest that there are 11 amino acids between the residues 149-168 on the Tie2 Ig2 domain that are involved in the interaction between Tie2 and Ang2 (Barton *et al*, 2006). Moreover, there are another 13 amino acids from residues 417-480 on Ang2 that are involved in the binding with Tie2 (Barton *et al*, 2006).

Tie2 can bind four ligands (Ang1-4), Ang1 and 2 are the best characterized ligands (Chung, 2014; Ray *et al*, 2000). Angiopoietin-1 is a ligand that has an agonistic action against Tie2 and can induce Tie2 signalling resulting in maintaining integrity of the vessels in the endothelial cells (Saharinen *et al*, 2005).

Tie2 activation can induce several signalling pathways including the PI3K/Akt pathway that promotes survival-associated pathways and inhibits apoptotic pathways (Kontos *et al*, 2002). In addition Tie2 phosphorylation triggers MAPK (mitogen activated protein kinase), Dok-R pathways, induction of endothelial NO (nitric oxide) synthase and recruitment of ABIN-2, which is the A20-binding inhibitor of NF-κB that inhibits NF-κB activity (Sato *et al*, 1995; Brindle, Saharinen and Alitalo, 2006). Activation of Tie2 through phosphorylation occurs by induction of receptor oligomerization by tetrameric Ang1 ligand that results in trans-phosphorylation and activation of the enzyme (Yu, 2010; Fachinger *et al*, 1999). Both Ang1 and Ang2 have the ability to induce phosphorylation of Tie2 in non-endothelial cells (Teichert-Kuliszewska *et al*, 2001).



(A)



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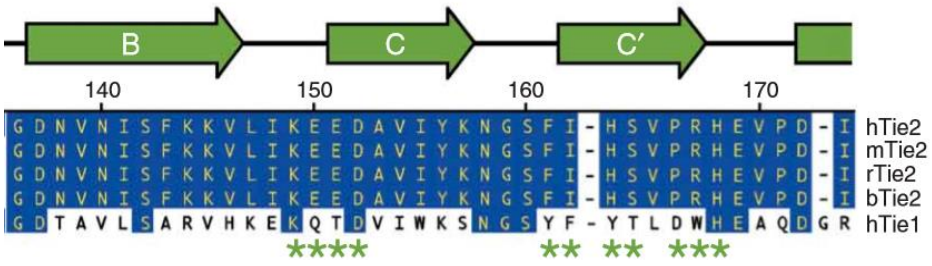

(C)

Figure 1.2: Sequence alignment of the binding domains of Ties and angiopoietins and Tie2 ectodomain.

The figure shows A: the extracellular domains of Tie2 that includes Ig-1, Ig-2 (Ig-2: is the binding site that bind with angiopoietin ligand), Ig-3 plus three EGF followed by three fibronectin type III (FNIII). B: angiopoietins domain: showing residues involved in binding with Tie2 ectodomain (between 417-480 residues, black stars). C: Tie2 extracellular domain (Ig2): showing residues involved in binding with angiopoietins (149-168 residues, green stars) (Barton *et al*, 2006).

1.1.2 Tie1 receptor

Tie1 is a member of the tyrosine kinase receptor family. The structure includes an extracellular domain involving two immunoglobulin-like domains, three EGF, and a third immunoglobulin domain, followed by three fibronectin III and the transmembrane and intracellular domains (Macdonald *et al*, 2006). It is expressed in the vascular system, mainly on endothelial cells, from the early stages of embryogenesis (Nakashima *et al*, 2001; Chan and Sukhatme, 2009). There is no specific ligand identified for the Tie1 receptor, however, in the presence of Tie2, Ang1 can activate Tie1 through interaction with Tie2 (Eklund and Saharinen, 2013; Gjini *et al*, 2011; Saharinen *et al*, 2005). In normal conditions (non-inflammatory conditions), Tie1 directly interacts with Tie2 where it regulates Tie2 signalling in response to Ang1 and Ang2 (Korhonen *et al*, 2016). Tie1 can limit the activation of Tie2 as a result of heterodimerization with Tie2 leading to suppression of Tie2 clustering (Marron *et al*, 2007). Under quiescent conditions of confluent cells Tie1 appears to be required for maximal activity of Ang1 through Tie2, and allows Ang2 to act as an agonist (Savant *et al*, 2015). In contrast, in sub-confluent growing endothelial cells Tie1 suppresses Ang1 action through Tie2 by limiting its ability to bind Tie2 (Brindle *et al*, 2006).

The presence of Tie1 is not essential for vasculogenesis, but it has a critical role in microvasculature maintenance during murine embryonic development. Thus, absence of the Tie1 gene results in embryonic lethality, oedema, and haemorrhage (Sato *et al*, 1995). Lack of Tie1 can lead to defects including an increased density of the vessels at the later stages of angiogenesis; this reflects the crucial role of Tie1 during blood vessel maturation (Salim, 2007). Moreover, Tie1 has a role in the formation of atherosclerotic plaques, shown by absence of Tie1 decreasing plaque formation (Woo *et al*, 2011).

Up-regulation of Tie1 occurs in hypoxic states (McCarthy *et al*, 1998). Tie1 has a pro-inflammatory characteristic that could result from its ability to enhance the Vascular Cell Adhesion Molecule-1 (VCAM-1), E-selectin, and Intercellular Adhesion Molecule-1 (ICAM-1) (Ahmed and Fujisawa, 2011; Chan *et al*, 2008). Tie1 acts alongside Tie2 to control the responsiveness of the Tie2 receptor to the ligand Ang1 (Eklund and Saharinen, 2013b). Although the Tie1 receptor is not activated directly by Ang1, it could play a role in mediating Ang1 effects on endothelial survival (Chong *et al*, 2004; Milner *et al*, 2009).

1.1.3 Tie1 cleavage

Several structural studies refer to the important role of Tie1 in maintaining the vascular endothelial cells by regulating Tie2 functions, vascular remodelling and formation. The complete length of Tie1 is important as a regulator required to activate Tie2 phosphorylation and to maintain vascular stability (Savant *et al*, 2015). Endothelial Tie1 silencing could result in impaired formation of vasculature (Loos, 2013).

Tie1 shedding during inflammation leads to switching of Ang2 action from a weak agonist to antagonist (Kim *et al*, 2016). In addition, Tie1 cleavage is associated with decreased Tie2 phosphorylation, Ang2 agonist activity, Tie2 and Ang1 expression and shows increased Ang2 expression which results in vascular instability (Korhonen *et al*, 2016). Decreased Tie2 phosphorylation also results in decrease forkhead box O1 (FOXO1) phosphorylation and leads to transcriptional upregulation of FOXO1 which induces expression of genes that have a crucial role in mediating vascular destabilization (Daly *et al*, 2004; Potente *et al*, 2005).

Tie1 overexpression plays a crucial role in inflammatory diseases such as atherosclerosis (Chan and Sukhatme, 2009). This is probably due to the fact that it possesses a pro-inflammatory characteristic and has the ability to upregulate VCAM-1, ICAM-1 and E-selectin (Chan *et al*, 2008). Previous studies have demonstrated that Tie1 expression is upregulated in several tumor diseases such as gastric cancer, breast carcinomas, angiosarcomas, leukemia (Yang *et al*, 2015; Rees *et al*, 2007; Buehler *et al*, 2013).

Endothelial cell stimulation with TNF-α or Phorbol-myristate-acetate (PMA) leads to release a 100kDa fragment of the extracellular domain of Tie1 (Yabkowitz *et al*, 1997; Yabkowitz *et al*, 1999) (Figure 1.3).

The ADAMs (disintegrin and metalloproteases) are one of the possible proteases responsible on Tie1 cleavage. The ADAM family are anchored glycoproteins with different regulating functions such as protein shedding and angiogenesis. They are classified as one of the major groups with sheddase activity responsible for cleavage of variety protein ectodomains such as anchored GF (growth factors), receptors, cytokines, TNF-α and adhesion proteins (Arduise *et al*, 2008; Schlondorff and Blobel, 1999; Moss *et al*, 2001). The disintegrin (cysteine-rich) domain has adhesive properties that enable it to have a crucial role in regulating different types of cell interaction such as cell–cell and cell–extracellular matrix interactions (White, 2003).

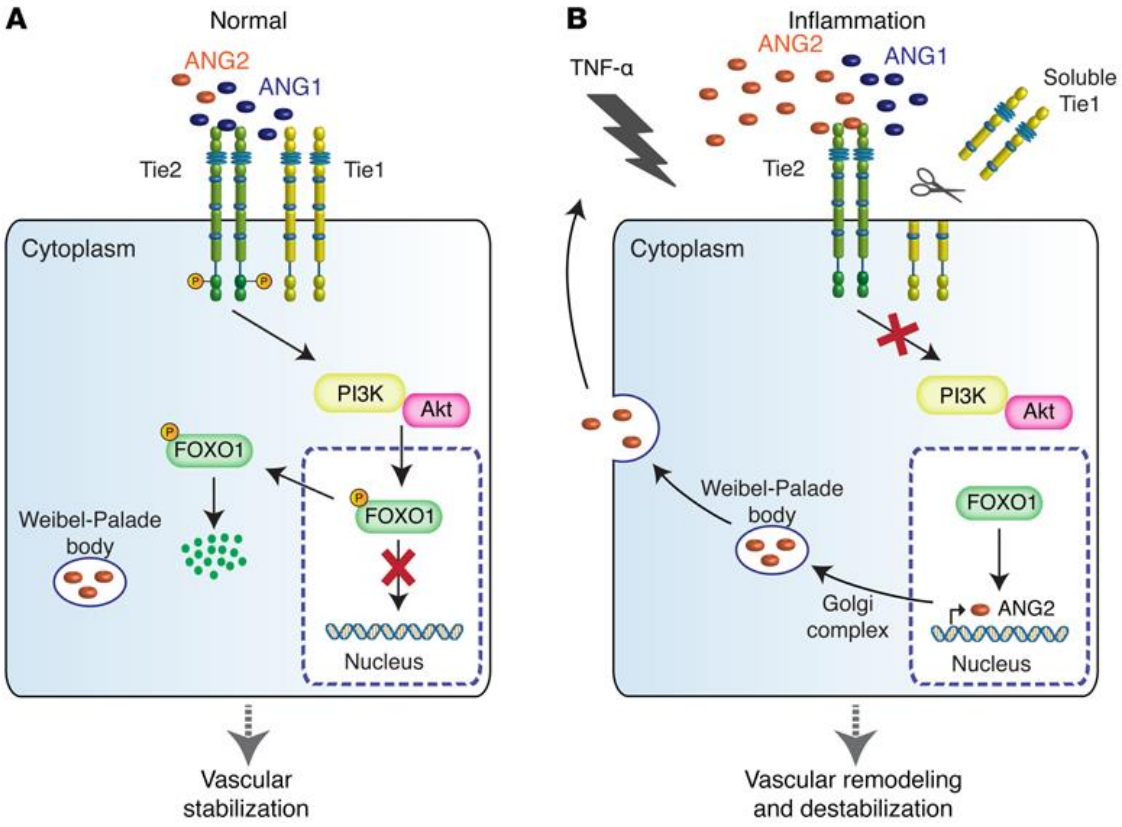

Figure 1.3: Possible role of Tie1 in inflammatory conditions

(A): In basal conditions Ang1 phosphorylates Tie2 and triggers the PI3K/Akt pathway which leads to FOXO1 phosphorylation and inhibits gene transcriptional activation including Ang2 and other vascular destabilising proteins. (B): Inflammatory conditions result in shedding of Tie1 and decrease Tie2 phosphorylation. This will promote FOXO1 gene transcriptional activation and increase Ang2 expression which sustains PI3K/Akt inactivation and keeps the vascular endothelial cells in an unstable state (Kim *et al*, 2016).

1.2 Angiopoietins

Angiopoietins are secreted glycoprotein ligands for Tie2 receptors and take part in angiogenesis and stabilization of the vessels (Oike *et al*, 2004). Both Ang1 and 2 are considered as important regulators for embryonic and postnatal neovascularization, and they participate in maintaining the physiological integrity of the microvasculature (Asahara *et al*, 1998; Suri *et al*, 1996). The angiopoietins share the same basic structure (Figure 1.4) (Moss, 2011). There are four ligands, Ang1, Ang2, Ang-3, and Ang-4 (Ahmed and Fujisawa, 2011; Chung, 2014). In human Ang1, 2 and 4, in mouse Ang1, 2 and the orthologue for Ang4 is Ang3 (Chung, 2014). These angiopoietins have the ability to bind to Tie2, but none of them bind to the Tie1 receptor (Salim, 2007). Several studies show the vital role of the Ang1 and Ang2 in the stabilisation or regression of vessels during angiogenesis (Rasul *et al*, 2012). Ang1 has higher affinity for Tie2 than Ang2 (Yuan *et al*, 2009). Ang-4 can activate the Tie2 receptor in human endothelial cells, whereas Ang-3 has an agonistic action on the endothelial receptor of mice (Lee *et al*, 2004).

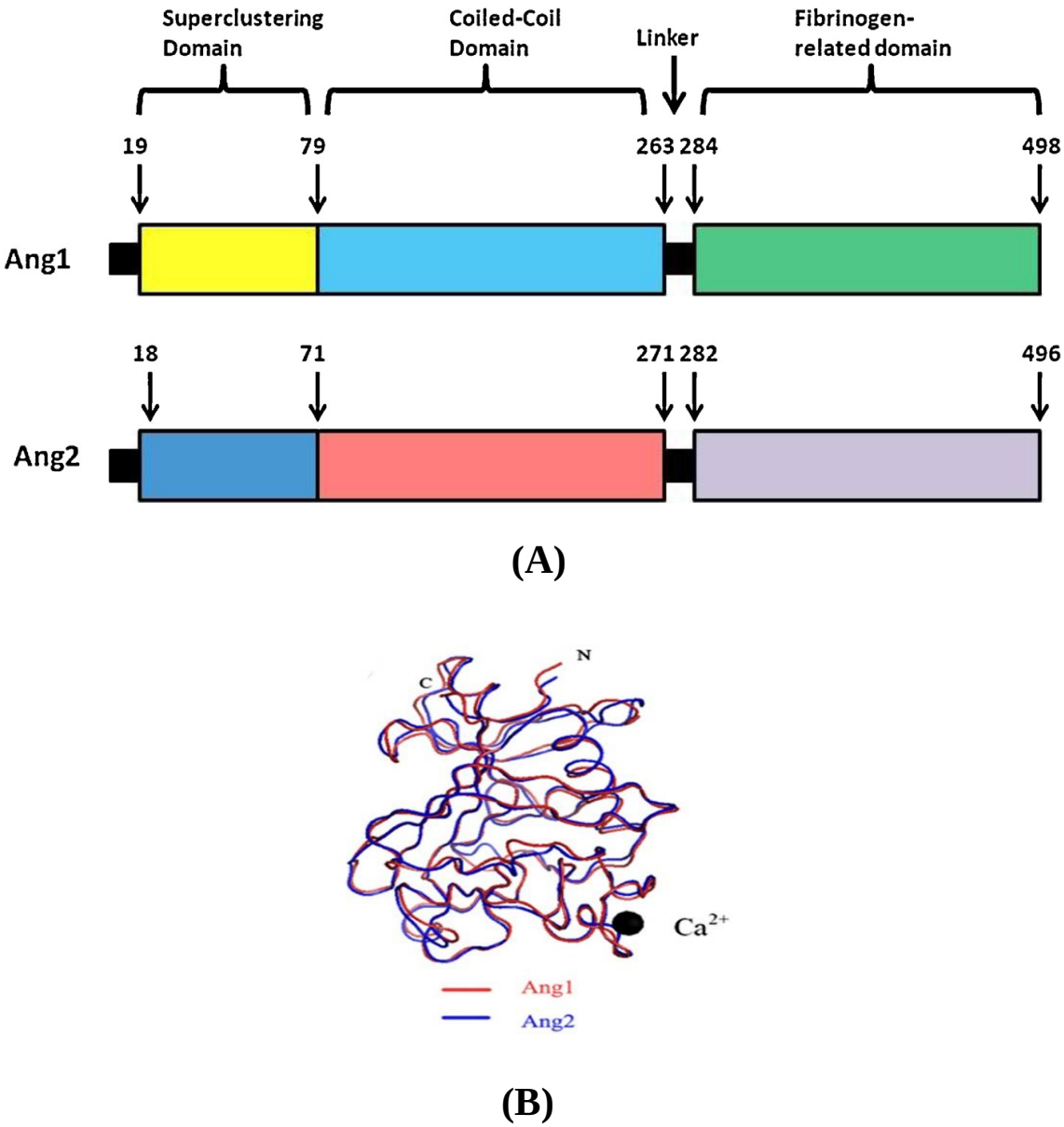

Figure 1.4: Basic structures of Ang1 and Ang2 ligands.

A: Schematic diagram outlining the basic similarities between Ang1 and Ang2. Both include a short peptide signal sequence followed by a supercluster domain (SCD) between residues 20 to 79 and 19 to 71 in Ang1 and Ang2 respectively. The coiled-coil domains (CCD) start from residues 80–263 and 72–271 in Ang1 and Ang2 respectively. Then short linkers are followed by fibrinogen binding domains between residues 285–498 and residues 283–496 (Moss, 2013). B: crystal structure for receptor binding domain of Ang1 and 2 in coil representation overlying each other (Yu *et al*, 2013).

1.2.1 Angiopoietin-1

Angiopoietin-1 (Ang1) is an agonist ligand that binds to the Tie2 receptor and is expressed by the perivascular cells (Audero *et al*, 2001). It is secreted by the endothelial supporting cell-like pericytes, SMC, tumor cells and fibroblasts (Syrjälä, 2014). Secretion of Ang1 from the vascular pericytes leads to activation of the Tie2 receptors and maintains junctional integrity by increasing endothelial accumulation of the adhesion proteins such as cadherin at cell-cell junctions (Uchida *et al*, 2014).

Ang1 consists of a fibrinogen-like domain that is responsible for binding to Tie2, and the N-terminal domain. Downstream of the fibrinogen liked domain is a flexible linker followed by a coiled-coiled domain and the N-terminal superclustring domain (Figure 1.4). The coiled-coiled domain is a dimerised domain, the supercluster domain brings the dimerised coiled-coiled domain into tetramer or higher order structures (Figure 1.4). The N-terminal domain is essential for Tie2 activation, through which it can form higher order oligomers (Procopio *et al*, 1999; Davis *et al*, 2003).

Ang1 usually exists as a multimeric ligand including trimeric, tetrameric and pentameric homo-oligomers. The multimeric form is important for Tie2 activation (Brindle *et al*, 2006). The multimerization of Ang1 leads to Tie2 clustering. This promotes transphosphorylation of the receptor.

Ang1 promotes several actions during development and maturation of the embryonic vascular system. It induces angiogenesis over the course of development (Ghosh *et al*, 2012). Decreased Ang1-Tie2 activation in a mice animal model resulted in fatal vascular defects due to decrease vascular sprouting (Audero *et al*, 2001). In contrast, Ang1 overexpression during heart development of a mouse model resulted in cardiac haemorrhage between the E12.5 and E15.5 and ninety percent of mice were dead (Ward *et al*, 2004).

In endothelial cells, Ang1 inhibits apoptosis, inflammatory gene and tissue factor expression and promotes monolayer integrity. The *in vivo* translation of these actions indicates suppression of inflammation in the vessels, decreased vascular permeability and maintainence of cells in a quiescent state (Moss, 2011; Audero *et al*, 2001; Chen and Stinnett, 2008; Brindle *et al*, 2006) (Figure 1.5). The anti-leakage characteristic of Ang1 is achieved by regulating VE-cadherin phosphorylation that could result in reducing the gaps between the endothelial cells (Baffert *et al*, 2006; S. Lee *et al*, 2011). Ang1 is thought to promote vessel survival by up-regulating survivin in ECs (Chong and Lip, 2004). All these actions, shown in Figure 1.5, are consistent with the important role of Ang1 during maturation and remodeling of the vessels (Rasul *et al*, 2012; Audero *et al*, 2001).

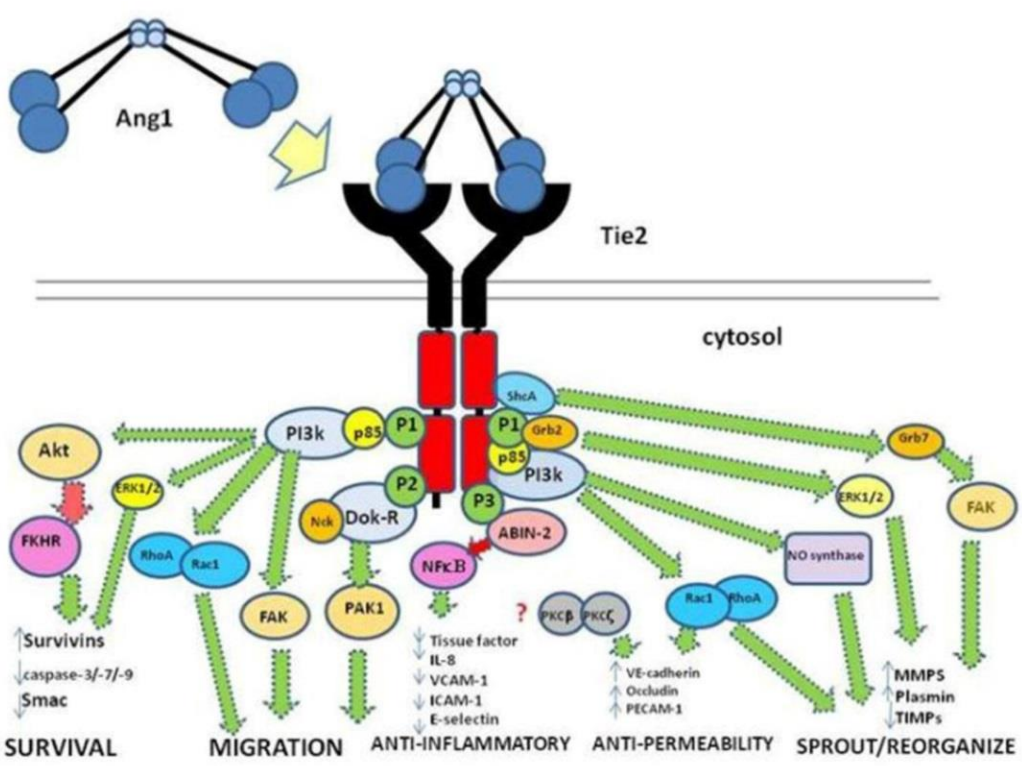

Figure 1.5: The actions of Ang1: Tie1 system

The figure summarises some of the key activities of Ang1 acting through Tie2 receptor on the endothelial cells. In addition, there are signalling pathways shown for some of the key events. These involve the phosphotyrosines p1 (1101 residue), p2 (1106 residue) p3 (1113 residue) and and DOK-r site required to initiate downstream signalling (Moss, 2011).

1.2.2 Angiopoietin-2

Angiopoietin-2 is an angiogenic growth factor that is expressed by endothelial cells, and Ang2 expression is increased in hypoxic blood vessels as well as in response to TNF-α (Eklund and Saharinen, 2013; Kim, *et al*, 2000). Studies on mice show that although mice can be born without requiring Ang2, they soon show chylous ascites and may die postnatally (Syrjälä, 2014). This suggests that Ang2 does not have a crucial role in embryogenesis but is important later in development.

Post-development, the Ang2 level is decreased and only expressed in vascular remodelling areas (Gurnik, 2015). Ang2 has context-dependent antagonistic effects as a result of its ability to induce or suppress Tie2 receptor under varying conditions. Ang2 has agonistic actions in different circumstances, such as when it is found at high concentrations and also if it present for prolonged periods (Kim, *et al*, 2000; Teichert-Kuliszewska *et al*, 2001). Furthermore, this action was observed in stressed endothelial cells (Daly *et al*, 2006). The Ang2 context-dependent effects also rely on the vascular endothelial protein tyrosine phosphatase (VEPTP) which can switch Ang2 to a potent activator of Tie2 when inhibited (Souma *et al*, 2018).

Ang2 has a long half-life (about 16 hours), and is stored in the Weibel-Palade bodies within the cytoplasm of endothelial cells (Fiedler *et al*, 2004; Fiedler and Augustin, 2006; David *et al*, 2009). There is a rapid release of Ang2 in response to stimulators such as VEGF-A (Vascular endothelial growth factor A) and IGF-1 (Insulin-like growth factor-1) (Eklund and Olsen, 2006; Chung, 2014; Augustin *et al*, 2009; Moss, 2011). Moreover, there are several stimulators that can induce the secretion of Ang2 from its storage such as hypoxia, thrombin, and leptin (Wang *et al*, 2012; Oh *et al*, 1999). Hypoxia is one of the important factors that regulates Ang2 expression and results in increased levels of intracellular Ang2 (Oh *et al*, 1999). Furthermore, Ang2 can be released in response to cytokine activators such as histamine, vasopressin, and its effect on the Tie2 receptor has an autocrine signalling mode (Fiedler *et al*, 2004).

Ang2 is found as trimeric, tetrameric, and pentameric oligomers (Brindle, Saharinen and Alitalo, 2006). Ang2 consists of an N-terminal Ang-specific super-clustering domain containing two Cys molecules, an amino-terminal coiled-coil domain, and a carboxy-terminal fibrinogen-like domain (Chung, 2014; Ward and Dumont, 2002). The Ang2-Tie2 binding occurs through binding of the fibrinogen-like domain of the Ang2 to Ig-2 part of the Tie2 (Ward and Dumont, 2002; Chung, 2014; Augustin *et al*, 2009). Studies recognized 13 amino acids on the Ang2 FRD (Fibrinogen-like Domain) binding site from residues 417-480 that participate in the interactions with Tie2 (Barton *et al*, 2005) .

Ang 1 and Ang2 compete for the same binding site on the Tie2 receptor that can result in either activation or suppression of the receptor, depending on the ratio of the angiopoietins (Chung, 2014). Under normal conditions when Tie2 and Tie1 are present, Ang1 is a better agonist and Ang2 is an agonist, while losing Tie1 results in converting Ang2 to an antagonist and Ang1 becoming a weaker agonist (Eklund and Saharinen, 2013). When Ang2 act as an antagonist, Tie2-Ang2 binding inhibits the anti-inflammatory effect of Ang1 (Augustin *et al*, 2009). Also, Ang2 can induce vascular leakage by blocking the anti-leakage effect of Ang1; this was seen when Ang2 was given to healthy adult mice (Ghosh *et al*, 2012).

Ang2 is likely to exert two actions, facilitating angiogenesis in the presence of VEGF and promoting vessel regression in the absence of the vascular endothelial growth factor (VEGF) (Yin *et al*, 2018; Fiedler *et al*, 2004; Fiedler *et al*, 2004; Chong and Lip, 2004). Previous research suggested that hypoxia induced Ang2 secretion and resulted in retinal neovascularization in the presence of VEGF, while when hypoxia decreased and VEGF was inhibited, it caused vascular regression (Pichiule *et al*, 2004).

In many inflammatory-related diseases, Ang2 is increased in the circulation and the level of Ang2 correlates with occurrence and severity of the disease. Patients with severe sepsis or sepsis-associated coagulopathy have higher levels of Ang2 compared to patients with mild sepsis (Wang *et al*, 2012; Statz *et al*, 2017). Ang2 is an essential mediator of the action of TNF-α on the endothelial cell inflammatory activation (Fiedler and Augustin, 2006; Wang *et al*, 2012). In addition, Ang2 has the ability to up-regulate ICAM-1 and VCAM-1 in response to TNF-α (Chen *et al*, 2012). One study illustrated that Ang2 deficient mice show no response to inflammatory mediators due to failure in stimulating the expression of the adhesion molecule (Augustin *et al*, 2009). Moreover, the study of Imhof & Lions (2006) indicated that up-regulation of TNF-α during inflammation lead to increased Ang2 levels. In addition, inhibition of NF-kB by Ang1can reduce inflammation resulting from TNF-α up-regulation (Figure 1.6). Consequently, competitive inhibition of the Tie2 receptor results in suppression of the anti-inflammatory effect of Ang1 (Imhof and Aurrand-Lions, 2006). Furthermore, Michalska *et al*. (2010) demonstrated that there was a correlation between the inflammatory parameters such as Erythrocyte Sedimentation Rate (ESR), C-reactive protein, and Ang2 in patients with systemic sclerosis and rheumatoid arthritis (Michalska-Jakubus *et al*, 2011).

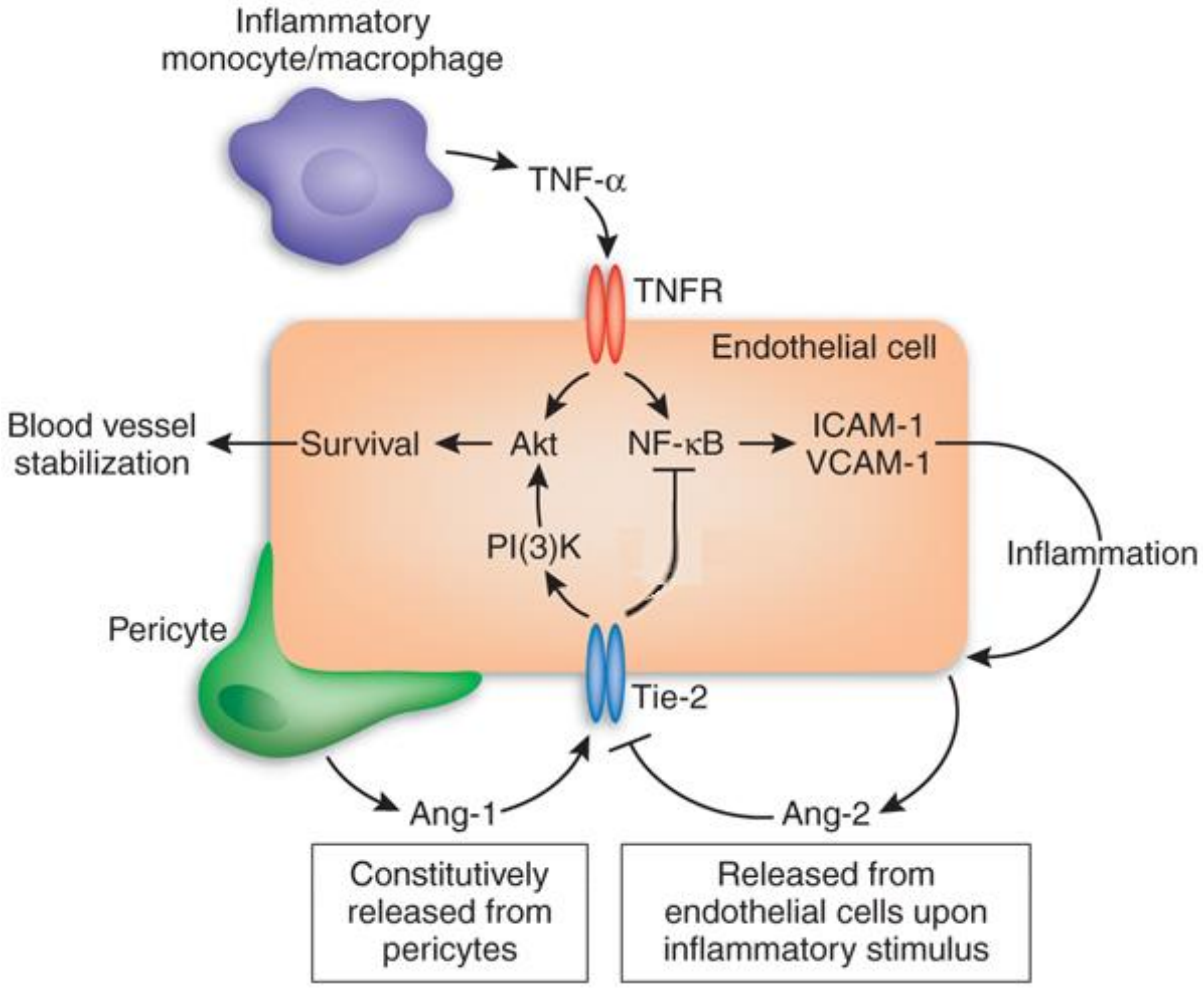

Figure 1.6: A scheme showing the effects of Ang1 and Ang2 in inflammation.

Binding of Ang1 to Tie2 enhances vessel stabilization through activation of the PI3K-Akt cell-survival pathway and inhibition of the NF-kB pathway. Increased Ang2 levels suppress the stabilizing effect of Ang1. The image was reproduced after (Imhof and Aurrand-Lions, 2006).

1.3 Post developmental role of Ang1 and Ang2.

Angiopoietin expression has a crucial role in maintaining endothelial homeostasis and concurrently, it can play a negative role by promoting vessel regression especially post-angiogenic development. Several studies illustrated the positive effects of Ang1 on the adult vasculature, including anti-permeability effects, anti-inflammatory effects, and enhancement of vessel survival (Brindle *et al*, 2006).

1.3.1 Apoptosis

Ang1 can block endothelial cell apoptosis in microvascular endothelial cells required to maintain the cells (Dallabrida *et al*, 2005; Harfouche *et al*, 2002) (Dallabrida *et al*. 2005, Harfouche *et al*. 2002). The anti-apoptotic effect of Ang1 is mediated by activating the phosphatidylinositol 3-kinase (PI3K) /Akt pathway (Papapetropoulos *et al*, 2000; Kim, *et al*, 2000). On the other hand, Ang2 can block this effect and induce cell apoptosis (Li *et al*, 2018).

1.3.2 Inflammatory effect

Ang1 can protect vessels through its ability to suppress inflammatory mediators (Roviezzo *et al*, 2005). The anti-inflammatory effect of Ang1 can decrease the pro-inflammatory effects resulting from TNF-α and VEGF stimulation (Kwak *et al*, 2000; Kim *et al*, 2001; Kim *et a*l, 2000). Using Ang1 in an animal model with septic shock was associated with lower inflammatory response (Witzenbichler *et al*, 2005). Ang1 has the ability to decrease expression of several inflammatory adhesion molecule such as, Eselectin, ICAM1and VCAM1. In addition, Ang1 enhances recruitment of A20 binding inhibitor of NFκB-2 (ABIN2) to Tie2 which inhibits expression of inflammatory genes (Brindle *et al*, 2006; Hughes *et al*, 2003). Ang2 has pro-inflammatory properties that enable it to modulate the endothelial barrier controlling the integrity of vessels (Huang *et al*, 2011).

Furthermore, the antagonistic actions of Ang2 were evident in vascular remodelling and inflammatory conditions as a result of increased levels of Ang2 relative to Ang1 (Augustin *et al*, 2009; Scholz *et al*, 2015). Ang2 plays an essential role in mediating inflammation through activating ECs, neutrophils and increasing sensitivity of endothelial cells to TNF-α (Wang *et al*, 2012).

1.3.3 Survival effect

Ang1-Tie2 binding results in Tie2 phosphorylation and activation of Akt/PKB (protein kinase B) pathway. This induces survivin upregulation and protects the endothelial cells (Papapetropoulos *et al*, 2000). Lack of Ang1 secretion in congestive heart failure (CHF) might be one of the factors leading to endothelial damage. This is due to a lack in the survivin pathway stimulated by Ang1, as survivin can protect cells from death-inducing stimuli (Kubo *et al*, 1991). Moreover, Ang2 promotes vessel regression through its ability to inhibit the pro survival effect of Ang1 (Gurnik, 2015).

1.3.4 Leaking effect

Ang1 is characterized by an anti-leaking effect (section: 1.14.1), as a result, a lack of Ang1 can lead to leaky capillaries (Chong and Lip, 2004; Lim *et al*, 2004). Studies illustrated the important role of Ang1/Tie2 in preserving the stability of the vessels through its ability in the recruitment of pericytes and smooth muscle cells (Gurnik, 2015b). Moreover, the study of Thurston (1999) showed that Ang1 stimulation following VEGF or a chronic inflammatory state can reduce leakage of microvascular diseases (Thurston *et al*, 1999).

Ang2 inhibits the protective effects of Ang1, consequently causing extensively leaky capillaries leading to serious pathological conditions as a result of oedema and swelling (Sharma, 2011; Chong and Lip, 2004).The study of Roviezzo *et al.*, (2005) demonstrated that Ang2 increases cellular infiltration and vascular permeability in tissues (Roviezzo *et al*, 2005).

1.3.4.1 VE-cadherin cleavage

VE-cadherin is one of the important proteins that has a crucial role in maintaining the vascular barrier function required for keeping the vessels in homeostasis and is also important for the formation of vascular lumenization during vasculogenesis (Dejana *et al*, 2008; Carmeliet *et al*, 1999; Harris and Nelson, 2010; Montero-Balaguer *et al*, 2009). The cytoplasmic tail of VE-cadherin binds to the junctional adherent protein β-catenin, p120 and plakoglobin to form a complex adherent protein. The α-catenin connects this complex to the actin filaments which supports the formation of a strong cytoskeleton (Millán *et al*, 2010; Bazzoni and Dejana, 2004; Dejana and Vestweber, 2013). The cytoplasmic domain of VE-cadherin involves nine tyrosine groups (Shasby *et al*, 2002). Phosphorylation of β-catenin by src kinase causes a loss of the connection between VE-cadherin and β-catenin, leading to a weak cell-cell junction and increased cell permeability (Lilien and Balsamo, 2005). Confluent, tight monolayer cells are more stabilized and have more tight junctions due to less VE-cadherin tyrosine phosphorylation. In this case, the VE-cadherin will bind primarily to plakoglobin and to a lesser extent with β-catenin and p120 (Lampugnani *et al*, 1997).

VE-cadherin plays a central role in maintaining vascular endothelial integrity. It organises the gene expression and differentiation of other molecules that maintain the barrier stability, such as claudin-5, endothelial-protein tyrosine phosphatase (VE-PTP) and von Willebrand factor (Morini *et al*, 2018).

Claudin-5 is a one of the specific components required for a tight cell-cell junction and also regulates endothelial permeability, proliferation and migration and plays a crucial role in brain cancer metastasis (Morita *et al*, 1999; Ma *et al*, 2017). Previous research suggested that in sepsis associated with organ failure, increased permeability due to endothelial barrier disruption could be as a result of decreases in expression levels of the adhesion and tight junctional proteins such as VE-cadherin and claudin-5 (Aslan *et al*, 2017). VE-cadherin upregulates the expression of the tight junction adhesive claudin-5, which supports formation of a tight endothelial junction through the FOXO1 phosphorylation–Akt pathway and by limiting β-catenin translocation (Taddei *et al*, 2008; Morini *et al*, 2018). It also mediates vascular stability via a Tie2 signalling pathway by regulating VE-PTP, which decreases the VE-cadherin tyrosine phosphorylation required to maintain the vascular stabilization (Nawroth *et al*, 2002). For these reasons, downregulation in the level of VE-cadherin influences the confluency of the cell and results in leaky vessels, inflammation, and increased tumor metastasis (Zanetta *et al*, 2005; Cappelli *et al*, 2018). Upregulation of VE-cadherin level can improve the T-cell infiltration to the solid tumors and enhances responses to the immunotherapy (Zhao *et al*, 2017). VE-cadherin can undergo internalization which is regulated by vascular endothelial growth factor receptor type 2 (VEGFR-2) (Lampugnani *et al*, 2006).

ADAM10 activation was associated with increased cellular permeability. ADAM10 is the disintegrin and metalloprotease responsible for VE-cadherin shedding resulting from increased influx of intracellular Ca^{2+} (Schulz *et al*, 2008). The study of (Sidibé *et al*, 2012) demonstrated that TNF-α can mediate VE-cadherin cleavage and results in release of the extracellular domain of VE-cadherin (Sidibé *et al*, 2012). High levels of TNF-α in a patient with rheumatoid arthritis as well as hereditary angioedema are associated with the release of the VE-cadherin extracellular domain (Sidibé *et al*, 2012; Bouillet *et al*, 2011). Ang2 can increase expression and secretion of MMP-2 as a result of activating αvβ1 and inducing glioma cell invasion by signalling through FAK/ $p130^{Cas}$/ERK1/2 and the JNK-pathway (Hu *et al*, 2006). ADAM10 has been proven to cause VE-cadherin cleavage resulting in weakening of endothelial cell-cell junctions and increased permeability (Schulz *et al*, 2008). Previous data reported that increased influx of intracellular Ca^{2+} activates the shedding property of ADAM10, while Ca^{2+} releases inhibition resulting from ADAM10 depletion leads to cell detachment (Nagano *et al*, 2004). Exposing

endothelial cells to radiation leads to VE-cadherin cleavage by ADAM10 as a result of intracellular release of Ca^{2+}. This can result in cardiovascular diseases such as atherosclerotic plaques (Kabacik and Raj, 2017).

1.4 Roles of Ang-1 and Ang-2 in Angiogenesis

Angiogenesis is an essential process that forms new vessels from the already present ones to ensure the survival of the cells located near to the vascular system; it involves sprouting of small capillaries from the larger one (Thurston, 2003; Staton *et al*, 2011). It has a crucial role in maintaining the homeostasis of the vasculature system, wound healing and tissue regeneration. In addition, it plays an important role in mediating pathogenic effects in cancer (Welti *et al*, 2013). Up-regulation of this vital process occurs in response to angiogenic molecules and hypoxia, which can occur after acute, sub-acute or chronic phases following a myocardial infarction (Lee, Lip and Blann, 2004; Sato *et al*, 1995).

Ang-1 has pro-angiogenic properties, binding to Tie-2 receptor leads to activation and subsequent autophosphorylation of the Tie-2 receptors (Chong and Lip, 2004). It maintains the integrity of the vessel through its ability to control growth, proliferation, and maturation of the vessels (Ray *et al*, 2000). It plays a significant role in angiogenesis by its ability to facilitate the formation of new vessels from the pre-existing vessels (Wakui *et al*, 2006). Moreover, Ang-1 has a remodelling effect as a result of its ability to increase the diameter of the vessels and increase branching of small vessels required to decrease progression of ischemia (Chen and Stinnett, 2008). Studies demonstrate that high levels of Ang-1 in transgenic mice can result in the formation of new vessels with larger diameters (Chen and Stinnett, 2008).

On the other hand, Ang-2 has a significant role in the process of neovascularization and vascular remodelling (Huang *et al*, 2002; Kumpers *et al*, 2008). Ang-2 has pro-inflammatory properties that enable it to modulate the endothelial barrier controlling the integrity of vessels (Huang *et al*, 2011). The inflammatory state accompanying tissue injury is considered as the main characteristic that differentiates between the pathological and physiological angiogenesis (Chen *et al*, 2004). The angiogenic effect of Ang-2 results from its antagonist action on Tie-2, and is dependent on the availability of VEGF that preserves the structure of the vessel (Orfanos *et al*, 2007; Augustin *et al*, 2009; Michalska-Jakubus *et al*, 2011). Ang-2 promotes increased diameter, basal lamina remodeling, growth, and sprouting of new vessels via proliferation from the pre-existing vascular endothelial cells (Chong *et al*, 2004). However, in the absence of the VEGF, Ang-2 can induce vessel regression and lead to endothelial cell death due to blocking of the pro-survival signals from Ang-1-Tie2 binding (Lee *et al*, 2004; Chong and Lip, 2004). It is evident that VEGF is considered as an important regulator of the Ang-2 action (Fonseca *et al*, 2015). Ang-2 could reduce angiogenesis as a result of reducing pericyte coating on endothelial vessels; this leads to conversion of the vessels to a further plastic state (Theelen *et al*, 2015). Angiogenesis has a crucial role in the pathophysiology of cardiovascular disease (CVD) (Felmeden *et al*, 2003). Cardiovascular-related research showed that impaired angiogenesis could contribute to ischemic heart disease and atherosclerosis, in addition to its contribution in cancer diseases (Welti *et al*, 2013; Felmeden *et al*, 2003).

1.5 Role of Ang2 in Cardiovascular Disease

1.5.1.1 Angiopoietin level in cardiovascular diseases

Patients with cardiovascular diseases (CVD) including acute and chronic congestive heart failure, hypertension, diabetes and atherosclerosis show abnormally high levels of Ang2 (Tsai *et al*, 2018; Lukasz *et al*, 2013; Fiedler *et al*, 2004; David *et al*, 2009). High levels of Ang2 are considered as a marker for oxidative stress, macro and micro-vascular related diseases (Chaves *et al*, 2018). In a community-based sample study, Lorbeer *et al*, (2014) found that high concentrations of Ang2 were associated with a higher incidence of cardiovascular diseases (Lorbeer *et al*, 2014). Moreover, clinical studies have reported that increased levels of Ang2 might be considered as a marker for myocardial infarction recurrence (S. Chen *et al*, 2013). Furthermore, the study of Patel et *al*., (2008) demonstrated that increased levels of Ang2 among hypertensive patients was considered a predictor of myocardial infarction (MI) (Patel *et al*, 2008). High Ang2 levels in patients with coronary heart disease (CHD) might reflect the severity of the disease (Wang *et al*, 2012a). Moreover, disequilibrium in the level of Ang2 can occur in diabetes mellitus, cardiac allograft arteriosclerosis, acute coronary syndrome, vasculitis, sepsis, and glomerulonephritis (David *et al*, 2009). Myocardial ischemia plays a significant role in the regression of cardiac vessels that end with heart cell injury, inflammatory reactions and endothelial dysfunction (Turer and Hill, 2010). Moreover, coronary heart disease is considered as one of the inflammatory diseases that leads to endothelial dysfunction (Wang *et al*, 2012). Reduced tissue perfusion in coronary arteries might occur as a result of endothelial dysfunction and could conclude with myocardial ischemia (Drexler and Hornig, 1999).

The study of Stinnett (2008) showed that Ang1 gene therapy enhances stability and maturation of the vessel and improves myocardial angiogenesis and remodelling in a diabetic mouse model (Chen and Stinnett, 2008). Overexpression of Ang1 stimulates recruitment of SMC in a diabetic mouse with induced myocardial ischemia, which leads to increased maturity and density of the vessels within the infarcted area and decreases interstitial fibrosis (Chen and Stinnett, 2008). Consequently, these effects can be inhibited by increased levels of Ang2 that antagonizes the effect of Ang1 leading to destabilization and regression of the vessel (Stoeltzing *et al*, 2003).

Up-regulation of Ang2 is usually controlled by several pro-inflammatory stimuli that lead to increased leakage of the vessel. Therefore Ang2 participates in the inflammatory progression that occurs in coronary heart disease (Wang *et al*, 2012). Furthermore, decreased capillary density could lead to cardiac dysfunction and subsequent myocardial ischemia (Yoon *et al*, 2005). During the healing phase (proliferative phase) of myocardial damage, the necrotic area is extremely cellular and involves many metabolic activities that require vascular supply. As healing begins, the vascular granulated tissue is replaced by collagen-rich scar and angiogenesis in the infarct area is inhibited (Ren *et al*, 2002; Dobaczewski *et al*, 2004). The healing process requires a muscular coat for the formation of infarct neovessel; otherwise neovessel regression may occur (Zymek *et al*, 2006).

Several studies show that overexpression of Ang2 occurs in animal models with cardiovascular and cancer related diseases (Holopainen *et al*, 2012). Hypertensive patients show excessive stretching of the arterial wall of endothelial cells which causes intracellular calcium release and subsequently release of Ang2 from the native Weibel Palade bodies by exocytosis. This was suggested by a previous study which used an animal model to show the Ang1 effect on blocking intracellular calcium release (Korff *et al*, 2012).

Using several animal models, studies have shown that increasing Ang2 by transgenic or adenoviral approach drives cardiovascular pathologies. The study of Chen et al., (2012) showed that overexpression of Ang2 in diabetic mouse leads to increased endothelial apoptosis, sensitivity to inflammation in the microvasculature of the heart and induction of fibrosis (Chen *et al*, 2012). Moreover, the study of Chen, Zeng et al. (2012) demonstrated that increased levels of Ang2 in a diabetic heart of a mouse model has a crucial impact on the progression of myocardial fibrosis and could result from impaired angiogenesis, increased endothelial apoptosis, increased microvascular inflammation, and loss of capillary density (Chen *et al*, 2012). Ang2 ligand-traps on endothelial cells stimulated with inflammatory molecules were found to inhibit the adhesion of platelet/leucocyte aggregates to endothelial monolayers and suppress LPS-induced oedema (Omeed *et al*, 2018).

In an animal model of myocardial infarction, adenoviral vectors were used to introduce Ang2 to the infarcted area causing increases in the infarct size (S. Chen *et al*, 2013). On the other hand, using Ang1 for the same model can protect the heart by preventing the inflammatory process stimulated by Ang2 (Chen *et al*, 2013). Using an Ang2 inhibitor after myocardial infarction restores integrity of the endothelial barrier, reduces cell hypoxia and the size of infarction (Lee *et al*, 2018). In hypercholesterolemic mice models using Ang2 blockage resulted in a decreases in the fatty streak (cells with high content of lipid) formation and plasma triglyceride (Theelen *et al*, 2015). Using Ang2 blocker in a xenograft metastatic mice models with high levels of Ang2 resulted in blocking dissemination of the tumour through enhancing junctional integrity between endothelial cells (Holopainen *et al*, 2012).

1.5.2 Pathogenic effects of high level of Ang2

1.5.2.1 Ang2 pathogenic effects through Tie2 dependent pathway

Balancing between levels of Ang1 and Ang2 is the main regulator for Tie2 receptor. The anti-inflammatory effects of Ang1 have a crucial impact on the stability of endothelial cells. However, an excessive amount of Ang2 can suppress Tie2 phosphorylation mediated by Ang1 and lead to endothelial destabilization (Hakanpaa *et al*, 2015). In normal homeostatic conditions, Ang2 has an agonist action (Yuan *et al*, 2009). The data of Kim et al, (2016) suggests that Ang2 promotes vascular enlargement and stability in non-leaky vessels, while in inflammatory conditions Ang2 blocks Tie2 and promotes vascular destabilization through the FOXO1 signaling pathway (Kim *et al*, 2016).

Recent data demonstrated that Ang1 induced-Tie2 phosphorylation maintains the endothelial cells in a quiescent state and retains the gaps between the cells-cell junctions. This could happen through a mechanism that does not involve a reduction in endothelial cell contraction nor a decrease in the actin stress fibre formation (Rokhzan *et al*, 2018).

High levels of Ang2 block the anti-inflammatory, anti-permeability and anti-apoptotic effects of Ang1 (Figure 1.7) (Brindle *et al*, 2006). The study of (Ghosh *et al*, 2012) shows that introducing Ang2 to healthy adult mice leads to increased cell permeability (Ghosh *et al*, 2012). Increased levels of Ang2 in the retina of an animal diabetic model causes increased endothelial permeability as a result of VE-cadherin phosphorylation (Rangasamy *et al*, 2011). Several studies demonstrated that there is a correlation between metastatic cancer diseases and integrity of the endothelial cells (Imanishi *et al*, 2007). The study of (Holopainen *et al*, 2012) showed that blocking Ang2's effect can decrease cell's metastatic dissemination by enhancing a tight junction between the endothelial cells (Holopainen *et al*, 2012).

High levels of Ang2 block the anti-inflammatory actions of Ang1 through blocking the inhibiting effect of Ang1 on NF-kB, enhancing interlukin-1 secretion, and increasing sensitivity of ECs to TNF-α (Fiedler and Augustin, 2006; Wang *et al*, 2012; Imhof and Aurrand-Lions, 2006). Ang2 levels show upregulation during inflammatory disease such as sepsis associated coagulopathy and can be used as a biomarker in patient with sepsis diseases (Statz *et al*, 2018; Orfanos *et al*, 2007).

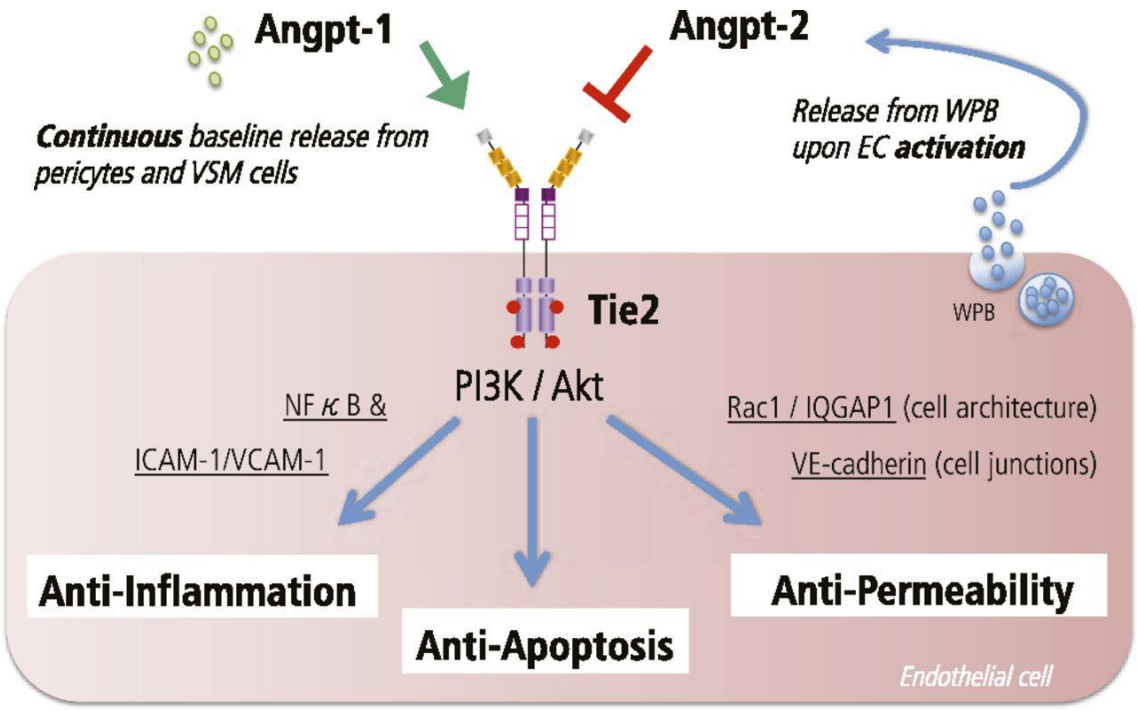

Figure 1.7: The vascular stabilising and regressive effects of Ang-Tie2 system

A scheme showing the crucial role of the Ang-Tie2 system. Ang1 stimulates Tie2 phosphorylation and propagates signalling pathways through phosphatidylinositol 3-kinase (PI3K)/Akt which mediates anti-inflammatory, anti-permeability, and anti-apoptotic effects. The anti-inflammatory effects are mediated by blocking the expression of ICAM-1, VCAM-1 and transcription nuclear factor-kB. The anti-permeability effects are mediated by Rho kinase inhibition and activation of the small GTPase Rac1 through binding to the IQ-motif containing GTPase activating protein-1 (IQGAP1). In addition to the effect of the VE-cadherin through the src. The anti-apoptotic effect is mediated by PI3K/Akt signaling pathway which stimulates pro-survival signals. All these effects can be antagonised by high levels of Ang2 Angpt-1=Ang1, Angpt-2=Ang2. (David *et al*, 2013).

1.5.2.2 Ang2 Pathogenic effects through Tie2 independent pathway

In addition to binding Tie2 receptor, angiopoietins can also bind integrin (Carlson *et al*, 2001). Integrin is a heterodimeric glycoprotein that composes of alpha and beta subunits that are non-covalently bound to each other. They act as adhesion proteins to enable the cells to bind extracellular matrix to the cytoskeleton through recruiting proteins such as talin, tensin, paxillin, vinculin and α-actinin to support the integrity and stability of the endothelial cells (Desgrosellier and Cheresh, 2010; Lal *et al*, 2009). Integrin lacks kinase activity. Binding of integrin to surrounding ligands leads to integrin activation and conformational changes. The conformational changes cause recruitment of the adaptor signalling molecules and subsequent formation of focal adhesions. Clustering of integrins leads to activation of FAK, Src kinases and scaffold molecules (Desgrosellier and Cheresh, 2010).

It has recently been shown that Ang2 can have effect through integrin $\alpha_5\beta_1$ to promote endothelial permeability (Hakanpaa *et al*, 2018). Increased levels of Ang2 have the ability to activate the β1-integrin via the Ang2 N-terminus (Kim *et al*, 2016; Hakanpaa *et al*, 2015). The study by (Lee *et al*, 2018) reported that high levels of Ang2 in a mouse model with myocardial infarction (after chronic phase), promotes vascular regression and inflammation via $\alpha_5\beta_1$ signalling. This could occur due to vascular leakage, pericyte detachment and increased expression of adhesion molecules (Lee *et al*, 2018).

In addition to $\alpha_5\beta_1$, Ang2 has also been showed to bind and signalling through other integrins specifically $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Yun *et al*, 2017; Imanishi *et al*, 2007; Felcht *et al*, 2012). Previous data suggested that during inflammatory conditions, the endothelium shows upregulation of $\alpha_v\beta_3$ (Johnson *et al*, 1993). Ang2 induces $\alpha_v\beta_3$-integrin-Tie2 complex formation which results in FAK recruitment to integrin and subsequent phosphorylation of focal adhesion kinase (FAK) leading to talin dissociation, $\alpha_v\beta_3$-integrin internalization and degradation by lysosomes (Thomas *et al*, 2010).

On the other hand, activation of $\alpha_5\beta_1$ by fibronectin results in increased formation of $\alpha_5\beta_1$-Tie2 complex (Serini *et al*, 2008). In such cases Ang1 can phosphorylate Tie2 at a lower concentration than required to activate the free Tie2. Complex activation by Ang1 also results in a prolonged Tie2 phosphorylation compared to the free Tie2. Ang1 stimulates biochemical signals to recruit the p85 subunit of PI3K and FAK (Serini *et al*, 2008).

1.6 Research project aim

Growing evidence supports a role of Ang2 in promoting cardiovascular and cancer related diseases. The aims of this project are twofold:

Project aims

1. To further improve an Ang2 ligand-trap to block the effect of this ligand acting via the Tie2 receptor.
2. Characterise the potential pathogenic effects of Ang2 acting via a Tie2 independent pathway and test the ability of integrin inhibitors to block them.

Objectives

For aim one: one approach to block Ang2 action through Tie2 is to use a ligand trap. A ligand-trap could block the ability of Ang2 to bind to the Tie2 receptor. The first selective Ang2 ligand-trap was described in 2013 (Brindle *et al*, 2013) and consisted of a mutant verson of the Tie2 extracellular domain that selectively binds Ang2. As a soluble protein this ligand-trap can bind Ang2 preventing its action on cell surface Tie2. Attempts will be made to further improve this trap by:

1. Testing additional mutant versions of the trap for increased affinity for Ang2.
2. Engineering an oligomerized version of the trap and testing for binding to Ang2 and Ang1.

For aim two: this study will investigate potential pathogenic pathways through which Ang2 could exert effects without acting through Tie2. Specifically, this study will examine Ang2 effects on VE-cadherin and Tie1 cleavage by:

1. Testing Ang2 pathogenic effects on VE-cadherin and Tie1 cleavage.
2. Testing involvement of ADAMs in Ang2-induced VE-cadherin and Tie1 cleavage.
3. Testing involvement of the Tie2 receptor in Ang2-induced VE-cadherin and Tie1 cleavage.
4. Testing involvement of integrins in Ang2-induced VE-cadherin and Tie1 cleavage.
5. Testing the possibility of blocking these Ang2 pathogenic effects.

Chapter 2: Materials and methods

2.1 Reagents

All the reagents were obtained from Fischer Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK) unless otherwise stated. Consumable plastics were obtained from the Nalgene NUNC International (Denmark). The solutions were diluted using deionized distilled water and the pH was adjusted using 4M NaOH and 5M HCl.

Table 2.1: Reagents and media

Reagents
Coomassie stain: 10% acetic acid, 40% ethanol, 0.1% (w/v) coomassie blue
Destain solution: 10% (v/v) acetic acid, 40% (v/v) ethanol
Developer: solution A: 198µM p-Coumaric acid in DMSO, solution B: 1.25mM Luminol (5-amino-2,3-dihydro-1,4-pthalazinedione) in DMSO.
Distilled water: DNAse-RNase free (Gibco, Invitrogen)
Dithiothreitol (1M): Dissolve 15 mg DTT into 100µl of H2O
EA.hy926 complete media: DMEM (Life Technologies, UK), 10% (v/v) FCS
Ethanol 70%: Ethanol:H_2O 70:30 (v/v)
Ethidium Bromide (10 mg/ml, Invitrogen)
Freezing medium: 50% FBS, 40% media, 10% DMSO
HCMECs complete media: Endothelial cell growth medium (MV), (PromoCell, Catalog number:22002), supplementmix endothelial cell growth medium (PromoCell, Catalog number:C-39215)
HUVECs complete media: Medium 200 (Life Technologies, UK), 10 ml FCS, 2ml Low Serum Growth Supplement (LSGS)
LB Agar: for 1L: 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar
LB: for 1L: 16g tryptone, 10g yeast extract, 10g NaCl
Propanol (Sigma)
TAE (1x): 40mM Tris-acetate, 1µM EDTA
Tryple 10X (Life Technologies, UK). **Catalog number:** A1217701 Add 1ml of Tryple 10x plus 9 ml of sterile PBS into a falcon tube under sterile condition.

2.1.1 Buffers

Table 2.2: List of buffers

Buffers
2x sample buffer: 50mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA, 100mM DTT
BSA blocking buffer: 3% BSA in TBS – 0.05% Tween-20
DNA gel-loading dye (6X): 0.25% bromophenol blue, 0.25% (W/V) xylene cyanol FF, 40% w/v sucrose in H2O
Milk blocking buffer: 1XTBS containing 0.1% (v/v) Triton-X 100 and 5% (w/v) non-fat dry milk.
PBS: 140mM NaCl, 2.7mM KCl, 10mM Na2HPO4 (pH 7.4)
SDS-PAGE running buffer: 25mM Tris pH 8.3, 192mM glycine, 0.01% SDS
TBS: 50mM Tris, 150mM NaCl
Transfer buffer: 25mM Tris pH 8.3, 192mM glycine, 20% methanol
Wash buffer: TBS – 0.05% Tween-20
Zeba Spin Desalting columns 7K MWCO, 2ml (Fisher Scientific, UK)

2.1.2 KITS

Table 2.3: Kits

Kits	Source
EndoFree Plasmid Maxi Kit	Qiagen: Crawley, UK(12362)
Plasmid Mini prep Kit	Qiagen; Crawley, UK(27104)
NEB Turbo Competent *E-Coli* (Cacl2 method)	Homemade (New England Biolabs, Inc)
NEB Turbo Competent *E-Coli* (High Efficiency)	New England Biolabs, Inc(C2984H)
Quick Ligation kit	Biolab kit (M2200S quick ligation kit)
Restriction Enzymes	Roche Applied Sciences (UK) *Not* I: 13826621
Restriction Enzymes	New England Biolabs, Inc (US *Eco*R I: R0101S *Nhe* I: R3131S
QIAquick® Gel Extraction kit	Qiagen; Crawley, UK,(28115)
Polyethylenimine (PEI)	Linear, Sigma-Aldrich(764582)
Talone Metal Affinity Resin	(Clonteck; Crawley, united stat/Canada) Cat. Nos. Many PT1320-1 (031716)

2.1.3 Antibodies

Table 2.4: Antibodies used for immunoblotting test and binding assays

Primary Antibody	Dilution	Secondary Antibody	Dilution
Anti-h Tie2 100µg (RD system: AF313)	1:1000	anti-goat-HRP antibody 0.5g/L (Polyclonal rabbit anti-goat IgG (Dako))	1:1000
VE-Cadherin Antibody (RD system) Antigen Affinity-purified Polyclonal Goat IgG Catalog Number: AF938	1:1000	anti-goat-HRP antibody (Dako)	1:1000
1. Human TACE/ADAM10 ectodomain antibody-Monoclonal Mouse IgG1(MAB9301-SP:RD) 2. Anti-ADAM10 antibody (ab1997)	1:1000	anti-rabbit-HRP antibody (Polyclonal goat anti- rabbit Ig/HRP (Dako), REF: P0448	1:1000
1. Human TACE/ADAM17 ectodomain antibody-Monoclonal Mouse IgG28 (MAB1427-SP : RD) 2. Anti-ADAM17 antibody (ab39162)	1:1000	anti-rabbit-HRP antibody (Dako), REF: P0448	1:1000
Vinculin Monoclonal Antibody Catalog #:14-9777-82, Thermofisher	1:2000	anti-mouse-HRP antibody	1:2000
Beta-actin	1:1000	anti-mouse-HRP antibody	1:2000
Anti-Angiopoietin 2 antibody (AF623:RD)	1:2000	anti-rabbit-HRP antibody (Dako), REF: P0448	1:1000

Table 2.5: materials used for Bradford

Protein assay material (Bradford)	**Source**
Bradford Protein Assays(dye reagent): Phosphoric acid and methanol	Bio-rad
Bovine serum Albumin (BSA):	Sigma
Washing buffer: (1XTBS with 10 % (v/v) glycerol).	
Blocking buffer: 1XTBS containing 0.1% (v/v) Triton-X 100 and 5% milk (w/v).	

Table 2.6: Cells

Cell line	Company	Name of media	Company
HEK cell	Life Technologies, UK),	Free style (HEK293 media)	(Life Technologies, UK),
HUVECs	PromoCell	Endothelial cell growth medium.	PromoCell, Catalog number:22010
EAhy cell	(Edgell *et al*, 1983)	Demem (1x)+glutamax	(Life Technologies, UK), Ref:31966-021
HCMEC	PromoCell, cryopreserved cells, C-12285	Endothelial cell growth medium(MV)	PromoCell, Catalog number:22002
Endothelial Cell Growth Medium MV Kit: C-22120			

Table 2.7: siRNA and Transfection reagent used in knockdown experiments

siRNA	Source	Transfection reagent	Source	Dilution reagent	Source
ADAM10	Dharmacon, Human ADAM10 gene (102) siRNA	Lipofectamine (RNAiMAX Transfection Reagent)	Invitrogen, lot no: 1920861	Optemem media	Catalog number: 31985 062
ADAM17	Dharmacon, Human ADAM17 gene (6868) siRNA	Lipofectamine (RNAiMAX Transfection Reagent)	Invitrogen, lot no: 1920861	Optemem media	Catalog number: 31985 062
Ang2	Dharmacon, Mouse Ang2 gene (11731) siRNA	Lipofectamine (RNAiMAX Transfection Reagent)	Invitrogen, lot no: 1920861	Optemem media	Thermofisher, Catalog number: 31985 062
Tie2 siRNA	Dharmacon, Human TEK gene (7010) siRNA	Lipofectamine (RNAiMAX Transfection Reagent)	Invitrogen, lot no: 1920861	Optemem media	Catalog number: 31985 062

Table 2.8: Stimulants and blockers used in Ang2 stimulated cleavage and inflammatory experiments

	Source
Recombinant Human Tumor Necrosis Factor-α (TNF-α)	Catalog number: PHC3015 Gibco™
Recombinant Ang2	RD system, Catalog number: 7186-AN
Integrin α5β1	Purified Rat Anti-Human CD29 (Mab 13 monoclonal antibody or Integrin beta-1 antibody) Catalog No :552828 :BD Pharmingen TM
MAB	Anti-Integrin alpha V beta 3 antibody [LM609] (ab190147)
ATN161 (α5β1 integrin receptor antagonist)	ATN161, Tocris Bioscience, Cat. No. 6058/10
Cilengitide	Adooq, Catalog No. A12372
Blebbistatin	Sigma, Product Number B 0560

2.2 Microbiological media

2.2.1 Luria Bertani (LB) Broth

The broth was prepared by dissolving 5g yeast extract, 10g tryptone, 10g NaCl in 1 litre of distilled water and mixed, then autoclaved for about 20 minutes at 120^{o}C and stored at room temperature. Antibiotic (Ampicillin: 100µg/ml or Kanamycin: 50µg/ml) was added after cooling the solution. The broth was stored at 4°C in a refrigerator.

2.2.2 Preparing LB with ampicillin plates

The melted media was poured out into sterile plastic petri dishes (10ml per plate). The media was allowed to cool down for about 20 minutes, then stored in the cold room. The plates were labelled with date and initials.

2.3 Preparation and storage of plasmid DNA

2.3.1 Transformation of plasmids into *E.Coli*.

50µl competent cells were taken out of -80°C and placed on ice for 20-30 minutes. The DNA was used for transforming the cells and incubated on ice for 30 minutes with 50µl of the competent cells followed by a heat shock for 30 seconds at 42°C. The reaction then proceeded on ice for 5 minute incubation. Then 250µl of pre-heated (42°C) LB broth or antibiotic free SOC media was added. The reaction was incubated at 37°C for 1 hour on a shaker at 225 rpm. The cells were spread on 10cm LB agar plates containing ampicillin (100ng/µl) or kanamycin (50 ng/µl) and incubated at 37°C for 18 hours.

2.3.2 Agarose Gel Electrophoresis

Gel electrophoresis of DNA was carried out using 0.5-1% agarose-TAE gel. The agarose was added to 50 ml of 1x TAE and heated using a microwave to allow the agarose to dissolve, then allowed to cool to 50°C. The final concentration of 0.2 µg/ml Ethidium Bromide was added and mixed then placed in a gel tray to solidify. The samples of DNA were mixed with 3µl of 6 x loading dye and loaded into the wells. Electrophoresis was performed at 120V in running buffer using 1X TAE. The gel was placed under ultraviolet light to visualise and to capture the images for the DNA bands. The size of DNA was identified by comparing with 1 KB plus of the Invitrogen DNA ladder.

2.3.3 DNA Gel Extraction

The DNA of interest was run on an agarose gel by electrophoresis. The correct size band was excised from the gel using a sharp scalpel. The excised gel involving the DNA was purified with gel extraction kit according to the manufacturer's instruction (Qiagen). Briefly, the excised gel was weighed, dissolved in a three gel volume of the QG buffer and incubated at 50°C for 10 minutes to allow complete dissolution of the gel. One gel volume of isopropanol was added to the sample and mixed well. The sample was added to the QIAquick column and spun for 1 min to allow the sample pass through the column. The flow-through was discarded and QIAquick column returned back into the tube before adding 0.5ml of QG buffer and spun for 1 minute. Washing was performed by adding 0.75ml of buffer PE to the column before eluting the DNA sample with 50µl of sterile water. The purified DNA was analysed by running on an agarose gel to check the correct band size.

2.3.4 Restriction-Digestion for screening purposes

Diagnostic double restriction digestion of the DNA was performed using appropriate buffers according to the manufacturer's instruction (Roche). 2µg of the DNA was digested using 1 unit of each enzyme (*Nhe*-I and *Eco*R) and 5µl of 10x Buffer 4. The volume was topped up with dH2O to 50µl and mixed by pipetting. The digested mixture was incubated at 37°C for 1 hour. Agarose gel electrophoresis was performed to visualize the results.

2.3.4.1 Restriction-Digestion for ligation purposes

DNA Restriction digestion for ligation purposes was performed according to the manufacturer's instruction (NEB). 2µg of the DNA was digested using 1µl of each enzyme (*Nhe* I and *Not* I) or (*Nhe* I and *Eco*R I) plus 3µl of cutsmart Buffer 4. The volume was topped up with dH2O to 30µl and mixed by pipetting. The digested mixture was incubated at 37° C for 3 hours. Agarose gel electrophoresis was performed to visualize the results.

2.3.5 DNA Quick ligation

A ligation was performed according to the manufacturer's instructions provided with the kit (M2200S NEB) and made up of 3:1 ratio for the insert and vector. 5µl of the insert was mixed with 4µl of the vector and the volume was completed to 10µl with ddH2O. Then 10µl of the 2X ligation buffer and 1µl of quick T4 DNA Ligase was added and mixed by pipetting. The reaction was incubated at 25°C for 3 hours.

2.4 Plasmid DNA preparation

After transformation, a colony of bacteria was picked using a sterile pipette tip. Then inoculated with a starter culture of 5ml LB broth containing 5µl of antibiotics (Ampicillin: 100µg/ml or Kanamycin: 50µg/ml) and incubated overnight at 37°C with vigorous shaking at 300 rpm.

2.4.1 Bacterial Culture growth

In a sterilized flask the starter culture was diluted 1 in 1000 using 1000ml selective LB medium (for high-copy plasmids) with 1000µl of ampicillin (100µg/ml). The cells were incubated to grow at 37°C overnight with shaking at 300 rpm.

2.4.2 Plasmid purification

2.4.2.1 Mini Prep kit for plasmid purification

A single colony of *E. coli* was picked and inoculated into a starter culture of 5ml LB broth containing kanamycin (50µg/ml). The cells were incubated at 37ºC overnight on a shaker at 300 rpm. Cell harvesting was performed by centrifugation at 3000 rcf for 15 minutes. Then, the bacterial pellet was re-suspended using buffer P1, P2 and N3 according to the manufacturer's instructions and the plasmid extracted using the QIAprep spin column to which the DNA plasmid bound. Then, the DNA was eluted using 50µl of the elution buffer and stored at -20ºC.

2.4.2.2 Maxi Prep kit for plasmid purification

The bacterial cells were harvested using the QIAprep Maxi Prep kit (Qiagen) according to the manufacturer's protocol (by centrifugation at 6000 rcf for 15 min at 4°C.). The pellet was then re-suspended in 10 ml buffer P1 and pipetted until no clumps remained. 10ml buffer P2 was added and mixed vigorously by inverting the sealed tube 4–6 times until a complete homogeneous suspension was achieved and incubated at room temperature for 5 minutes. 10ml of pre-chilled buffer P3 was added and mixed instantly by inverting the sealed tube 4–6 times, and incubated on ice for 20 minutes. Centrifugation at 20000 rcf was performed for 30 min at 4°C. After washing the column with 10 ml QBT, the precipitate was discarded. The supernatant was run through the column to which the DNA is bound. The column was washed by using 60 ml QC buffer. DNA was eluted with 15ml QF buffer into a clean 50ml tube or flask. The DNA was precipitated by adding 10.5ml of isopropanol and mixed. Then, centrifugation was performed at 15000 rcf for 30 minutes at 4°C to complete precipitation. Precipitated DNA was washed with 1-5ml ethanol 70% and centrifuged at 15000 rcf for 10 minutes. The supernatant was discarded carefully, then the pellet was dried for 5-10 minutes. The DNA sample was re-suspended in 100-200µl TE buffer and stored at -20°C. Nanodrop was used to determine the concentration of the DNA and agarose electrophoresis was applied to assess the DNA.

2.4.2.3 Mega plasmid purification.

One litre of bacterial cells culture was harvested by centrifugation at 6000 rcf for 5 minutes at 4°C. The pellet was re-suspended in 32ml solution I (Table 2.9) and pipetted until no clumps remained. 48ml of solution II (Table 2.9) was added and mixed gently by swirling the sealed tube for 3 min to allow formation of a viscous solution. Another gentle mixing was performed after adding 40ml of solution III (Table 2.9) until a white precipitate of chromosomal DNA and protein formed. Centrifugation at 6000 rcf was performed for 5 minutes at room temperature. The sample was filtered through Mira cloth to remove all traces of white precipitate contamination. An equal amount of isopropanol was added and mixed by inversion. Centrifugation at 6000 rcf was performed for 10 minutes at 4ºC to pellet the remaining nucleic acid. The supernatant was then discarded and the pellet re-suspended in 15ml of sterile water and transferred into a clean centrifuge tube (50 ml). Equal volume of 8M lithium chloride was added, vortexed to precipitate the RNA and spun at 10000 rcf for 10 minutes at 4ºC to precipitate the RNA species. The supernatant was transferred into a clean 50 ml centrifuge tube and equal amounts of isopropanol was added to the supernatant before it was vortexed for 10 seconds and spun at 10000 rcf for 5 minutes at room temperature. The supernatant was discarded and the pellet re-suspended into 5ml of sterile water and transferred into a 15ml Falcon tube. 40 µl of a 1mg/ml RNAse was added, then vortexed and incubated for at least 1 hour at 37^0C. 1ml phenol chloroform was added followed by a brief vortex and spinning at 1800 rcf for 5 min at 4^0C. The aqueous layer at the top was preserved as it contains the plasmid and transferred into clean Falcon tube. 1ml of chloroform was added to extract any remaining phenol Chloroform. Then the sample was vortexed and spun at 2300 rcf for 5 minutes at 4^0C and the aqueous layer transferred into another clean tube. To the aqueous DNA solution 1/10 volume of 3M Na acetate pH 5 (i.e. 0.5 ml) and 3 volumes ethanol 100% (i.e. 15 ml) were added. The sample was vortexed well and to precipitate the DNA by spun at 1800 rcf for 10 minutes at 4^0C, before the supernatant was discarded. Precipitated DNA was washed with 5ml ethanol 70% and centrifuged at 1800 rcf for 2 minutes at 4^0C. Then, the pellet was dried overnight. The DNA sample was re-suspended in 2ml TE buffer or sterile water and stored at -20°C. The concentration of the DNA was determined using Nanodrop and agarose electrophoresis was applied to assess the DNA.

Table 2.9: Solutions used for Mega Plasmid purification

Solution I Glucose 50 mM Tris-HCl pH 8.0 25 mM EDTA pH 8.0 10 Mm Autoclaved 15 min at 120^{o}C	**Solution II** 0.2m NaOH 1%W/V SDS
Solution III Potassium acetate 117.77g Glacial acetic acid 46 ml Autoclaved 15 min at 120^{o}C	
50 ml of 3M sod acetate pH.5.2	
100ml of 70% ethanol (v/v)	
100ml 8M Li CL	

2.4.3 Glycerol stock storage of bacterial culture

To facilitate long-term storage of successfully transformed bacterial cultures, 2ml of culture, which had been grown overnight in LB media containing antibiotics (100ng/µl ampicillin or 50ng/µl kanamycin) was transferred to a sterile freezing vial under sterile conditions and 2ml of 80% glycerol was added to the vial and vortexed. The mixture was stored at -80^{o}C to be used again when needed.

2.5 PCR & Cloning

2.5.1 PCR plan to replace the native secretory leader with CD5

As a result of the low yield of the oligomerised protein, a trial to increase the yield of the protein was performed by replacement of endogenous native secretory leader of the oligomeric construct with CD5 leader which has been reported to have a better expression (Harmsen *et al*, 2013).

PCR amplification reaction was used for this purpose to amplify the wanted nucleotide involving the Tie2-COMP and part of the new CD5 secretory leader. Two oligonucleotide primers designed then ordered from (Eurofins/Genomics) were used in the PCR reaction (Table 2.12). The amplified oligonucleotide was run on an agarose gel to confirm the size. Additional steps were performed by PCR extension reaction to add the rest of the CD5 secretory leader using two primers (Eurofins/Genomics) to proceed the PCR reaction. An agarose gel was performed to confirm the size of the extended oligonucleotide.

Further experiments were performed to digest and extract (section 2.3.4.1) the purified form of the PCR-2 oligonucleotide (Tie2 containing CD5 leader sequence) using *Nhe*-I/*Not* I and *Nhe* I/*Not* I with *Eco*R I for the R3-COMP-pcDNA3.1 and the Wt-FC-pcDNA3.1. The size of the extracted (Section 2.3.3) insert and the vector were confirmed by running another agarose gel and the concentrations were determined by NanoDrop 2000 (Section 2.8). Further steps proceeded by ligation to incorporate the insert including CD5 with the vector to form ectodomain containing CD5 (Section 2.3.5). The ligated

plasmid was transformed to prepared starter culture and mini prepped (section 2.4.2.1), and Nanodrop was used to determine the concentration. The Tie2 ectodomain containing CD5 was transfected into the HEK 293 cells and the expressed proteins were harvested after 4 days. The protein was purified using Ni-NTA via the His-tag and eluted by imidazole containing buffer. Successful protein expression and purification were confirmed by SDS-GEL. Bradford assay was used to determine the concentration of the protein.

2.5.2 PCR: plasmid DNA

PCR reactions were conducted using KOD Hot Start DNA Polymerase (Novagen) to amplify the specified gene. The PCR component set up for each reaction is shown in Table 2.10. The Thermocycler (Perkin Elmer) was used to conduct the PCR reaction. Cycling conditions are shown in (Table 2.11).

Table 2.10: PCR components for 100µl reaction volume

Component	Volume	Final cons
dNTPs (2 mM each)	10µl	0.2 mM (each)
10X Buffer for KOD Hot Start DNA Polymerase	10µl	1x
$MgSO_4$	4µl	1.5mM
DMSO	5µl	
Forward primer 10µM	1µl	250 ng/µl
Reverse primer(BGH-R) 10µM	1µl	250 ng/µl
Template DNA (10ng/µl)	1µl	10 ng/µl
KOD Hot Start DNA Polymerase (1 U/µl)	2µl	0.02U/µl
PCR Grade Water	66µl	
Total reaction volume	100µl	

Table 2.11: PCR condition

Step	Temperature	Time	Cycles
Polymerase activation	94°C	5 min	1X
Denature	94°C	30 s	30 X
Annealing FW primer Tm > 75 C^0, BGH-R primer Tm 54.8°C.	50°C for	30 s	
Extension	72°C	2 min	
Final Extension		10 min	
Hold	4°C		

Table 2.12: list of primers use in PCR reaction

Primers	Name	Sequence	Tm
5->3 Sequence primers	CD5-Tie2-5	GATGCTGGTCGCTTCCTGCCTCGGA**GCAATGGACCTGATACTCATTAACTC** CD5 **Tie2(R3)**	> 75 C^0
3->5 Sequence primers	BGH-R	TAGAAGGCACAGTCGAGG	54.8C^0.
5-> 3 Sequence primers	CD5	**TTCACCGCTAGC**ATGCCCATGGGGTCTCTGCAACCGCTGGCCACCTTGT ACCTGCTGGGGATGCTGGTCGCTTCCTGCCTCGGA	

2.6 Sequencing

Sequencing was performed using Light Run sequencing service from GATC.

2.7 Gene synthesis

Gene syntheses was performed by Eurofins following sequence optimization using the geneius system.

2.8 Determination of DNA concentration

The concentration of the DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). 1µl of DNA was used to determine the concentration at OD260 and OD280. The purity of the DNA was assessed by the ratio of OD260/OD280 (pure DNA has 1.8-2.0 purity).

2.9 Cell culture

2.9.1 Culture of HUVECs

Medium 200 containing LSGS was used as a culture for the HUVECs. Cells were incubated at 37 °C and 5% CO_2. For cell maintenance, fresh culture media was used to replace the HUVEC media every 48 hours. To subculture HUVECs, the media was discarded and the cells washed with sterile PBS prior to the addition of 1.4ml of the 1x Tryple (Trypsin/EDTA) solution. The flask was gently shaken so the Tryple covered the entire surface. The flask was incubated for 2 minutes at 37^0C.

The cells were viewed using a microscope to ensure complete detachment of the cells. 3.6ml of complete media was added to neutralise the action of the 1x Tryple. Centrifugation at 250 rcf for 5 minutes was performed to collect the cell lysate and discard the supernatant. The lysate was re-suspended using complete media. HUVEC cultures seeded at 2 x 10^3 cells per cm^2 of growing area (using 6, 24 well or T25, 75 Flask). The cells need 4-5 days to reach (80%-100%) confluence before sub-culturing again. 6 or 12 well plates were used to be seeded at $2x10^5$ for each well and cells were grown to reach at least 30-80% confluency before using again.

2.9.2 Culture of EA.hy 926 cells

DMEM media with 10% (v/v) FCS was used for culturing the EA.hy 926 cells. The cells were incubated at 37°C and 5% (v/v) CO_2. Prior to using the cells in experiments, cells were detached using the 5x Typle (Trypsin/EDTA) and neutralised with complete medium. Pelleted cells were collected by centrifugation at 250 rcf for 5 minutes. Cells were re-suspended in fresh complete medium before culturing at $2x10^5$ per each well and at least 30%-80% confluency needed prior using for any experiments.

2.9.3 Human Cardiac Microvascular Endothelial Cells (HCMEC)

Endothelial cell growth media containing growth factor (complete media) was used as a culture for the HCMECs. Cells were incubated at 37 °C and 5% CO_2. For cell maintenance, fresh culture media was used to replace the HCMECs media every 48 hours. To subculture HCMECs, the media was discarded and the cells washed with sterile Hepes BSS Solution prior to the addition of the Trypsin/EDTA solution. The flask was gently shaken so the Trypsin/EDTA solution covered the entire surface. The flask was incubated for 2 minutes at 37^0C. The cells were viewed using a microscope to ensure that there was complete detachment of the cells. Trypsin neutralization solution was added to neutralise the action of the Trypsin/EDTA solution. Centrifugation at 250 rcf for 5 minutes was performed to collect the cell lysate and discard the supernatant. The lysate was re-suspended using cell growth medium. HCMECs cultures seeded at 2 x 10^3 cells per cm^2 of growing area (using 6, 24 well or T25, 75 Flask). The cells need 4-5 days to reach (80%-100%) confluence before sub-culturing again. 6 or 12 well plates were used to be seeded at $2x10^5$ for each well and cells were grown to reach at least 30-80% confluency before using again.

2.10 Concentration of conditional cell culture media

Culture media samples (0.3 or 0.5 ml) were collected from the cell lines and place into centrifugal filter unit (Amicon ultra-0.5ml, ultracel-30k, Ref: UFC503096). The samples were centrifuged for 7 minutes then the concentrator removed and placed upside down into a new collection tube, spun for 3 minutes at 5000 rcf to get final volume 20-40µl concentrate. Sample buffer with or without DTT was added to the concentrated medium and carefully pipetted into small microfuge tube before being boiled for 3 minutes and spun at full speed for 5 minutes to be run on a SDS gel and blotted using western blot test.

2.10.1 Cell lysate sample preparation

Cell lysate samples were collected from the cell lines after removing the media by adding 60-100µl of sample buffer with or without DTT. The sample was boiled for 4 minutes at 95℃ and sonicated and centrifuged at maximum speed for 5 minutes then loaded into SDS gel and blotted using western blot test.

2.11 Cell culture experiments

2.11.1 Ang2 stimulating Tie1 and VE-cadherin cleavage test

Six-well plates containing 80-100% confluent cells of (HUVECs or HCMECs) were used to test the effect of Ang2 on the cells. The media was discarded and washed twice with serum free media (Demem media) before adding 0.3 ml of serum free media to each well. The treated cells were incubated at 5% CO_2, / 95% O_2 at 37^{o}C for 1 hour. Media samples were collected after incubation then concentrated to be run on a gel and blotted using western blot test to determine the cleaved protein in the media.

2.11.2 B-integrin blocking effect on Ang2 stimulating cleavage

One day before the experiment, the cells (HCMECs or HUVECs) were passaged and plated out into a 24 well plate to reach (80-100%) confluency the next day. The media was discarded and washed twice with serum free media (Demem media) before adding 0.3 ml of serum free media to each well. The antibody blockers were added to the relevant wells and incubated for 10 min at 37^{0}C before treating the cells with Ang2 or any other stimulant, followed by incubation for 1 hour at 37^{0}C and 5% (v/v) CO_2. Media and cell lysate samples were prepared (Section 2.10, 2.10.1) and treated with DTT sample buffer to be run on SDS gel and blotted using western blot test.

2.11.3 siRNA cell transfection (Tie2, Ang2 , ADAM10 and 17)

One day before transfection, the cells (HCMECs or HUVECs) were passaged and plated out into 24 well plate to reach 20-30% confluency. The siRNA was diluted to 2µM and two eppendorf tubes were prepared for each well. Tube A involved 7.5µl siRNA plus 117.5 µl serum free medium (Optemem media) and was mixed by pipetting. Tube B involved 2.5 µl Dharmafect plus 117.5µl serum free medium (Optemem media) and was mixed by pipetting. Both tubes A and B were incubated for 5 minutes at room temperature then combined and incubated for 20 minutes at room temperature. The media was removed from the cells and replaced with 500µl antibiotic free complete media (Optemem media) plus the 100µl of combined A and B tube and mixed by pipetting, after 5 hours replace media with complete media then incubated for 48-72 hours. After 48-72 hours, the media was washed twice with 500µl SFM (serum free media: media 200) then replaced with 300µl SFM for each well. Then the cells were stimulated (Ang2, TNF-α or Ang2 plus TNF-α), before being incubated for 1 hour. Samples (0.3ml) were collected and concentrated (Section 2.10) and treated with or without DTT containing buffer so they could be run on an SDS gel then blotted. Cell lysate samples were also collected using 60µl of 1.5x sample buffer with or without DTT (Section 2.10.1).

2.12 Protein Expression

HEK293 were transfected with the plasmid-encoding gene of interest and expressed protein purified from condition medium as detailed below.

2.12.1 Protein expression using HEK293 cells (300 ml transfection)

Freestyle media was used for growing the HEK293 cells in 5% CO_2 and at 37°C using a shaking incubator. When the cell density reached about $2.4x10^6$ the cells were passaged, and transfection was performed at the cell density of $1x10^6$. The transfection proceeded using a specified amount of DNA that was diluted in 30ml of sterile PBS and vortexed, then 600µl of PEI (1mg/ml) was added and vortexed again. The transfection tube was incubated for 20 minutes before adding to the HEK293 cells. The cells were placed in a shaking incubator at 5% CO2, 37°C and at 122 rpm for 4 days then harvested.

2.12.2 Haemocytometer for Cell counting

10µl transfected cells were mixed with 10µl trypan blue dye and transferred into a Neubauer hematocytometer. The cells were counted in the four squares of one hematocytometer grid. Cell counting was performed using microscope at 20x magnification and using the following equation to find the total number of cells. Counted cell number 4×10^4 = cell number/ ml.

2.12.3 Protein purification

Gravity-Flow chromatography was used to purify the Poly His-tagged protein using Ni-NTA Agarose (Qiagen) as beads to extract the protein that collected as fractions and was stored at 4^0C. The cells were harvested by centrifugation at 1400 rcf for 5 minutes, then filtered using mira cloth into large bottle. For every 40 ml of media the following were added: 5 ml glycerol, 20 mM imidazole and 5ml of 10x PBS. Then 0.5 ml/100 ml of re-suspended slurry (QIAGEN-NI) was washed according to the manufacturer's instructions then incubated with the media on a rocker at room temperature for at least 40 minutes. The beads were collected by centrifugation at 1000 rcf for 3 minutes. The Ni column was prepared by washing it with 5ml washing buffer before adding the beads and washing it with 15-20 ml of washing buffer. Then the protein was eluted with 5 ml elution buffer the eluted protein was and collected directly into Eppendorf tube as fraction. SDS-PAGE and Coomassie Blue staining were used to identify the fractions that involved the protein (Table 2.13).

Table 2.13: Buffers used for protein purification

1- Washing buffer: 50mM Tris-Hcl PH 8 300 mM NaCl Glycerol 10% 20 mM imidazole	**2- Elution buffer:** 50mM Tris-HCl PH 8 300 mM NaCL Glycerol 10% 250 mM imidazole
3- Preservation buffer: 1X TBS with Glycerol 10 % Each buffer made by adding the solutions then topping up to 100ml using distilled water in a duran bottle then stored at room temperature.	

2.12.3.1 Buffer Exchange to remove imidazole from protein sample.

High concentration of imidazole salt in the elution buffer can affect the downstream applications. As a result Zeba Columns were used to exchange the imidazole from the purified protein. The columns were spun at 1000g for 2 minutes with 1ml TBSg (1x TBS with glycerol 10%). Then the TBSg was discarded and additional washing with 1ml TBSg was repeated three times and spun at the same speed and for the same period of time. The protein samples were added to the centre of the compact resin bed of the column and by spinning the column at 1000g for 2 minutes the sample was cleared from imidazole.

2.12.3.2 Ultrafiltration unit to increase protein concentration

Centrifugal ultrafiltration unit (Amicon Ultra-15) was used to increase the concentration of the protein. The unit was washed with 1ml of diluted 1X TBSg then centrifuged to discard the supernatant buffer. Washing was repeated three times. Then the protein sample was added to the filter unit and centrifuged at 3000g for 20 min. The sample was collected, and Bradford assay was performed to measure the new concentration.

2.12.4 Analysis of Protein Expression

2.12.4.1 Bradford assay (Standard Procedure for Microtiter Plates)

Bradford protein assay was performed according to the manufacturer's instruction (Bio-rad Bradford assay). The dye reagent was prepared by diluting 1 part of the dye reagent with 4 parts of DDI water. Five dilutions of the standard protein (Bovine serum albumin) were prepared. These standards were considered as representative for the tested protein solution. The standard linear range of the microtiter plate assay was between 0.05 and 0.5 mg/ml. The test was proceeded by pipetting 10µl of every standard and sample into separate microtiter plate wells. Then 200µl of the diluted dye reagent was added to each well. The sample and reagent were mixed using clean tips each time. Incubation was performed for at least 15 minutes and not more than 30 minutes at room temperature. The

absorbance of of each well was measured at 595nm and the result was calculated using Graphpad Prism 7 to determine the concentration of the samples.

2.12.5 ELISA

ELISA binding tests were performed to determine the relative binding affinity of recombinant proteins. The plate was coated with 20µl of the prepared Ang2 and incubated overnight at 4°C or 1 hour at room temperature. The Ang2 coating buffer was removed, and the wells were blocked with 300µl of blocking buffer and incubated at room temperature for 1 hour. Again, the buffer was removed, and 20µl of each concentration of the ectodomain protein (Tie2) was added to the wells in triplicate or duplicate and incubated for 2 hour at 37°C (Table 2.14). Further, three washes were applied by adding 300µl of washing buffer per well and rocking for 5 minutes. 50µl of diluted anti-Tie2 antibody (1:500 dilution) was added to each well and incubated for 2 hours hour at room temperature. Another three washes were carried out, 5 minutes rocking each time, using 300µl washing buffer for each wash. 50µl of anti-goat-HRP antibody was added (1:1000 dilution) and incubated for 1 hour at room temperature. Further four washings were performed in the same way mentioned before. The wells were developed by adding 50µl of Sigma Fast OPD; colour development was monitored via reading the absorbance at 450nm.

Table 2.14: ELISA-protein binding assay

ELISA-protein binding buffers
Coating buffer: 757.5 mg of Na2CO3 and 1500 mg of NaHCO3was dissolved in 250 ml of H2O and adjust the PH to PH 9.6. **10x-Tris buffered saline (10X TBS)**: TRIS BASE 61g 0.5M, NaCl 87 g, Add HCL to adjust PH TO 7.35, Complete with H2O up to 1000ml. **Blocking buffer**: 1x TBS containing 0.1 %(v/v) Triton-X and 5 % milk (w/v).

2.12.6 Protein separation by SDS-PAGE

2.12.6.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was used to separate proteins according to their molecular weight. Gel was prepared from the following components that are shown in (Table 2.15) and (Table 2.16) using a Bio-Rad apparatus. Sample buffer was added to samples before heated for 3 minutes at 95 °C and loaded together with 7µl of protein marker into the SDS gel and run using 1x running buffer at 120V.

Table 2.15: Component of resolving gel used for SDS-PAGE

Resolving gel constituents	Volume for 10% gel	Volume for 7.5 % gel
30% Acrylamide/0.8% Bisacrylamide	6.7 mls	5 mls
2M Tris pH 8.8	3.7 mls	3.7 mls
dH2O	9.6 mls	10.9mls
20% SDS	100 µl	100 µl
10% Ammonium Per Sulfate (APS): Prepared by dissolving 0.1 g of ammonium per sulfate in 1ml water	134 µl	134 µl
Tetramethylethylenediamine (TEMED)	14 µl	14 µl

Table 2.16: Component of stacking gel used for SDS-PAGE

Stacking gel constituents	Volume for 10% and 7.5% gel
30% Acrylamide/0.8% Bisacrylamide	3.3mls
1M Tris, pH 6.8	2.5mls
dH2O	13.7mls
20% SDS	200 µl
10% Ammonium Per Sulfate (APS)	200 µl
Tetramethylethylenediamine (TEMED)	20 µl

2.12.7 Western blotting and Immunoblotting

SDS-PAGE was used to separate the proteins and the separated proteins were transferred to a Hybond-ECL nitrocellulose membrane by electrotransfer at 100V using 1x transfer buffer. The membrane was blocked using 5% blocking buffer (5% w/v of non-fat milk with TBS-TX-100) and incubated on a rocker for 1 hour. Immunoblotting was performed for the protein by incubating the membrane at 37^{o}C for 1 hour with the primary antibody diluted with 5% blocking buffer. Further three washings of the membrane were performed with TBS-TX-100 buffer and each time was incubated for 5 minutes on a rocker plate at room temperature. The membrane was incubated again with a secondary antibody diluted with 5% blocking buffer for 1 hour. Another three washes were performed before using the chemiluminescent system to detect the secondary antibodies, which was already bound to a Horseradish peroxides (HRP). The membrane was immersed in a developer solution for 1 minute to allow the probe to be soaked. Emitted light was detected by exposing the nitrocellulose membrane to Kodak imaging film and enhanced by chemiluminescence (ECL) system (Amersham Pharmacia Biotech, UK).

2.12.7.1 Membrane Reprobing

The membrane was placed in TBSTX-100 and washed for 5 minutes with rocking. Subsequently placed in 1X reblot solution (Chemicon International: 1.5ml of the 10x stock solution diluted in 13.5 ml dH2O). Incubation proceeded for 15 minutes with rocking at room temperature. Later three washes without rocking were performed (twice with water and another one with TBSTX-100). Finally, the membrane was incubated with 5% blocking buffer at room temperature for 30 minutes.

2.13 Statistcal analysis

The data was collated using Excel 2016 and Graphpad Prism (version 7.04) for statistical analysis. The data was analysed by One Way ANOVA and Tukey's post hoc test, *p<0.05, **p<0.01, ***p<0.001 as indicated in results. In some expermints data was analysed by student t-test as indicated in the results. The results are expressed as an average ∓ SEM for at least three independent experiments. Differences were judge as statistically significant at p< 0.05. Statistics performed on original (non normalised data). In some figures and tables the data is shown as % of normalised data.

Chapter 3: Improving selective binding of Tie2 ectodomain variant to Ang2

Previous work demonstrated that directed evolution of the Tie2 ectodomain using a cell study resulted in identifying a mutant Tie2 ectodomain receptor specific to Ang2 called the R3 protein. The difference between the wild-type and the mutant R3 ectodomain are the replacement of F161 with I and deletion of R167 and H168. With these three amino acid changes, the R3 protein preferentially binds to Ang2 but not Ang1 (Brindle *et al*, 2013). The R3 ectodomain has a lower binding affinity to Ang2 compared to the wild-type affinity. The experiments described in this chapter were aimed at increasing the apparent binding affinity of mutated ectodomain for Ang2. This was approached by: (1) introducing additional changes into the ectodomains by performing further mutagenesis and (2) engineering a new multimeric protein construct.

In this study, the R3 ectodomain protein was used to carry out the experiments. In addition to the R3 protein, two other proteins were used, the wild-type protein and the RH mutant protein. The wild-type protein represents a positive control due to its ability to bind equally to the Ang1 and 2. In contrast, the RH mutant protein represents a negative control, as it is not able to bind to Ang2. With these two controls, the binding activity of the R3 protein could be assessed. Initial work was aimed at confirming the sequence of the wild type, RH and R3 construct prior to their use in this study. In addition, these constructs will be transfected into a mammalian expression system and the expressed protein will be purified. Furthermore, these proteins will be used in binding assays. These experiments will form the foundation for production and testing of the newly engineered ectodomains.

3.1 Confirmation of plasmid identity

The plasmids (Wt-Fc, RH-Fc and R3-Fc) were transformed into *E. coli* (section 2.3.1). *E. coli* was grown, plasmid prepared and verified by performing restriction digests. As shown in Figure 3.1, digestion of plasmid with *Nhe*-I and *Eco*R-I released a fragment of approximately 1334bp from each of the plasmids which represents the expected size for the insert. Then sequencing was performed to confirm the identity of these plasmids before starting any experiments.

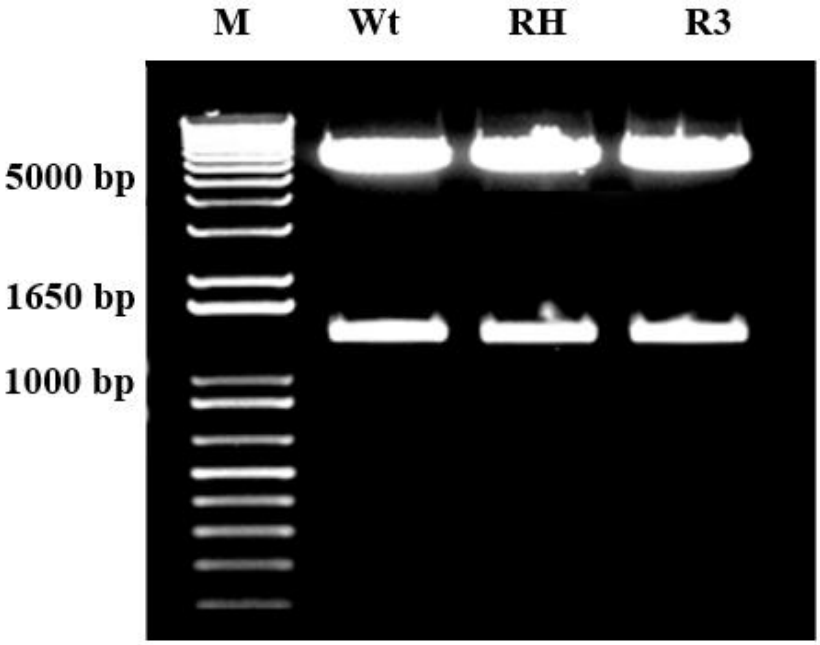

Figure 3.1: Agarose gel showing restriction digestion of Tie2 ectodomains.

The figure represent an agarose gel showing restriction digests with *Nhe*-I and *Eco*R-I of the extracellular Tie2 domains. Wt: Wt-Fc, R3:R3-Fc, RH: RH-Fc mutant ectodomains. The inserts of Wt-Fc, R3-Fc and RH-Fc were confirmed as it can be seen at 1334 bp. Positions of size markers (M) are shown in bp. This experiment was performed once.

3.2 Time course for the expressed protein

Protein expression was expected to be greater in the media than the cell lysate as the protein contains the signal peptide required to secrete the protein into the media. An experiment was performed to determine the time course of protein accumulated in the media using HEK293 transient transfection (Section: 2.12.1). To do this transfection, cells were transfected and sample of media taken at different time points. Secreted Tie2-Fc was detected by immunoblotting (Figure 3.2, A). The result of this experiment showed that maximum expression were between day (2-4) and the expression started to decrease at day (5-6). One possible reason for this decrease is that increasing cell death after day four cause releases of proteases due to cell lysis and this resulted in protein degradation (Figure 3.2, B).

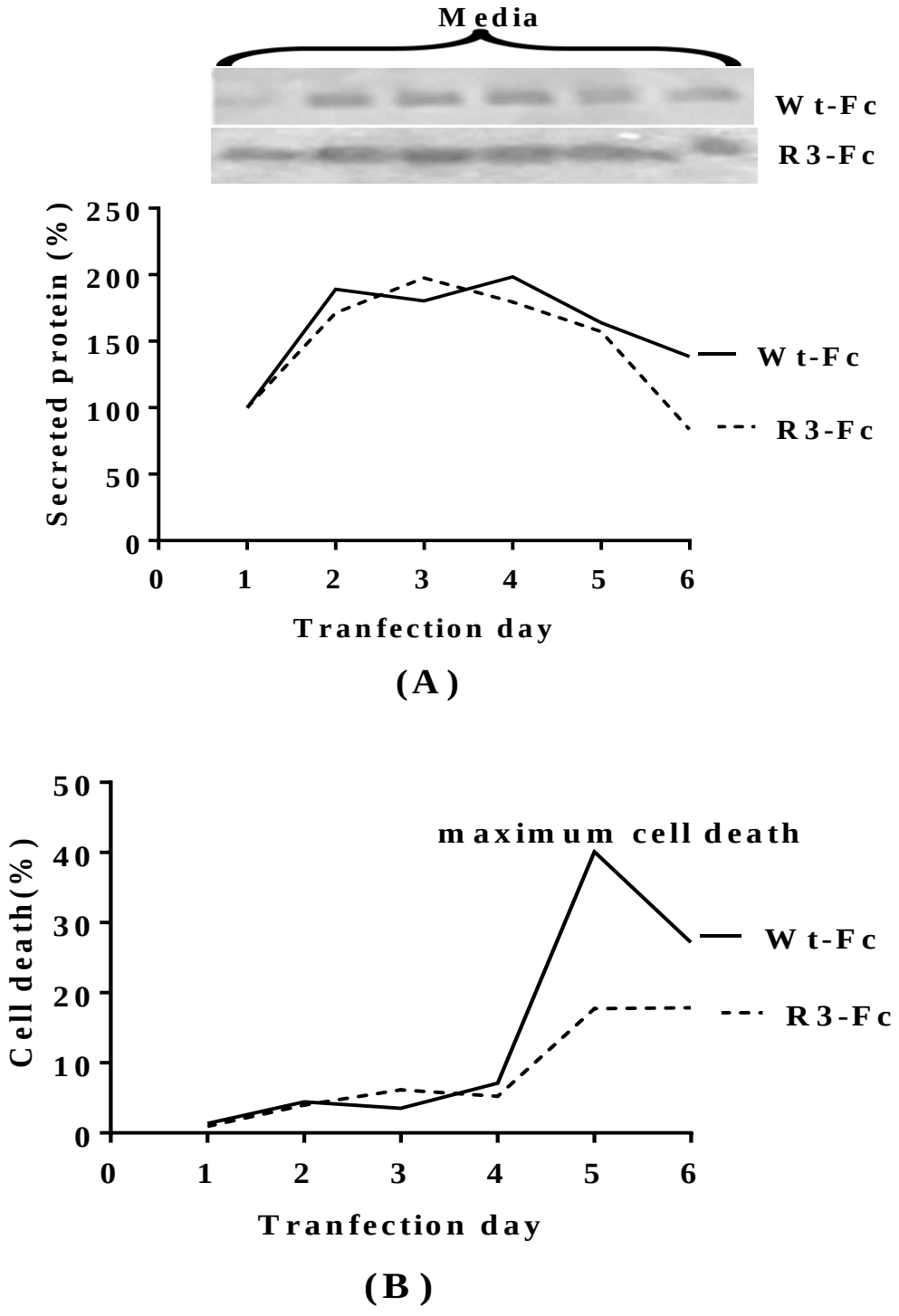

Figure 3.2: Time course of Accumulation secreted protein.

Accumulation secreted proteins of the dimeric Tie2 (Wt-Fc and R3-Fc mutant ectodomain) using HEK293 cell free medium following transient transfection. A: Western blot probed with anti Tie2 ectodomain shown time course for expression of Wt-Fc and R3-Fc mutant ectodomain after collecting media sample at days 1, 2, 3, 4, 5 and 6. ImageJ was used to quantitate the data. B: Media samples taken between days 1-6 to show percentage of cell death by counting the cells. This experiment was performed once.

3.3 Expression of ectodomains

Media was harvested after 3-4 days.The proteins were purified using TalonR nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices via the His-tag. As shown in Figure 3.3, a single 80 kDa band is evident, following SDS/PAGE and coomassie staining for RH-Fc, Wt-Fc and R3-Fc. This is consistent with the expected size of protein reported by Brindle et al .2013 (Brindle *et al*, 2013). Further, the gel revealed no contaminating protein detectable by Coomassie staining.

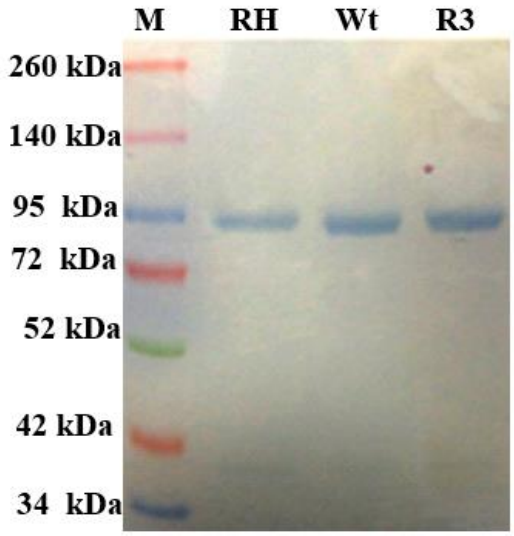

Figure 3.3: A– Coomassie Blue stain of purified Wt-Fc, R3-Fc and RH-Fc proteins following SDS/PAGE.

Coomassie Blue stained gel of RH-Fc, Wt-Fc and R3-Fc proteins expressed in HEK293 cells and purified from medium by Ni-NTA affinity purification. Proteins were resolved in the presence of DTT. The position of molecular mass marker was indicated. This experiment was performed once.

The Fc ectodomains were also resolved in the presence and absence of DTT in order to confirm the dimeric form of the ectodomains under non reducing conditions (Figure 3.4).

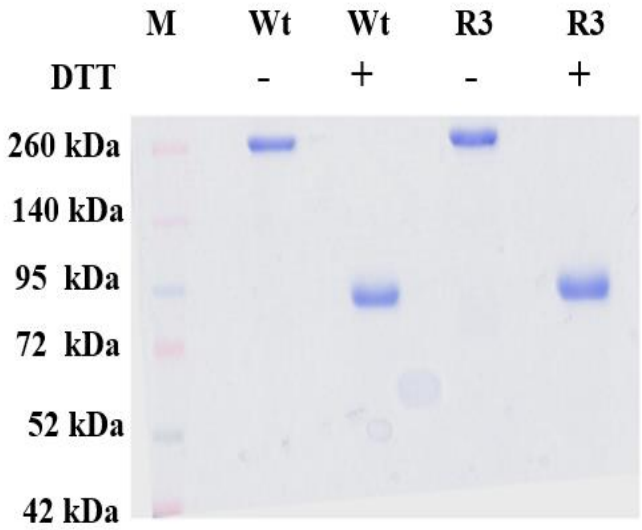

Figure 3.4: Coomassie Blue stain of dimeric and monomeric Wt-Fc and R3-Fc.

A –SDS 7.5% Coomassie Blue stain of His-tagged purified dimeric Wt-Fc, R3-Fc with and without DTT to show the monomeric and the dimeric form of the proteins. The position of molecular mass marker was indicated. This experiment was performed once.

In order to determine the average yield of purified ectodomain, proteins were quantified by Bradford assay following purification. Yield of Wt-Fc was 441.3 ∓ 30.9 ng/ml of transfection, $\text{mean} \mp \text{SEM}, n = 3$ (132.4 ∓ 9.3 µg/300 ml of transfection), yield of R3-Fc was 590 ∓ 82.9 ng/ml of transfected media, $\text{mean} \mp \text{SEM}, n = 4$, (177 ∓ 24.7 µg/300 ml of transfection).

3.3.1 Determination of Binding activity by ELISA

ELISA were carried out to validate the binding activity of the ectodomains to Ang1 and Ang2. Although using ELISA test cannot easily provide the actual Kd value, it can be used to assess the relative affinity of the ligand-trap by measuring the half-maximal binding concentration. Initially, the binding activity of the Wt-Fc and R3-Fc to Ang2 was determined (Figure 3.5). ELISA was performed at least three times and binding curve fitted to data points using Graphpad Prism. The concentration of ectodomains required for EC_{50} (concentration of half-maximal binding) was calculated from curve and is shown in Table 3.1. In each experiment performed binding to Wt-Fc was performed and the maximum binding activity of R3-Fc is expressed as a percentage of maximum binding to Wt-Fc (Table 3.1).

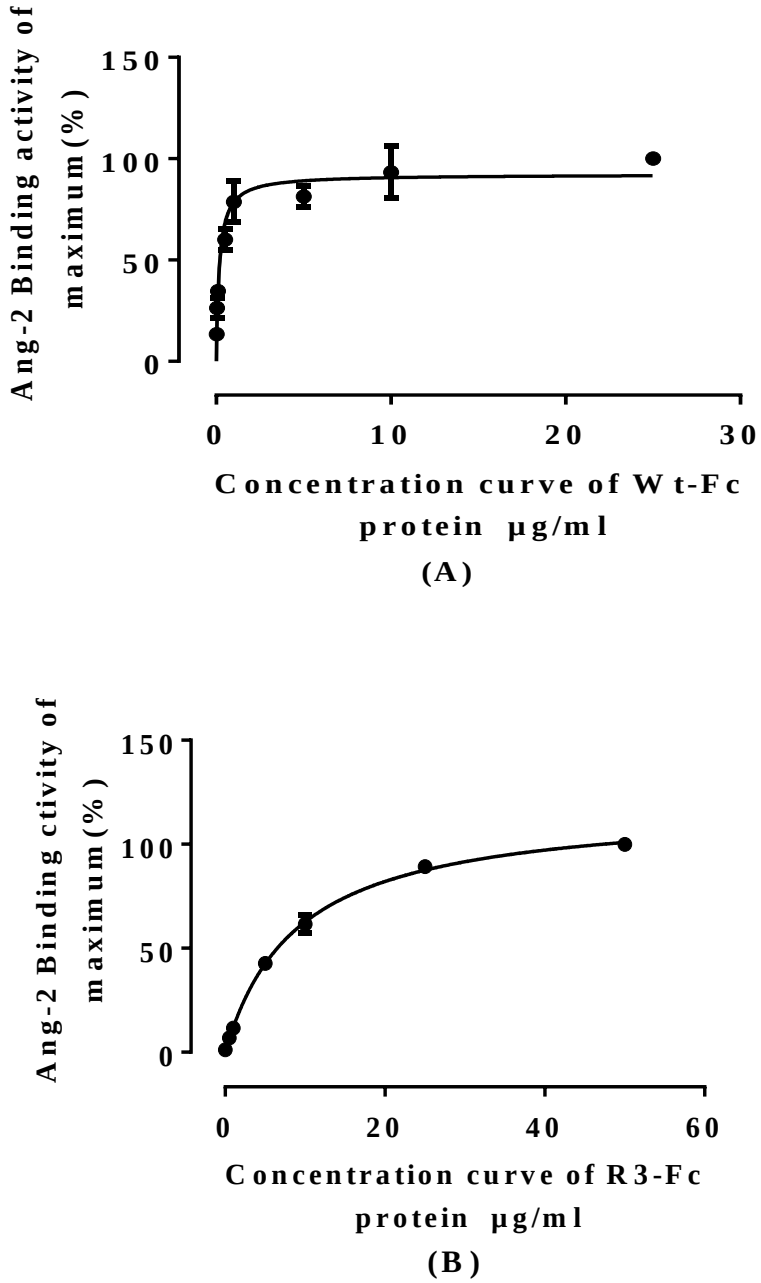

Figure 3.5: Binding activity curve of the Wt-Fc and R3-Fc to Ang2.

ELISA data of binding of Wt-Fc or R3-Fc to immobilized Ang2 are shown for a single experiment with duplicate determination. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism and shown as a mean and standard error of mean of duplicate measurements from a single representative experiment of nine.

Further ELISA tests were performed to assess the relative affinity of the R3-Fc ectodomain compared to the Wt-Fc. The EC_{50} of the R3-Fc to Ang2 (9.4∓1.7μg/ml) was significantly lower than the EC_{50} of the Wt-Fc to Ang2 (0.15∓0.07 μg/ml). Binding curve was also performed for Wt-Fc and R3-Fc binding to Ang1 (Figure 3.6). The maximum binding activity of the R3-Fc to Ang1 was significantly lower than the maximum binding activity of Wt-Fc (Table 3.1). These results are consistent with the previous data that has been reported by Brindle et al., 2013 (Brindle *et al*, 2013).

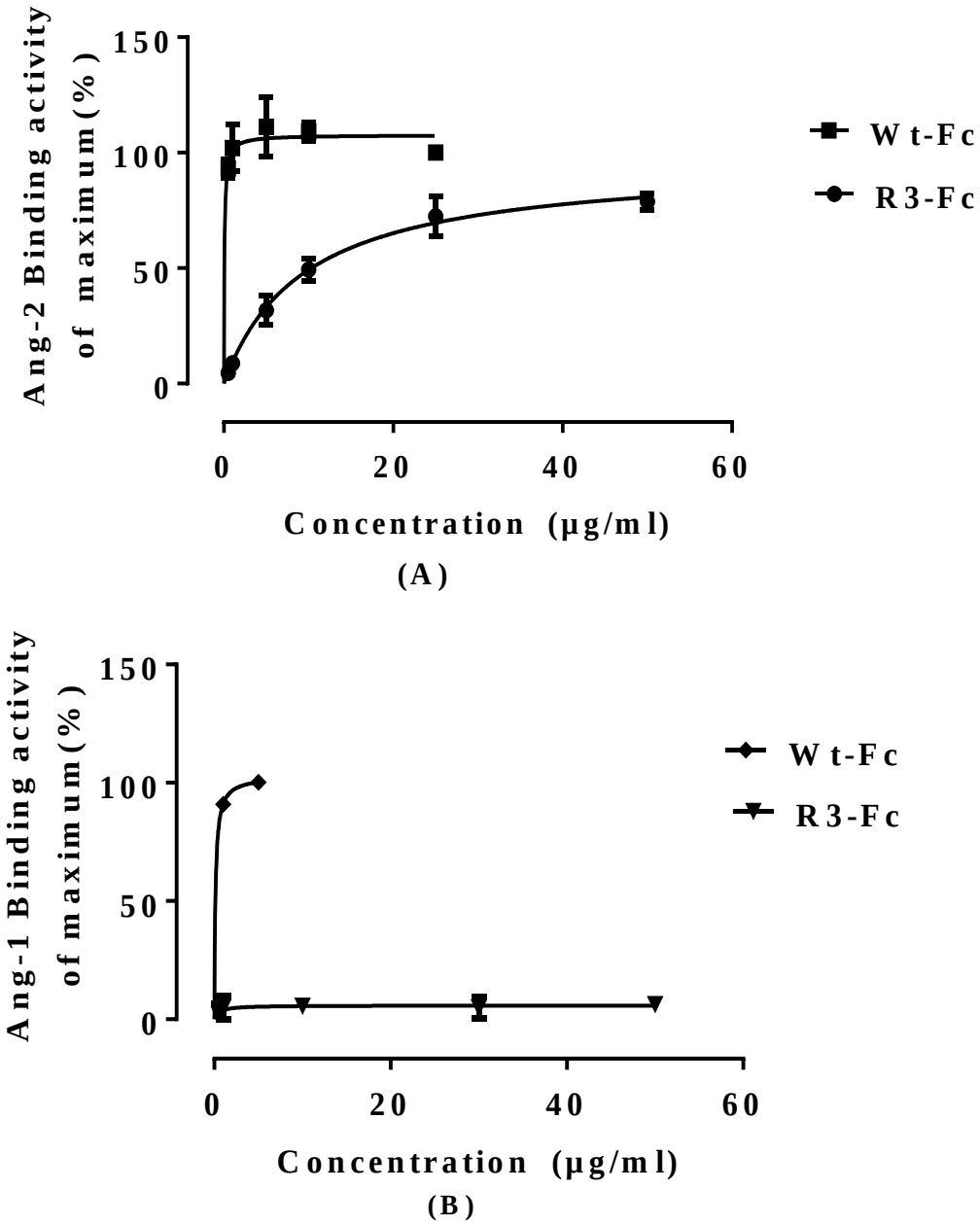

Figure 3.6: A comparison between binding activity of R3-Fc and Wt-Fc to show there affinity to Ang1 and Ang2.

ELISA data of binding of Wt-Fc and R3-Fc to immobilized Ang2 are shown for a single experiment with duplicate determination. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism and shown as a mean and standard error of mean of duplicate measurements from a single representative experiment of four. A: represents Ang2 binding activity of R3-Fc in comparison to Wt-Fc maximal binding concentration at 25µg/ml. B: represents Ang1 binding activity of R3-Fc in comparison to Wt-Fc maximal binding at 5µg/ml.

Table 3.1: Ang1 and Ang2 binding activity of R3-Fc in comparison to Wt-Fc

Ang2	**Wt-Fc**	**R3-Fc**
Bmax (%)	100 (n=4)	76∓3.5** (n=4)
EC_{50} (µg/ml): half maximum binding concentration	0.15 ∓0.07 (n=9)	9.4 ∓1.7*** (n=9)
Ang1	**Wt-Fc**	**R3-Fc**
Bmax (%)	100 (n=4)	4.5 ∓1*** (n=4)
EC_{50} (µg/ml): half maximum binding concentration	0.07∓0.04 (n=6)	ND (n=3)

Table 3.1 Shows the half maximum binding concentration for Wt-Fc and R3-Fc binding to Ang1and Ang2, as well as maximum binding capacity for R3-Fc compared to Wt-Fc. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed). **p<0.01, ***p<0.001(t test)

Additional ELISA were performed in order to confirm the lack of binding activity of the RH-Fc to Ang2 compared to the Wt-Fc ectodomain to Ang2. Two concentration of RH-Fc (10 µg/ml and 25µg/ml) were tested for binding to Ang2 in comparison with 0.05µg/ml Wt-Fc.

As shown in Figure 3.7, RH-Fc was unable to bind to Ang2 at either 10µg/ml or 25µg/ml. These data indicate that the RH protein can be used as a negative control and the wild protein as a positive control. Three experiments were conducted to compare the binding affinity of the wild-type with the RH ectodomain receptor using Ang2 for coating the well.

In conclusion, the wild-type has a concentration dependent binding to Ang1 and 2, the RH-Fc has no binding activity to Ang2, and the R3-Fc has lower binding activity to Ang2 than the Wt-Fc and at the same time shows no binding activity to Ang1. These results are consistent with the previous data that has been reported by Brindle et al., 2013 (Brindle *et al*, 2013).

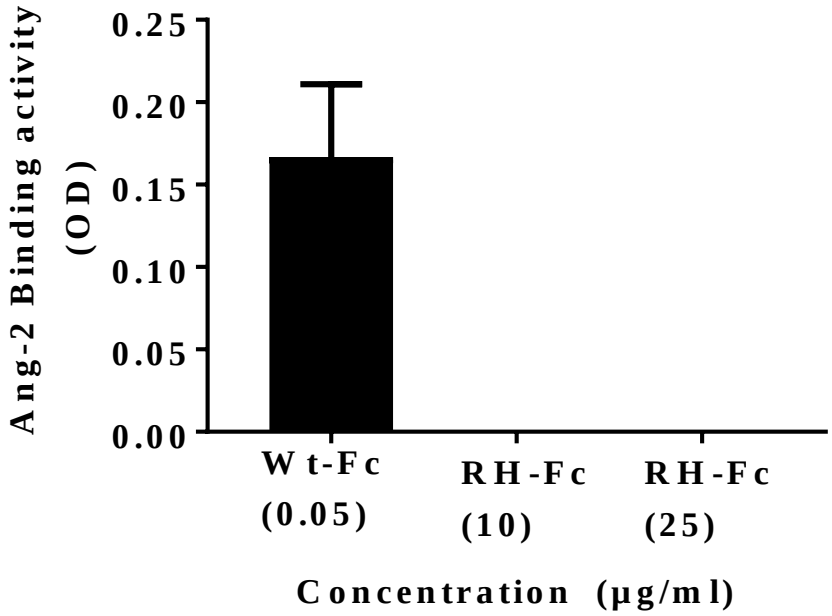

Figure 3.7: the binding activity of RH-Fc to Ang2 in comparison to Wt-Fc.

ELISA data of binding of RH-Fc and Wt-Fc to immobilized Ang2. Data are shown as a mean and standard error of the mean for three independent experiments (n=3). The data shows significant decreased binding affinity, $p< 0.001$ (Tukey's post hoc test) of the RH ectodomain compared to the maximum binding activity of the wild type.

3.4 Additional R3-Fc variants have improved binding to Ang2

Further sequencing of the pool of Ang2 selective variants of Tie2 ectodomains obtained by directed evolution (Brindle *et al*, 2013) revealed two variants with additional mutations. These variants have the following replacement (Table 3.2).

Table 3.2: R3-Fc additional variant

Variants	Mutations
TIE2-14	RH del ,V153/L and P169/A
TIE2-15	RH del , E169: D and V170/I.

In order to test whether these additional mutations improve Ang2 binding they were introduced into the R3-Fc design by site-directed mutagenesis. In addition a third variant was constructed (TIE2-16: RH del, V153/L, P169/A, E169: D, V170/I) combining all mutations in T14 and T15. This site-directed mutagenesis was performed by Dr Neil Bate in the laboratory.

3.4.1 Protein expression of R3-TIE-Fc mutagenesis

The T14, 15 and 16 plasmids were transfected into the HEK 293 cells and the expressed proteins were harvested after 4 days. The proteins were purified using TalonR nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices via the His-tag. Successful protein expression and purification were confirmed by SDS-GEL (Figure 3.8). As shown in the figure below there are three major visible proteins (TIE2-14, TIE2-15 and TIE2-16) of approximately 80 kDa as expected on the gel, which indicates the purity of the proteins. Bradford assay was performed to determine the concentrations of the purified proteins and sufficient protein was obtained to proceed with experiments.

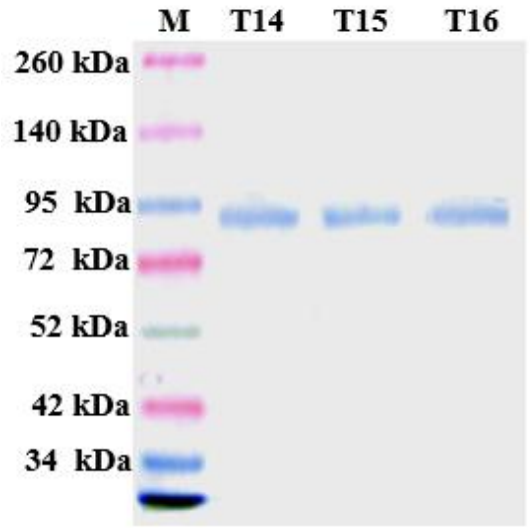

Figure 3.8: Coomassie stain of TIE2 14, 15 and 16 proteins.

Coomassie Blue stained gel of TIE2-14, TIE2-15 and the TIE2-16 expressed in HEK293 cells and purified from medium by Ni-NTA affinity purification. Proteins were resolved in the presence of DTT. T14: TIE2-14. T15: TIE2-15. T16: TIE2-16. The three bands were detected at approximately 80kDa. The position of molecular mass marker was indicated. This experiment was performed once.

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3.4.2 Binding affinity of TIE2-14, TIE2-15 and TIE2-16

ELISA tests were performed to validate the binding activity and specificity of these R3-Fc mutant ectodomains to Ang1 and 2. Initially, the binding activity of the TIE2-14, TIE2-15 and TIE2-16 proteins to Ang2 was determined and the concentration of maximal binding activity for these ectodomains was calculated to obtain the EC_{50}. The Figure 3.9, shows that there is a 2-4 fold improvement in the binding activity of the new mutant proteins {TIE2-14 (4.4∓ 0.18 μg/ml), TIE2-15 (5.3∓1.9 μg/ml) and TIE2-16 (2.2 ∓ 0.6 μg/ml)} to the Ang2 ligand compared to the binding activity of dimeric R3-Fc (9.4 ∓1.7μg/ml) (Table 3.3). The three variants have similar binding activity to Ang2 ectodomain. However, TIE2-16 probably has a higher affinity to Ang2 than the other dimeric mutants including the R3-Fc.

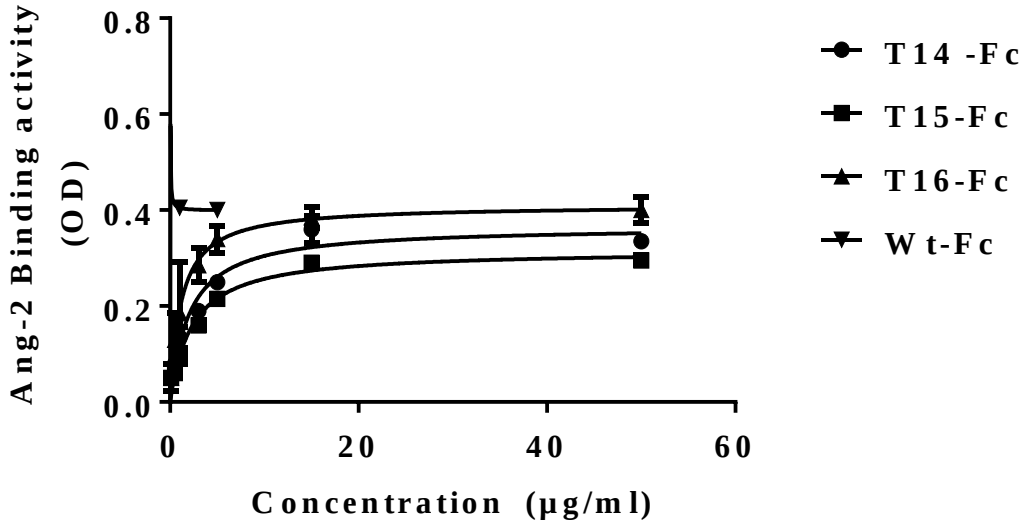


Figure 3.9: Binding activity curve of the T14, T15 and T16 to Ang2.

ELISA data of binding of T14-Fc, T15-Fc and T16-Fc to immobilized Ang2 at maximal binding concentration of Wt-Fc at 5µg/ml are shown for a single experiment with duplicate determination. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism and shown as a mean and standard error of mean of duplicate measurements from a single experiment.

Table 3.3: Ang2 binding activity of T14-Fc, T15-Fc and T16-Fc

	T14-Fc	**T15-Fc**	**T16-Fc**	**Wt-Fc**
Bmax (%)	76 ∓8* (n=3)	54∓3** (n=3)	85 ns_1 (n=1)	100 (n=3)
EC_{50} (µg/ml): half maximum binding concentration	4.3 ∓1.7 ** (n= 3)	5.3 ∓1.9** (n= 3)	2.2∓0.6 ns_2 (n= 3)	0.15 ∓0.07 (n=9)

Table 3.3 Shows the EC_{50} for T14-Fc, T15-Fc and T16-Fc binding to Ang2, as well as maximum binding capacity for T14-Fc, T15-Fc compared to Wt-Fc. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed.*$p<0.05$, **$p<0.01$, ***$p<0.001$, ns_1: $p=0.326$, ns_2: $p=0.227$ (Tukey's post hoc test).

Additional experiments were performed to show the specificity of the T14, T15 and T16 proteins to Ang1 using Wt-Fc as a control. As shown in (Figure 3.10), the maximal binding activity and EC_{50} of the T14, T15 and T16 to Ang1 were not detected as the curve did not reach a plateau (Figure 3.10). The T14, T15 and T16 show less than 10% binding activity to Ang1 compared to the maximal binding activity of the Wt-Fc (Table 3.4). These results indicate the specificity of these receptors.

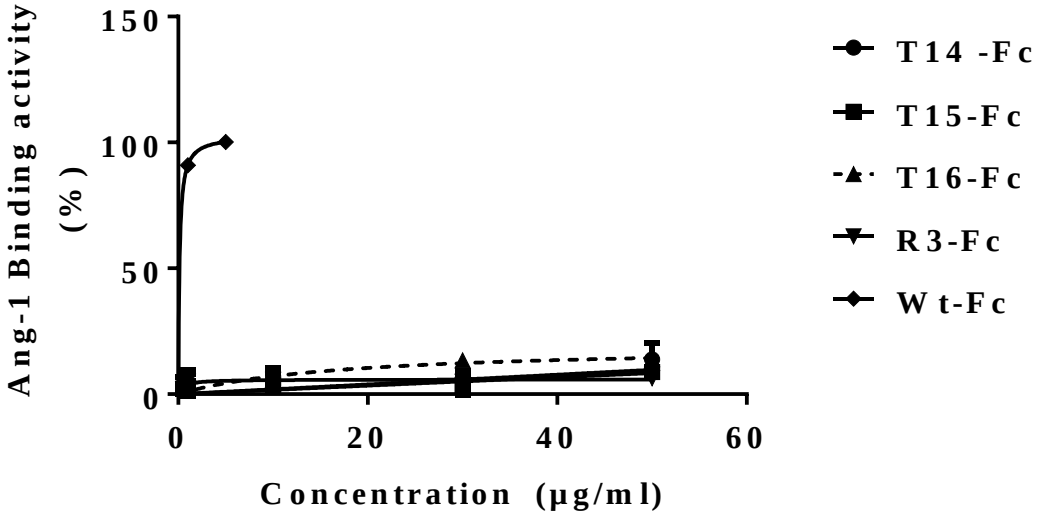


Figure 3.10: Binding activity curve of the T14, T15, and T16 to Ang1.

Data were shown as means and standard deviations from a single experiment with duplicate readings. Data shown from single representative experiment of two and fitted to non-linear concentration versus binding affinity using Graph Pad Prism for one site-specific binding model.

Table 3.4: Ang1 binding activity of T14-Fc, T15-Fc, T16-Fc, R3-Fc and Wt-Fc

	T14-Fc	**T15-Fc**	**T16-Fc**	**R3-Fc**	**Wt-Fc**
Bmax (%)	7 ∓4*** (n=3)	9∓3*** (n=3)	6 ∓4 (n=2)	8 ∓1.6 (n=2)	100 (n=3)
EC_{50} (µg/ml): half maximum binding	ND	ND	ND	ND	0.07 ∓0.04 (n=6)

Table 3.4 shows the EC_{50} for T14-Fc, T15-Fc, T16-Fc, R3-Fc and Wt-Fc binding to Ang1 as well as maximum binding capacity. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed, ND=not detected). ***p<0.001 (Tukey's post hoc test).

3.5 Protein oligomerization increases binding activity

It has already been established that protein oligomers bind with increased avidity compared to monomers or lower order oligomers (Köhling *et al*, 2019; Schirrmann *et al*, 2010). Several studies demonstrated the advantages of engineering pentameric protein to improve the avidity of the construct through making multivalent structure (Reddy *et al*, 2013; Terskikh *et al*, 1997). Increasing the binding sites of the R3 receptor by using the COMP (cartilage oligomeric matrix protein) to form the pentameric design can result in increasing frequency of binding to Ang2 and/or increase the binding strength between the R3-COMP receptor and Ang2. The multivalence provided by the COMP can positively affect the avidity of the receptor and result in full binding activity (Terskikh *et al*, 1997). The coiled–coiled domain of the COMP construct provides the structure with the stability required due to formation of a stable, strong disulphide ring at the carboxy-termini of the coil domains between two neighbouring cysteine chains (Issa *et al*, 2018).

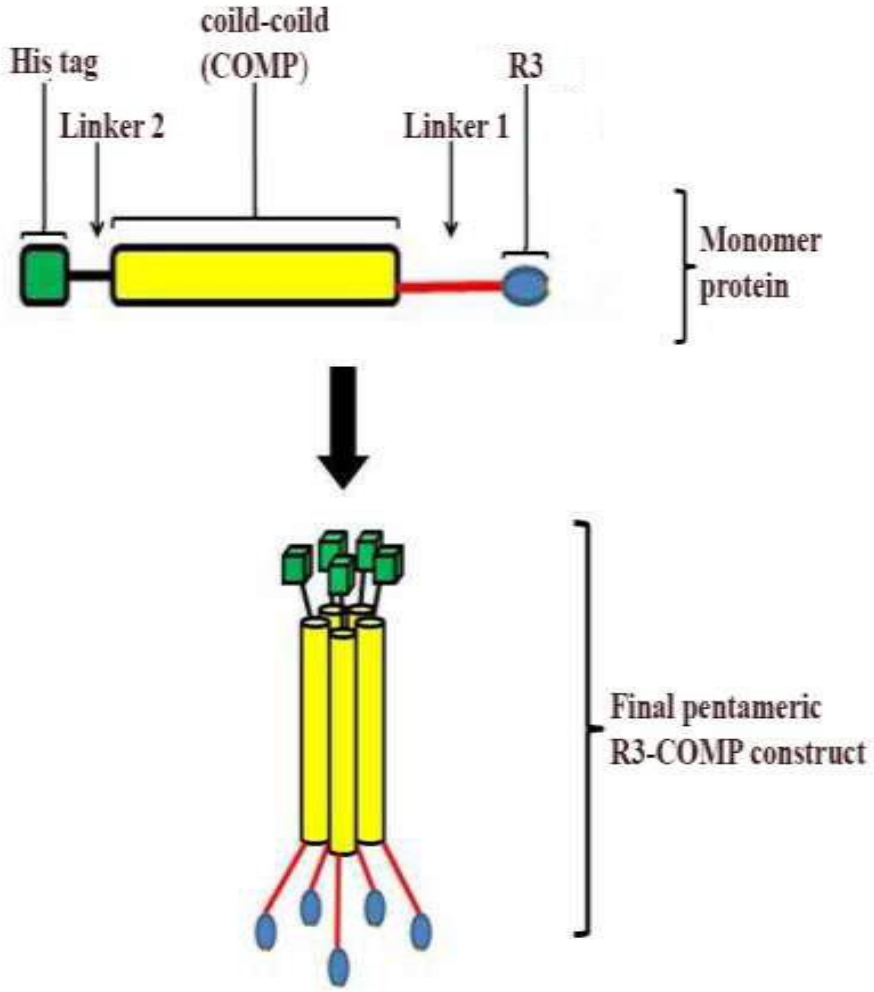
In addition, using short glycine linker residues to connect the R3 receptor with the coiled–coiled domain of the COMP construct can provide the final structure with flexibility and stability (Reddy *et al*, 2013; Trinh *et al*, 2004). These linkers can improve the characteristic of the fusion proteins through improving the biological activity, protein expression yield, and the pharmacokinetic property (X. Chen *et al*, 2013; Trinh *et al*, 2004). Glycine length of 11-19 residues is required for biological activity and to ensure that the protein will fold correctly (Robinson and Sauer, 1998; Argos, 1990).

Both the COMP and the glycine linker contribute to the formation of soluble and stable oligomeric proteins that have full binding activity (Terskikh *et al*, 1997a). This type of protein design could improve the binding activity of the receptor.

This study aims to increase the apparent affinity of the dimeric R3 mutant ectodomain by engineering an oligomeric protein construct. In order to perform that, a COMP construct was used to make the oligomeric R3.

3.6 Construction of the R3-COMP

The oligomeric construct was engineered by fusion of the C-terminal domain of the R3 ectodomain with the N-terminal coiled-coil domain of the COMP (DLAPQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMECDACG) using glycine linkers (Gly-Gly- Gly- Gly-Ser)$_3$. Then another short glycine linker (Gly-Gly-Gly-Gly-Ser) was used to fuse the C-terminal coiled-coil domain sequence of COMP with the N-terminal of the His-tag domain (Figure 3.11).



(A)

N.....Ig-I....C.....N(Gly-Gly-Gly-Ser)3.....

DLAPQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMECDACG....(Gly-Gly-Gly-Se)....HHHHHH

(B)

Figure 3.11: The R3-COMP design.

The figure represents a schematic diagram showing the approximate structure of R3-COMP in its pentameric form. A: represents a basic monomeric structure for the R3-COMP and the final pentameric protein construct, which composes of five binding sites. B: represents the initial design of the R3-COMP.

3.7 Engineering of the R3-COMP

After designing the R3-COMP amino acid sequence, codon optimization was performed to increase expression for HEK293 cells. The cDNA sequence was synthesised by Life Technologies and supplied in the cloning vector PEX-K4 (Appendix-8). Figure 3.12 below shows the steps taken after synthesis of the new construct to exchange the vector to the pcDNA3.1 to be used for the protein expression.

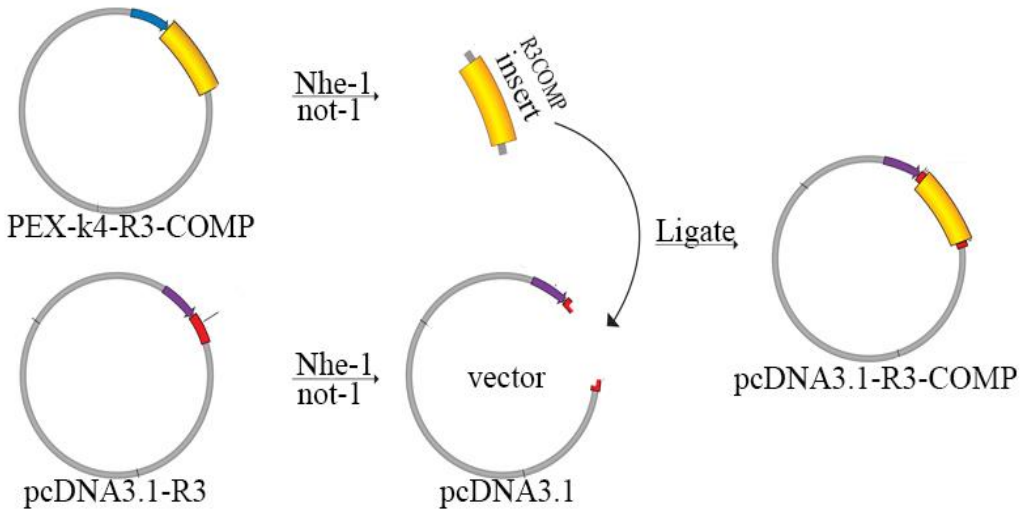


Figure 3.12: Preparation steps of the pcDNA3.1-R3-COMP constructs.

The scheme shows preparation steps of the pentameric pcDNA3.1-R3-COMP constructs: the pcDNA3.1-R3 was digested to release the vector (pcDNA3.1), then another digestion was performed for the PEX-k4-R3-COMP to release the insert (R3-COMP), then ligation was performed to obtain the pcDNA3.1-R3-COMP.

The R3-COMP plasmid design was transformed into *E.coli* (section 2.3.1). Restriction digests were performed to release the existing insert from the pcDNA3.1 by gel extraction technique. Then other digests were performed for the pEX-K4-R3-COMP to release the R3-COMP insert and for ligation the vector pcDNA3.1 to achieve the new pentameric design of the pcDNA3.1-R3-COMP. As shown in Figure 3.13, there is a band with size 5500bp and 1561bp, which represents the expected size of the vector (pcDNA3.1) and the insert (R3-COMP).

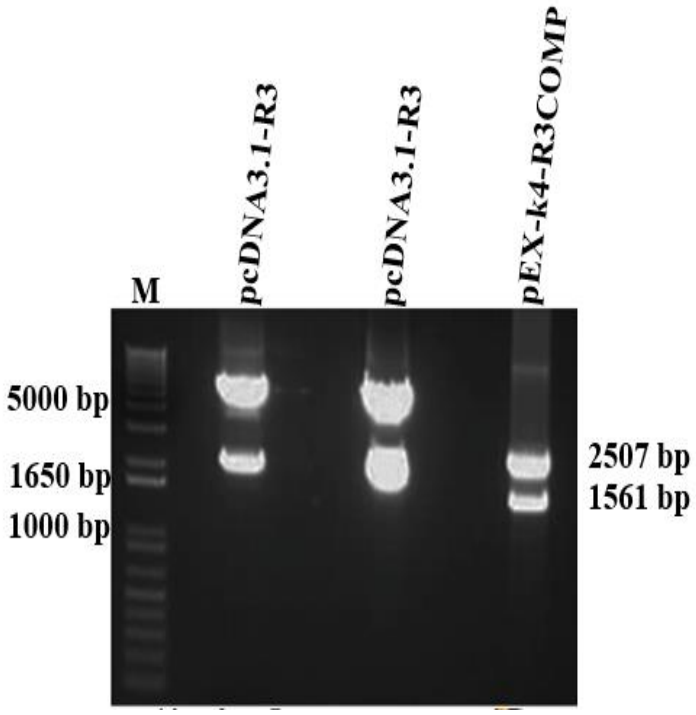


Figure 3.13: The restriction digestions of pEX-k4-R3-COMP and pcDNA3.1-R3 plasmids.

The figure shows the digestion of two plasmids using *Nhe* I and *Not* I enzyme to extract the R3-COMP insert and the pcDNA3.1 vector and to confirm the size of the expected bands at at 5500 bp and 1561 bp. M: size marker shown in bp. This experiment was performed once.

Then after the releasing of the insert and the vector, another gel was performed to confirm both identities. The correct bands for the insert and the vector were shown in Figure 3.14 as expected at 1561 bp for the insert and at 5500 bp for vector.

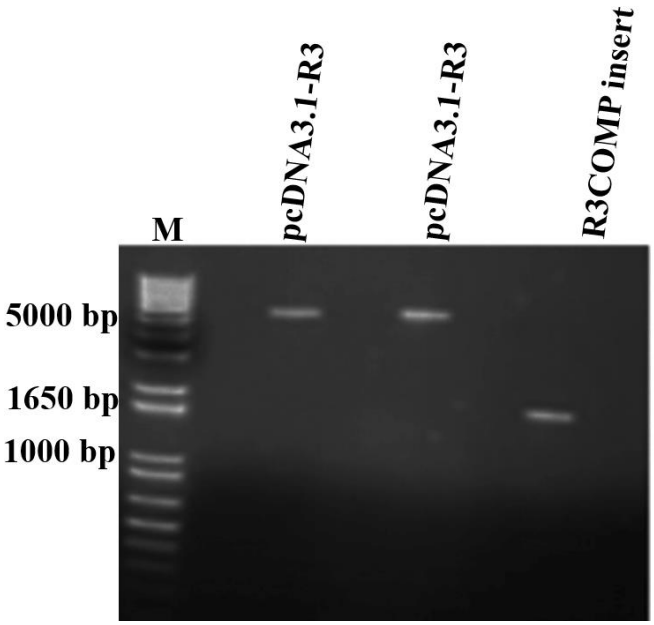


Figure 3.14: The R3-COMP insert and the pcDNA3.1 vector.

The figure shows the extracted R3-COMP insert and the pcDNA3.1 vector to confirm the size of both at 5500bp and 1561bp. M: size marker shown in bp. This experiment was performed once.

Ligation was performed for the R3-COMP insert and the pcDNA3.1 vector. The plasmid was transformed into *E.coli* and grown into broth culture. Then, the obtained plasmid was digested using *Nhe* I and *Not* I to confirm the size of the newly designed insert. As shown in Figure 3.15, a band at 1561 bp was detected which confirmed the correct size of the insert inside the correct vector (5500 bp). Sequencing was also performed for the ligated plasmid (pcDNA3.1-R3-COMP) using the T7 primer to compare the R3-COMP gene of the ligated pcDNA3.1-R3-COMP with the R3-COMP of the synthesized PEX-K4-R3-COMP. The result of the sequencing showed matching between the sequence of the engineered R3-COMP gene after optimization and the sequence of the ligated R3-COMP (Appendix-9).

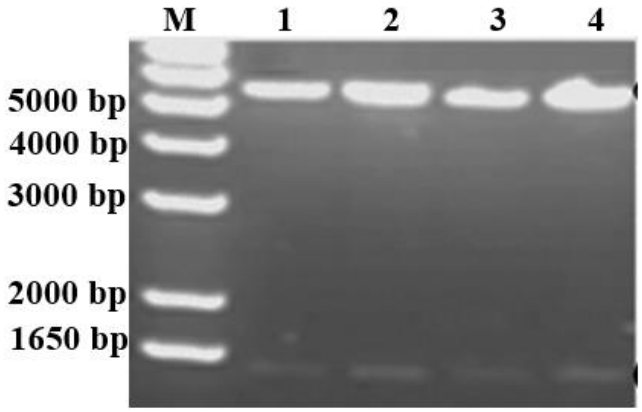


Figure 3.15: the pcDNA3.1-R3-COMP ligation.

The figure shows 1, 2, 3 and 4 which represent the restriction digestion of the ligated pcDNA3.1-R3-COMP. *Nhe* I and *Not* I were used to confirm the size of the R3-COMP insert at 1561bp and the vector at 5500bp. M: size marker shown in bp. This experiment was performed once.

3.8 Expression of R3-COMP

The oligomeric construct was transfected into HEK293 cells. Protein expression was confirmed by western blotting using the Tie2 ectodomain antibody to check the presence of the protein in the cell media. A time course of expression was performed (Figure 3.16) and peak secreted R3-COMP was detected three-four days post-transfection. The cells were also counted to determine the reason behind low expression post day four. The result of cell counting showed that post day four the increased percentage of the dead cells were associated with a decreased level of the secreted protein. One possible reason for that decrease is that the cell lysis resulted in protease secretion and this caused protein degradation.

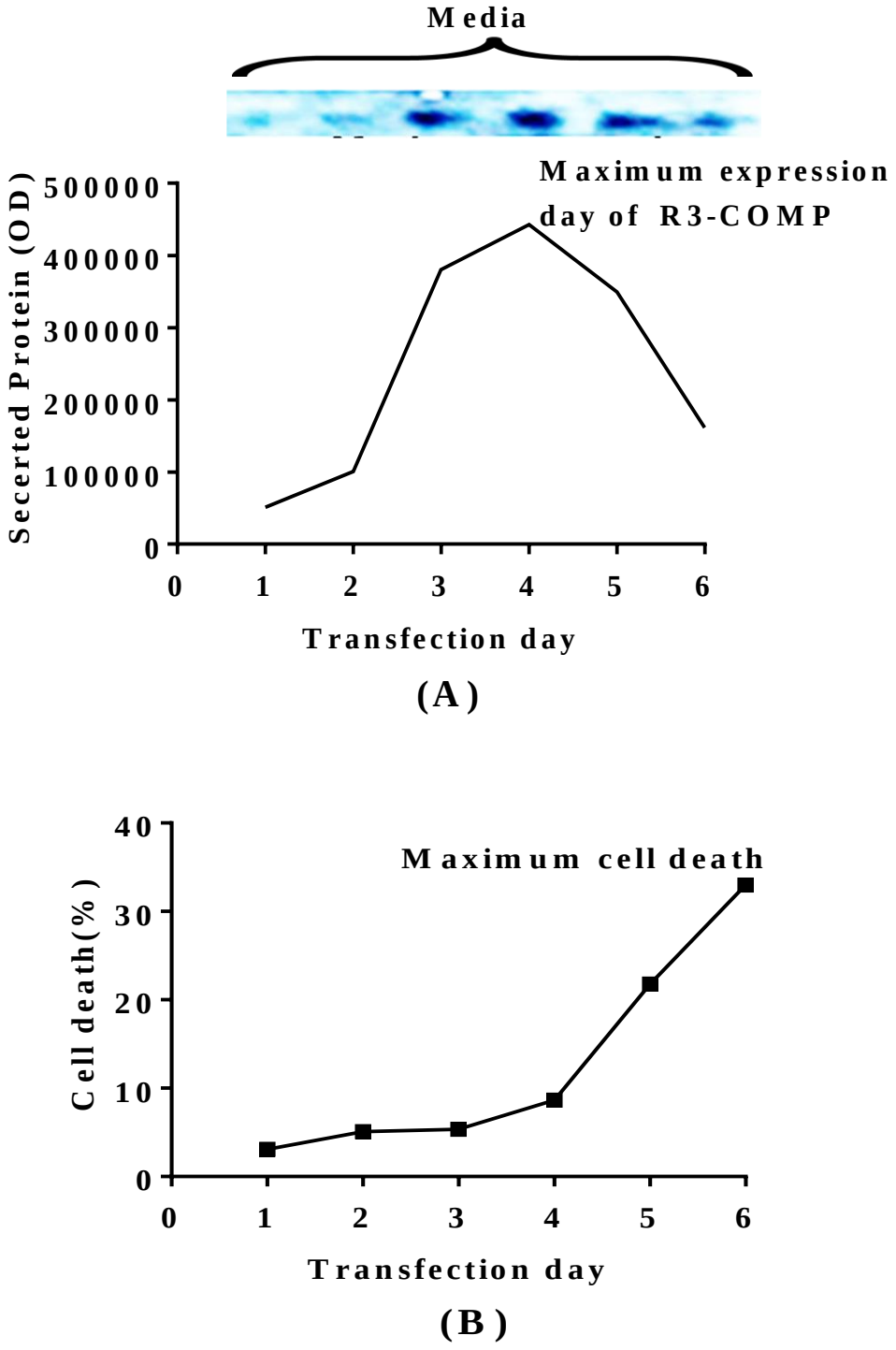


Figure 3.16: Time course of Accumulation secreted protein of R3-COMP.

Accumulation secreted proteins of the R3-COMP using HEK 293 cell free medium following transient transfection. A: Western blot probed with anti Tie2 ectodomain shown time course for expression of R3-COMP after collecting media sample at days 1, 2, 3, 4, 5,6. ImageJ was used to quantitate the data. B: Media samples taken between days 1-6 to show the percentage of cell death. This experiment was performed once.

The R3-COMP protein was purified using TalonR nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices via the His-tag and by using elution buffer containing different concentrations of imidazole (250 mM , 500 mM) to elute protein (Figure 3.17). As shown in Figure 3.18, there is only one major visible protein (monomeric R3-COMP) of approximately 65kDa as expected on the gel, which indicates purity of the protein. However, the total yield of oligomeric R3 protein was significantly lower than the yield of R3-Fc and Wt-Fc protein (Figure 3.19, Figure 3.20).

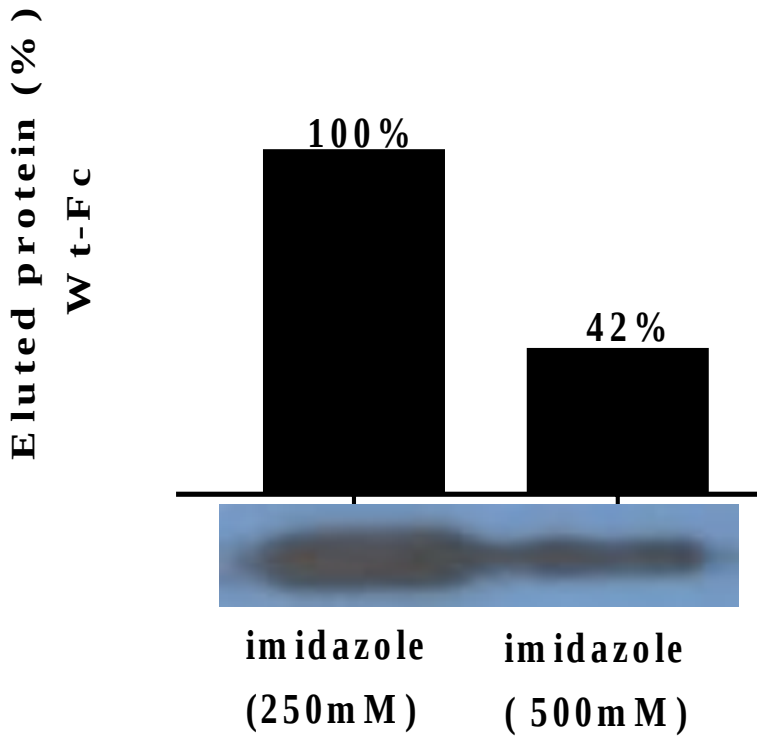


Figure 3.17: Western blot of HEK293 cells using anti-Tie2 and anti-goat-HRP.

The figure represents the protein purification of R3-COMP using elution buffer with different concentration of imidazole to elute protein. Fr1: fraction eluted by using 250mM imidazole. Fr2: fraction eluted by using 500 mM imidazole. Data shows the percentage of eluted protein of a single purification experiment. This experiment was performed once.

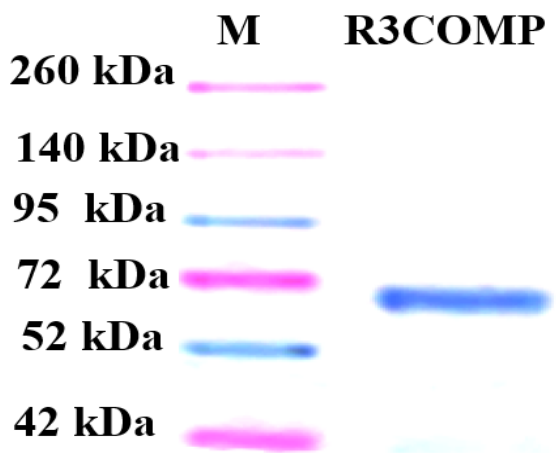


Figure 3.18: Coomassie Blue of R3-COMP protein.

Coomassie Blue stained gel of R3-COMP protein expressed in HEK293 cells and purified from medium by Ni-NTA affinity purification. Proteins were resolved in the presence of DTT. The R3-COMP band was detected at approximately 65kDa. The position of molecular mass marker was indicated. This experiment was performed once.

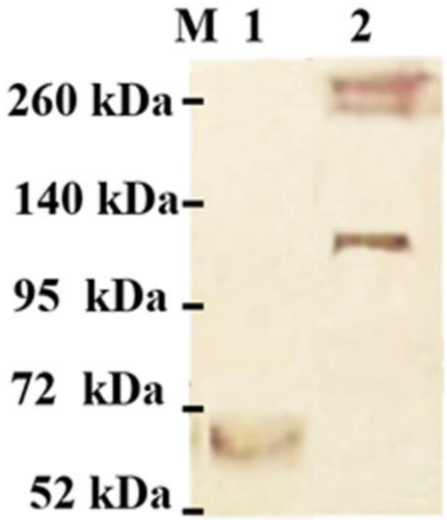


Figure 3.19: Western blot showing oligomerization, dimerization, and monomerization of R3-COMP.

Western blot probed with anti Tie2 ectodomain shown different forms of R3COMP after collecting media sample with and without DTT. ImageJ was used to quantitate the data. Monomeric: 65 kDa, dimeric: 130 kDa, tetrameric: 260 kDa, pentameric form: 325 kDa. 1: R3-COMP with DTT, 2: R3-COMP with no DTT. M: marker. This experiment was performed once.

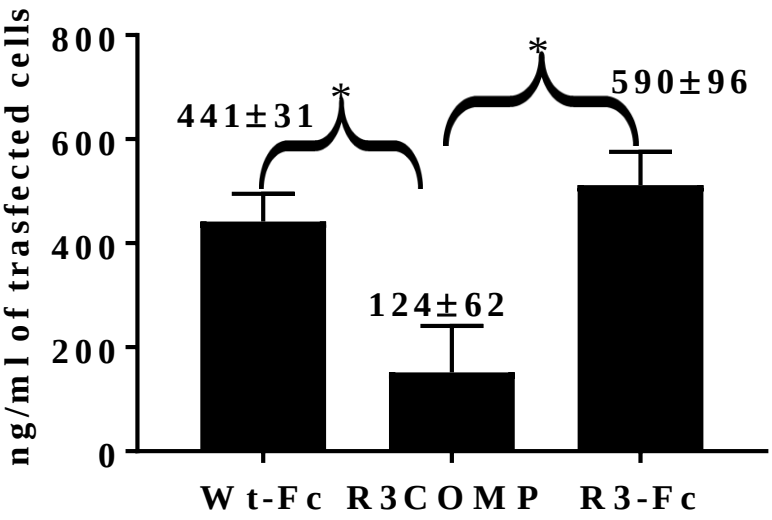


Figure 3.20: Protein yield of R3-COMP protein (ng/ml).

Protein yield following transfection and protein purification of Wt-Fc and R3-Fc. Data were shown as means and standard error for three independent transfection.*p<0.05 for R3-COMP compared with R3-Fc, .*p<0.05 for R3-COMP compared with Wt-Fc (Tukey's post hoc test).

3.9 Protein yields of R3-COMP

In order to determine whether the low yield of R3-COMP reflected poor secretion of the oligomeric protein, western blotting was performed on sample of media and cell following transfection with an equal amount of DNA including R3-Fc and R3-COMP. As shown in Figure 3.21 R3-Fc is seen in the media and the cells as a band of approximately 80 kDa and 75 kDa respectively. R3COMP is present as a band of approximately 65 kDa in the media and 55 kDa in the cells. Tie2 ectodomain is known to be glycosylated (Barton *et al*, 2006d) and the lower molecular mass form of ectodomain found in the cells most likely to represent the non-glycosylated ectodomains prior to secretion. Cellular R3-COMP was present at higher levels than cellular R3-Fc. This suggests the lower level of R3-COMP ectodomain found in media may reflect decreased ability of R3-COMP to be secreted.

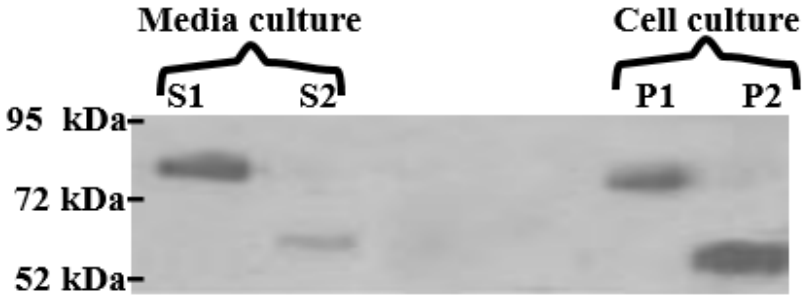


Figure 3.21: Western blot shown transient transfection of R3-COMP and R3-Fc.

Western blot probed with anti Tie2 ectodomain shows expression of R3-COMP and R3-Fc mutant in the cell media and cell lysate. S1 and P1 represent: media (S1) and lysate (P1) of R3-Fc. S2 and P2 represent: media (S2) and lysate (P2) of R3-Fc R3-COMP. This experiment was performed twice.

3.10 Attempts to increase soluble R3-COMP

An attempt was made to rupture the cells and to purify the cellular R3-COMP using lysis buffer and sonication. However none of these methods resulted in increased R3COMP in the soluble fraction.

3.10.1 Increasing R3-COMP secretion with CD5 secretory leader sequence

Further methods were used to increase the total yield of the R3-COMP protein. One possible solution was by replacement of endogenous native signal peptide of the oligomeric construct (R3-COMP) with a CD5 leader. Previous data has reported that the CD5 is considered as a strong secretory leader and can increase secretion of the protein into the cell media (Harmsen *et al*, 2013).

3.10.2 PCR plan to replace the native secretory leader with CD5

PCR amplification reaction was conducted to amplify the wanted nucleotide involving the R3-COMP and part of the new CD5 secretory leader sequence (Figure 3.22). Two oligonucleotide primers designed then ordered from (Eurofins/Genomics) were used in the PCR reaction (Table 2.3). The amplified product run on agarose gel to confirm the expected size at 1563bp (Figure 3.23).

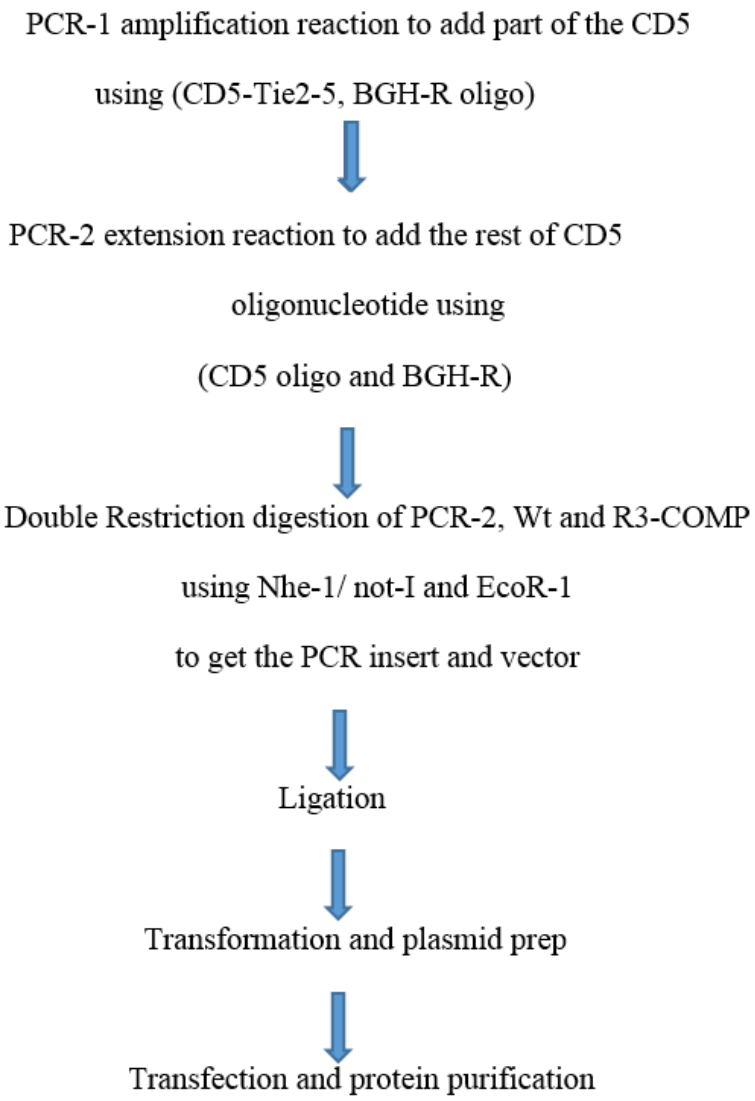


Figure 3.22: A scheme showing steps performed to replace native the secretory leader with the CD5 sequence

The steps performed to replace native the secretory leader with the CD5 sequence includes PCR amplification, PCR extension, digestion, ligation, transformation and transfection.

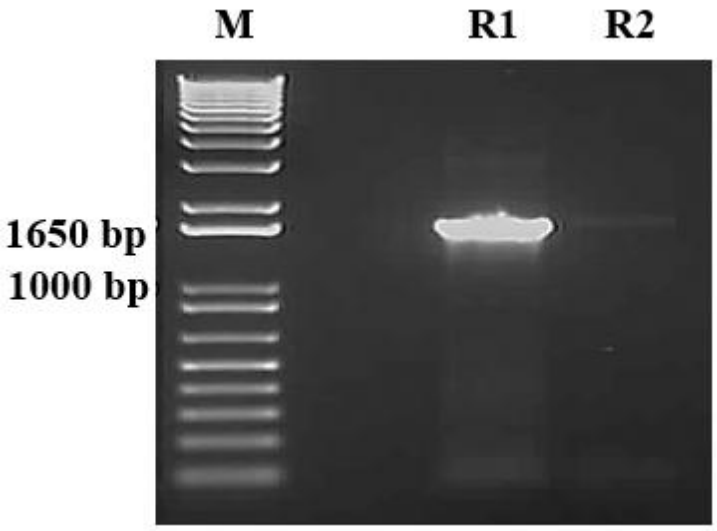


Figure 3.23: The PCR-1 amplification to amplify CD5 with R3-COMP.

The PCR-1 amplification to amplify part of the CD5 secretory leader with R3-COMP using CD5-Tie2-5 and BGH-R primers. R1: represents the PCR-1 reaction with DNA template. R2: PCR-1 reaction without DNA template. M: size marker shown in bp. This experiment was performed once.

Additional steps were performed by PCR extension reaction to add the rest of the CD5 secretory leader (Table 2.10). An agarose gel was performed to confirm the expected size of the extended oligonucleotide at 1617 bp. Furthermore, the differences between the amplified oligonucleotide of PCR-1 reaction (amplification) and PCR-2 reaction (extension) was confirmed by the differences between P1 and P2 as shown in Figure 3.24, which indicates successful addition for the rest of CD5. This was digested with *Nhe* I/*Not* I and ligated into pcDNA3.1. The plasmid was transformed into *E.coli*, amplified and sequence confirmed.

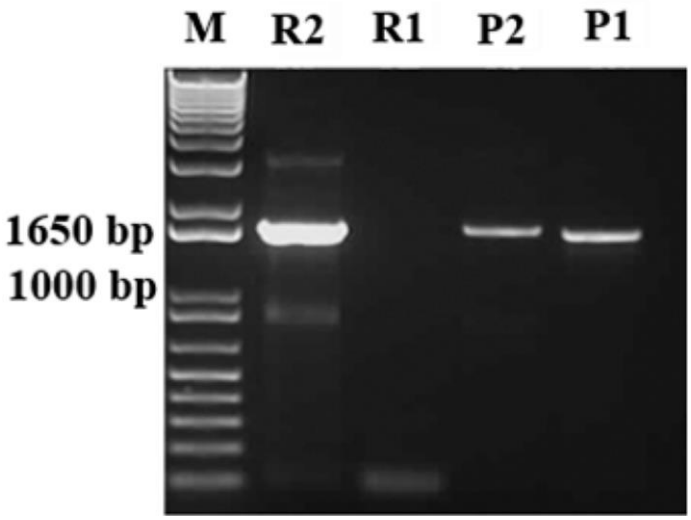


Figure 3.24: The PCR-2 extension of CD5 with R3-COMP.

The PCR-2 extension to add the rest of the CD5 leader to R3-COMP using CD5-Tie2-5 and BGH-R primers. R1: PCR-2 reaction without DNA template. R2: represents the PCR-2 reaction with DNA template. P1: amplified part resulted from PCR-1 reaction using DNA template. P2: extended part resulted from PCR-2 reaction using DNA template. M: marker. This experiment was performed once.

3.11 Protein expression of CD5-R3-COMP

The CD5-R3-COMP was transfected into the HEK293 cells and the expressed protein was harvested after 4 days (Section 2.12.1). The protein was purified using Ni-NTA via the His-tag and eluted by imidazole. Successful protein expression and purification were confirmed by SDS-GEL (Figure 3.25). Bradford assay was used to determine the concentration of the protein. The yield of the R3-COMP protein with CD5 leader increased 2-3 fold compared to the yield of R3-COMP protein with a native leader (Figure 3.26).

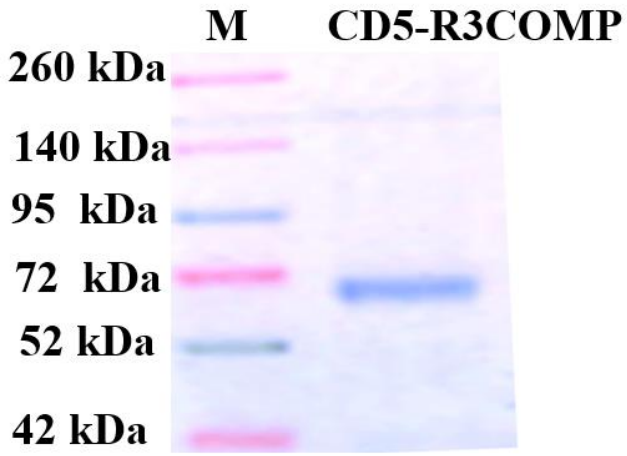


Figure 3.25:A – Coomassie Blue stain of purified R3-COMP proteins following SDS/PAGE.

Coomassie Blue stained gel of R3-COMP proteins expressed in HEK293 cells and purified from medium by Ni-NTA affinity purification. Proteins were resolved in the presence of DTT. The position of molecular mass marker was indicated. This experiment was performed once.

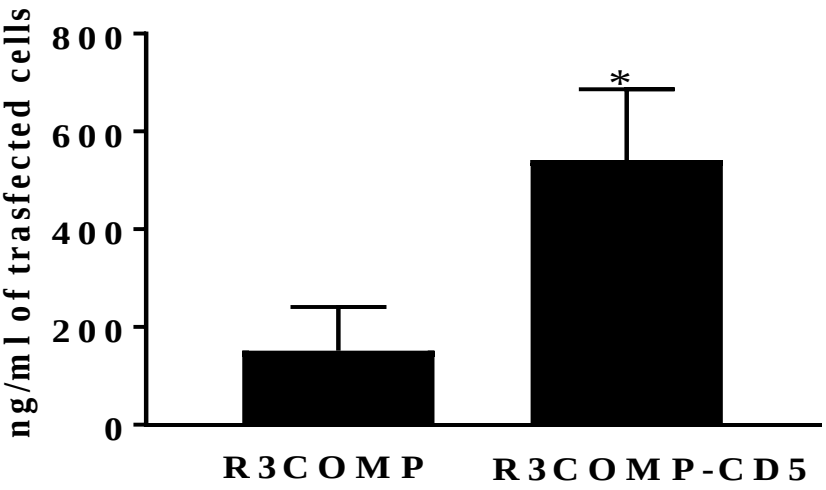


Figure 3.26 : Proteins yield of CD5-R3-COMP.

Protein yield following transfection and protein purification of R3-COMP with native leader and R3-COMP with CD5 leader. Data shown as means and standard error of the mean for three independent experiments.* indicates p= 0.0167 for CD5-R3COMP compared with native-R3-COMP.The CD5-R3-COMP protein shows significantly 3-fold higher yield compared to the R3-COMP with native leader (t test).

3.12 Binding of oligomeric R3 to Angiopoietins

ELISAs were performed to test the relative binding affinity of the oligomeric R3 ligand-trap to Ang2. As shown in Figure 3.27 below, there is an improvement in the binding affinity of the pentameric R3 to Ang2 ligand compared to the dimeric R3-Fc ectodomain. This was calculated from the average EC_{50} of the oligomeric R3 (0.5 ∓0.16μg/ml) compared to (9.4∓ 1.7μg/ml) of the R3-Fc ectodomain (Table 3.5).

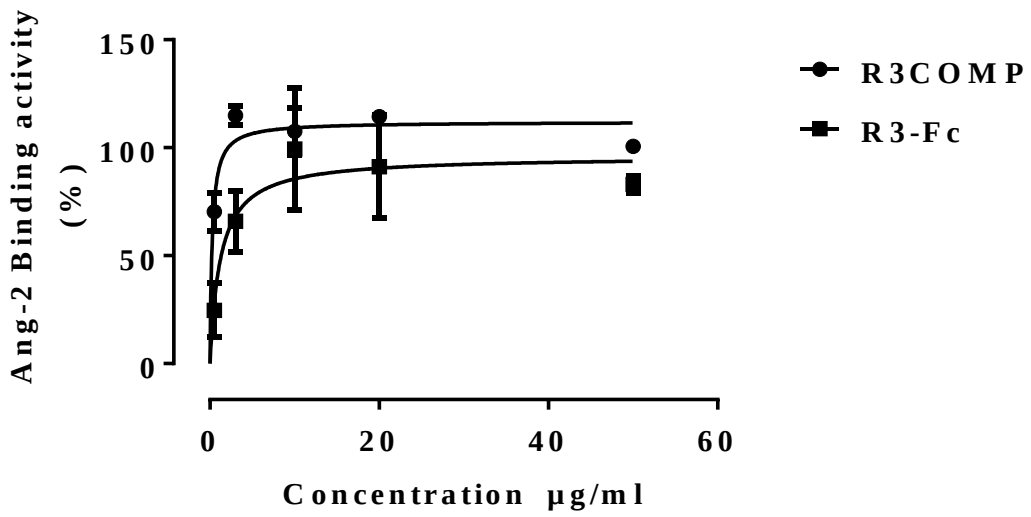


Figure 3.27: Binding activity curve of the R3COMP and R3-Fc to Ang2

ELISA data of binding of R3-COMP to immobilized Ang2 at maximal binding concentration of Wt-Fc at 50 µg/ml are shown for a single experiment with duplicate determination. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism and shown as a mean and standard error of mean of duplicate measurements from a single representative experiment of three.

Table 3.5: Ang2 binding activity of R3-COMP and R3-Fc

	R3-Fc	**R3COMP**
Bmax (%)	83. ∓5 n=(3) ns	100 ∓0.4 n=(3)
EC_{50} (µg/ml): half maximum binding concentration	9.4∓1.7 n=(9)***	0.5∓0.16 n=(6)

Maximum binding capacity of EC_{50} were calculated from ELISA assays by graph pad prism for at least 3 experiment as indicated. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed). *** indicates p= < 0.001, ns: p=0.093 (t test).

Further ELISA was performed to confirm the improvement in the binding activity of the R3-COPM to Ang2 by comparing its activity to the Wt-Fc (Figure 3.28). This was calculated from the EC_{50} of the oligomeric R3 (0.5 ∓0.16 µg/ml) compared to (0.15∓ 0.08 µg/ml) of the Wt-Fc ectodomain (Table 3.6).

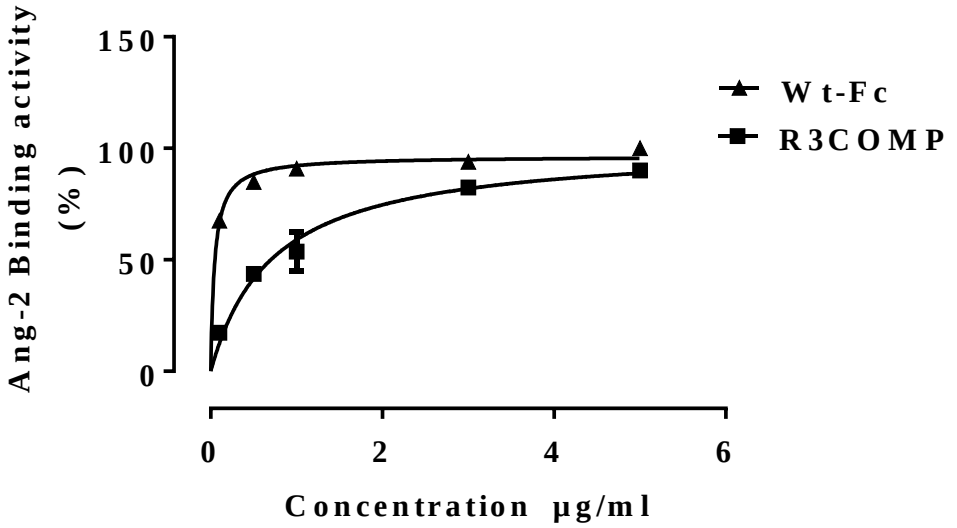


Figure 3.28: Binding activity curve of the R3COMP and Wt-Fc to Ang2

ELISA data of binding of R3-COMP to immobilized Ang2 at maximal binding concentration of Wt-Fc at 5µg/ml are shown for a single experiment representative of two with duplicate determinations. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism.

Table 3.6: Ang2 binding activity of R3-COMP and Wt-Fc

	Wt-Fc	**R3COMP**
Bmax (%)	100∓0 n=(2)	90∓0.4 ns n=(3)
EC_{50} (µg/ml): half maximum binding concentration	0.15 ∓0.077 n=(9)	0.5∓0.16 ** n=(6)

Maximum binding capacity of EC_{50} were calculated from ELISA by graph pad prism for at least 2 experiment as indicated. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed). **p<0.01, ns: p=0.143 (t test).

Further ELISA were performed to test the specificity of R3-COMP to Ang1. As shown in Figure 3.29 below, detection of R3COMP-Ang1 binding activity indicates an increases in Ang1 binding activity in comparison to the non-detected binding of the R3-Fc. This was calculated from the average EC_{50} of the oligomeric R3 (1∓0.2µg/ml) compared to non-detected binding of the R3-Fc (Table 3.7).

In conclusion, the pentameric design results in significant increases in the binding activity of the oligomeric R3 ligand-trap to Ang2 in comparison to R3-Fc. In addition, it increases in the binding activity of the oligomeric R3 ligand-trap to Ang1 compared to R3-Fc.

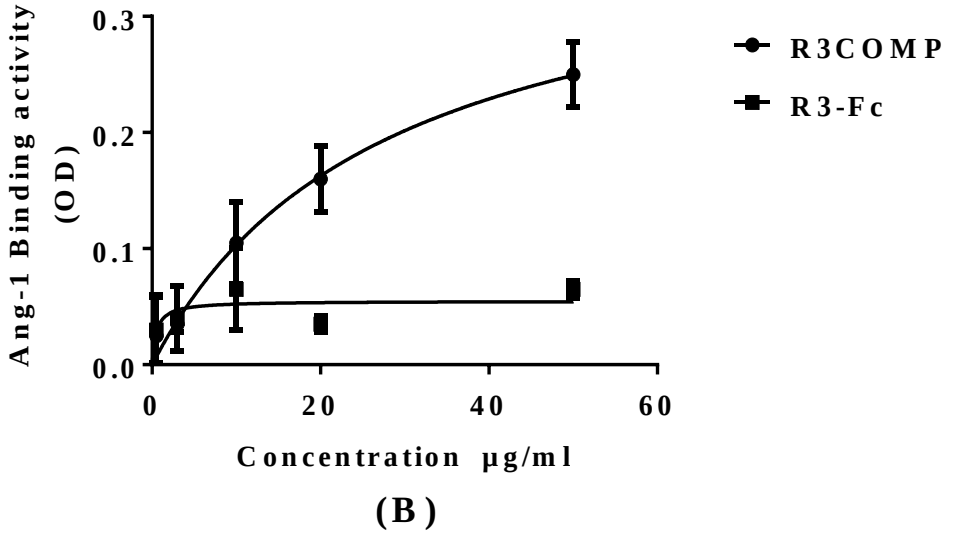


Figure 3.29: Binding activity curve of the wild-Fc, R3-Fc and R3-COMP to Ang1.

ELISA data of binding of R3-COMP to immobilized Ang1 at maximal binding concentration of R3-Fc at 50 µg/ml are shown for a single experiment representative of three with duplicate determinations. Data are shown following subtraction of binding to BSA and curve fitted using Graphpad Prism. Data are shown as a mean and standard error.

Table 3.7: Ang1 binding activity of R3-COMP

	R3-Fc	**R3COMP**
Bmax (%)	36 ∓19 *** n=(3)	100 n=(3)
EC_{50} (µg/ml): half maximum binding concentration	ND n=(3)	1 ∓0.2 n=(4)

Maximum binding capacity of EC_{50} were calculated from ELISA assays by Graph pad Prism. The Bmax and the EC_{50} was calculated as mean∓st.error as indicated (n= number of experiments performed). ***p<0.001(t test).

3.13 Discussion

The study of Brindle *et al*. 2013 has developed the extracellular domain of the Tie2 receptor by a directed evolution, which results in a new ligand-trap receptor called the R3 protein that preferentially binds to Ang2 but not Ang1. However, it has a lower binding affinity to Ang2 compared to the affinity of the wild-type ectodomain. The R3-Fc and the Wt-Fc ectodomains were expressed using mammalian HEK 293 cells and harvested after 96 hours. The yield obtained for the R3-Fc and the Wt-Fc ectodomains were sufficient to proceed with the experiments.

The aim of this study is to increase the binding affinity of a genetically engineered Ang2 ligand-trap (R3-Fc ectodomain) to block the adverse effects associated with high levels of Ang2 in CVD. This was approached by performing a series of steps. Firstly, by mutagenesis of the R3-Fc ectodomain to improve the affinity and obtain a ligand-trap with a higher affinity and specificity to Ang2. The proteins were expressed using mammalian HEK293 cells and harvested after 96 hours. The yield obtained for the T14-Fc, T15-Fc and T16-Fc ectodomains were sufficient to proceed with the experiments. The DT40-Tie2-R3 system was used by my colleague Dr. N. Bate to increase the affinity and specificity of the R3-Fc ectodomain. Using this system resulted in more mutants, designated T14-Fc and T15-Fc, as well as T16-Fc (which was made by incorporating the mutants of both T14-Fc and T15-Fc). This study found that these new mutant proteins have a higher affinity to Ang2 than the R3-Fc ectodomain by 2-4 fold, and show no detectable binding affinity to Ang1 compared to the Wt-Fc, indicating specificity of these proteins.

An alternative approach used, by engineering a new pentameric design to increase the affinity of the R3-Fc to the Ang2. Steps towards engineering of the new design have been taken by expressing the R3-COMP using mammalian HEK293 cells. The yield of the R3-COMP was very low but it was sufficient to proceed the tests. Previous laboratory work used the COMP and the efficient liposomal transfection to express a COMP-Ang2 protein and they obtained 400–500μg for every litre of the media (Kim *et al*, 2009). One of the possible reasons for the low R3-COMP yield is the inability of the protein his-tag to bind properly to the beads, as the his-tag may be imbedded into the structure of the protein. Another possible reason is the protein remaining in the cell lysate due to the large size of the pentameric protein (325kDa) and inability of the native signal peptide to secrete the protein into the culture media. The sonication technique was performed to release the protein from the cell lysate and resulted in very low yield of the protein. Another trial was used to increase secretion of the R3-COMP protein into the media, exchanging the Tie2 native leader with the CD5 leader, this resulted in better yield of the protein (Harmsen *et al*, 2013).

This study found that the binding activity of the oligomeric R3 to Ang2 was concentration dependent, therefore as the concentration increased the binding activity increased. The pentameric design resulted in 18-fold improvement in the binding activity of the R3-COMP to Ang2 (Reddy *et al*, 2013). This result suggested that the COMP design successfully increased the apparent affinity of the R3 protein to Ang2. The improvement in the R3COMP-Ang2 activity was associated with an improvement in R3COMP-Ang1 binding activity, and this means that the R3-COMP construct is not suitable for therapeutic uses as it will attenuate the protection effect of Ang1. Although, the mutant R3-Fc ectodomain preferentially binds to Ang2, it still retains some binding activity towards Ang1 which was not detected by the ELISA. Incorporating the R3 ectodomain into a COMP design resulted in an increase in the Ang1 binding activity, determined by the ELISA. One of the possible reasons for this is that the DT40 system developed the receptor to be more specific to Ang2 by compromising the binding interface residues i.e. by decreasing the number of binding residues between Tie2 and Ang1

and at the same time maintaining binding to Ang2 (DT40 system did not remove all the residues that allow binding between the Tie2 ectodomain and Ang1).

In conclusion, the identities of the plasmids were verified, The R3-Fc variant ectodomains show better binding activity to Ang2 than the R3-Fc ectodomain as well as show Ang1 binding activity which could limit their therapeutic use. The oligomeric R3 was prepared and expressed. However, lower protein yield of R3-COMP protein was obtained compared to previous analysis (Kim *et al*, 2009), and the problem was solved by changing the native leader with the CD5 leader.

This study found that the pentameric design successfully increased the binding activity of the R3 ectodomain to Ang2 by 18 fold compared to the R3-Fc ectodomain (Figure 3.30). However, the binding activity towards Ang1 was also increased which makes the construct impractical for therapeutic purposes as it leads to decreases in the Ang1 protective effect.

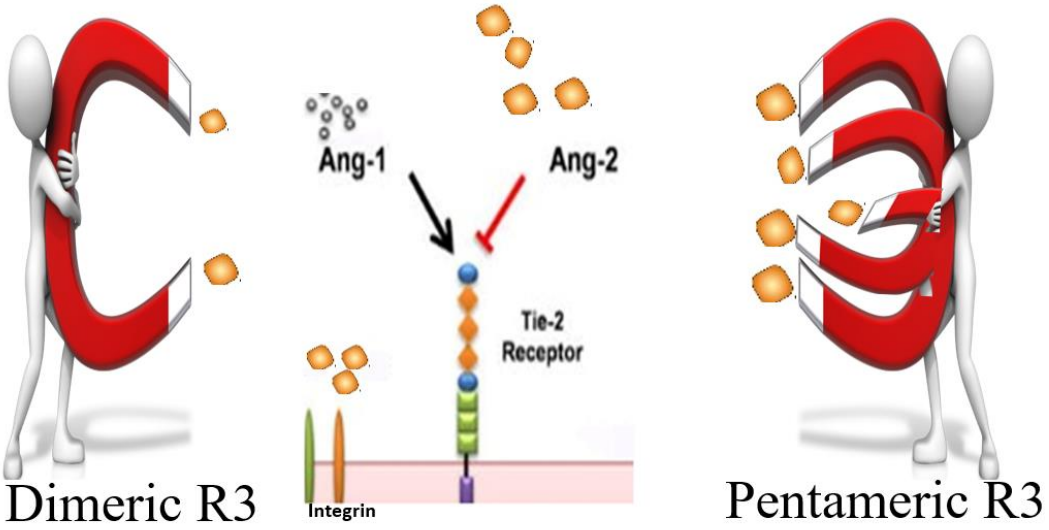


Figure 3.30: The R3-COMP increases affinity.

A schematic diagram depicting the proposed model to show the differences between the apparent affinity of the dimeric and pentameric R3 towards Ang2. The multivalent structure provided by the COMP construct will increase the apparent affinity of the ligand trap towards Ang2 by increasing frequency of binding or strength of binding between pentameric R3 and Ang2.

Chapter 4: Angiopoietin-2 induces VE-cadherin cleavage through a Tie2 independent pathway

The angiopoietins (Ang1 and Ang2) are vascular growth factors that bind to the Tie2 receptor and can promotes protection or regression effects of the vascular endothelial cells. Ang1 mediates the protective effects necessary for maintaining integrity of the vascular system (Fiedler and Augustin, 2006). On the other hand, several studies demonstrated the vascular destabilizing effect of Ang2 at high levels, which was associated with cardiovascular and cancer related diseases (Szarvas *et al*, 2008). Ang1 maintains monolayer integrity of the endothelial cell and prevent vascular permeability (Pizurki *et al*, 2003). The permeability effect induced by Ang2 in mouse was reduced by co-administration of Ang1 or soluble Tie2 (Roviezzo *et al*, 2005).

In addition to signalling through Tie2, it has been reported that Ang2 can mediate signalling through an integrin pathway. However, currently the pathogenic effects resulting from this pathway are unknown.

An important determinant of vascular permeability is VE-cadherin (Flemming *et al*, 2015). Previous studies reported that endothelial cell stimulation with thrombin and TNF-α resulted in VE-cadherin cleavage and increased permiability (Schulz *et al*, 2008; Menon *et al*, 2006). As TNF-α is known to induce Ang2 and Ang2 is known to be required to promote effects of TNF-α specifically on inflammation (Fiedler and Augustin, 2006; Wang *et al*, 2012), it is possible that Ang2 could have direct or indirect effects on VE-cadherin cleavage. ADAM10 has been implicated in cleavage of VE-cadherin *(*Schulz *et al*, 2008).

In this chapter therefore, HCMECs and/or HUVECs will be used in order to investigate the effects of Ang2 on cleavage of VE-cadherin.

4.1 Ang2 induces VE-cadherin cleavage

In order to examine the effects of Ang2 and TNF-α on the release of the extracellular domain of VE-cadherin, HUVECs were incubated with Ang2 and/or TNF-α for a period of one hour. The media was recovered and concentrated, run on SDS gel then transferred by nitrocellulose membrane and probed with antibodies that recognise the extracellular domain of VE-cadherin (Section: 2.11.1).

As shown in the Figure 4.1, in the presence of Ang2 a band of approximately 100 kDa was seen in the media. This corresponds to the reported size of the released extracellular domain of VE-cadherin from previous studies (Flemming *et al*, 2015). This indicates that Ang2 significantly increases releases of VE-cadherin from the surface, most likely by proteolysis as in comparison to control cells there is 6-fold increase in the activation. Vinculin probing of the cell lysate confirms that the there is no significant differences between cells number and this reflect that VE-cadherin releases from the same number of cells.

In the presence of TNF-α there was non-significant increase in the release of VE-cadherin. Nevertheless, when both Ang2 and TNF-α were incubated together they produced a significant synergistic effect, producing 2-fold stimulation in the release of extracellular domain of VE-cadherin in comparison to the Ang2, and 10-fold in comparison to control.

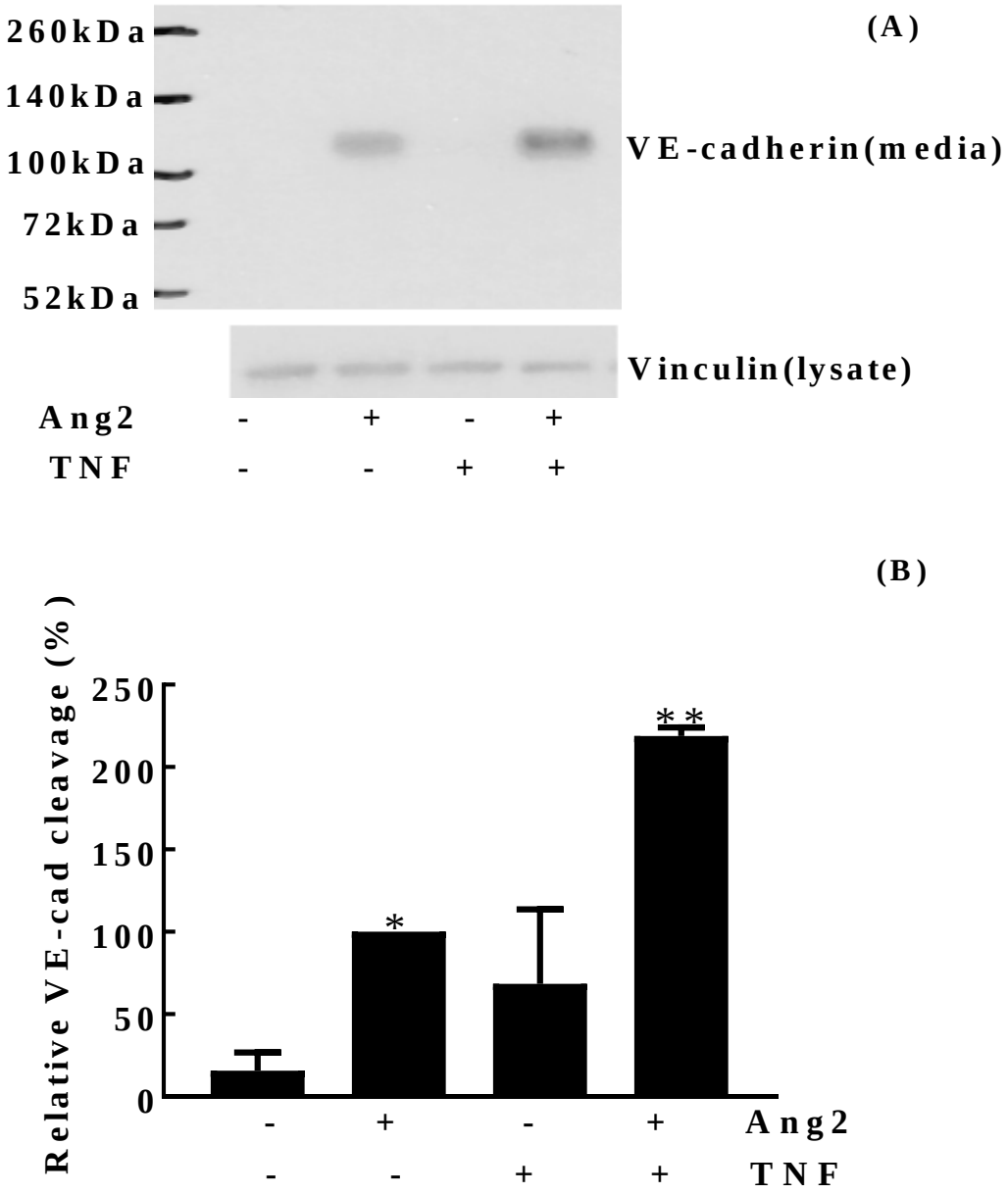


Figure 4.1: **Western blot showing Ang2 induce VE-cadherin cleavage in HUVECs.**

Cells were stimulated as follows: C: control. A2: 0.5µg/ml Ang2. TNF-α: 20ng/ml TNF-α. TA: 20 ng/ml TNF-α plus 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. *$p<0.05$ for Ang2 compared with control and **$p<0.01$ for Ang2 compared with TNF-α +Ang2, Tukey's post-hoc test.

In order to test whether or not the Ang2 effect was specific for HUVECs, the experiment was also performed in HCMECs. As showed in the Figure 4.2, when the cells were incubated with Ang2, one band of a proximately 100 kDa was identified in the media. Indicating that Ang2 significantly increased releases of VE-cadherin from the surface most likely by proteolysis as in comparison to control cells there is 6-fold increasing in the activation.

In order to test the pro-cleavage effect of TNF-α in HCMECs, a similar experiment performed in the presence of TNF-α and showed non-significant increase in the release of VE-cadherin in comparison to control (Figure 4.2). Ang2-induced significant increases in VE-cadherin cleavage in comparison to TNF-α cleavage effect. However TNF-α did not enhance the effect of Ang2 on VE-cadherin cleavage. These data are different from those found in HUVECs, when TNF-α enhanced Ang2 effects.

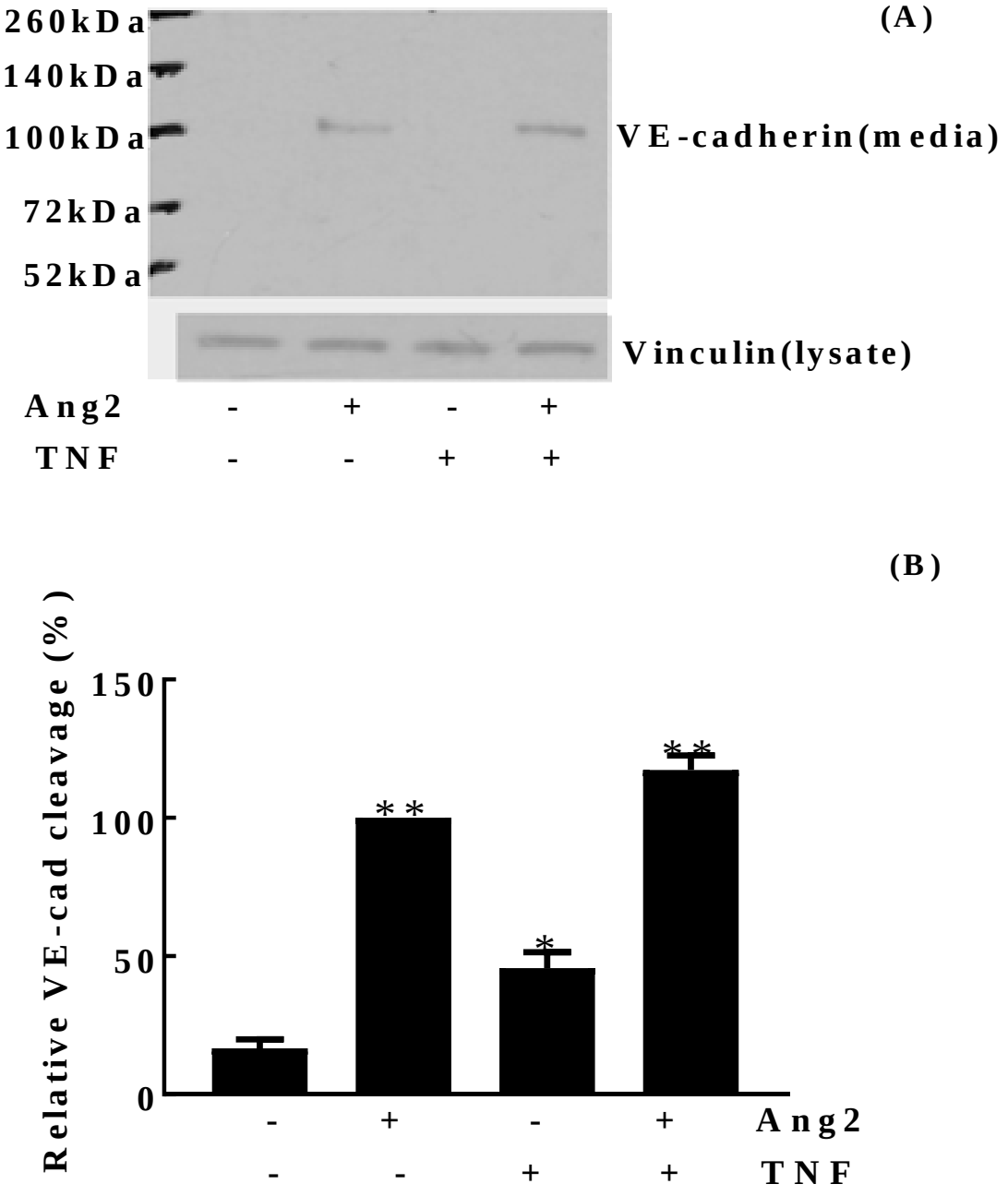


Figure 4.2: **Western blot showing Ang2 induce VE-cadherin cleavage in HCMECs.**

Cells were stimulated as follows: C: control. A2: 0.5µg/ml Ang2. TNF-α: 20ng/ml TNF-α. TA: 20 ng/ml TNF-α plus 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. **p<0.01 for Ang2 in comparison to control and *p<0.05 for TNF-α in comparison to Ang2. **p<0.01 for TNF-α plus Ang2 in comparison to control. ns: indicates p= 0.199 for TNF-α in comparison to control, Tukey's post hoc test.

4.2 TNF-α induces VE- cadherin cleavage requires Ang2

As previous data showed some of TNF-α effects are mediated through autocrine action of Ang2 (Fiedler *et al*, 2006), it is possible that TNF-α could exert effects on VE-cadherin cleavage through Ang2. In order to investigate this, the effect of silencing Ang2 expression on TNF-α induced VE-cadherin cleavage was tested.

HUVECs were transfected with Ang2 siRNA and forty-eight hours later were stimulated with TNF-α for a period of one hour (Section: 2.11.3). The media and the lysate sample were collected and prepared as described in section: 2.10 and 2.10.1. The prepared samples were loaded into SDS gel then transferred by nitrocellulose member and probed with antibodies against the extracellular domain of VE-cadherin (Section: 2.12.7).

As shown in the Figure 4.3, Ang2 silencing was performed and resulted in substantial decreases in the level of Ang2 in HUVECs. The Figure 4.3 also showed that in the presence of TNF-α, a band of approximately 100 kDa was seen in the media, and nothing else on the gel.

Analysis of the effect of TNF-α on VE-cadherin release showed that the ligand stimulates release in cells transfected with control siRNA. However TNF-α failed to stimulate VE-cadherin release above control levels in cells in which Ang2 expression has been supressed. Indicating that Ang2 mediates the pro-cleavage effect of TNF-α (Figure 4.3). The Figure 4.3 showed that vinculin has no significant effect on VE-cadherin cleavage. Indicating releases of VE-cadherin from the same number of cell. Two experiments were performed and presented as additional experiments failed to supress Ang2.

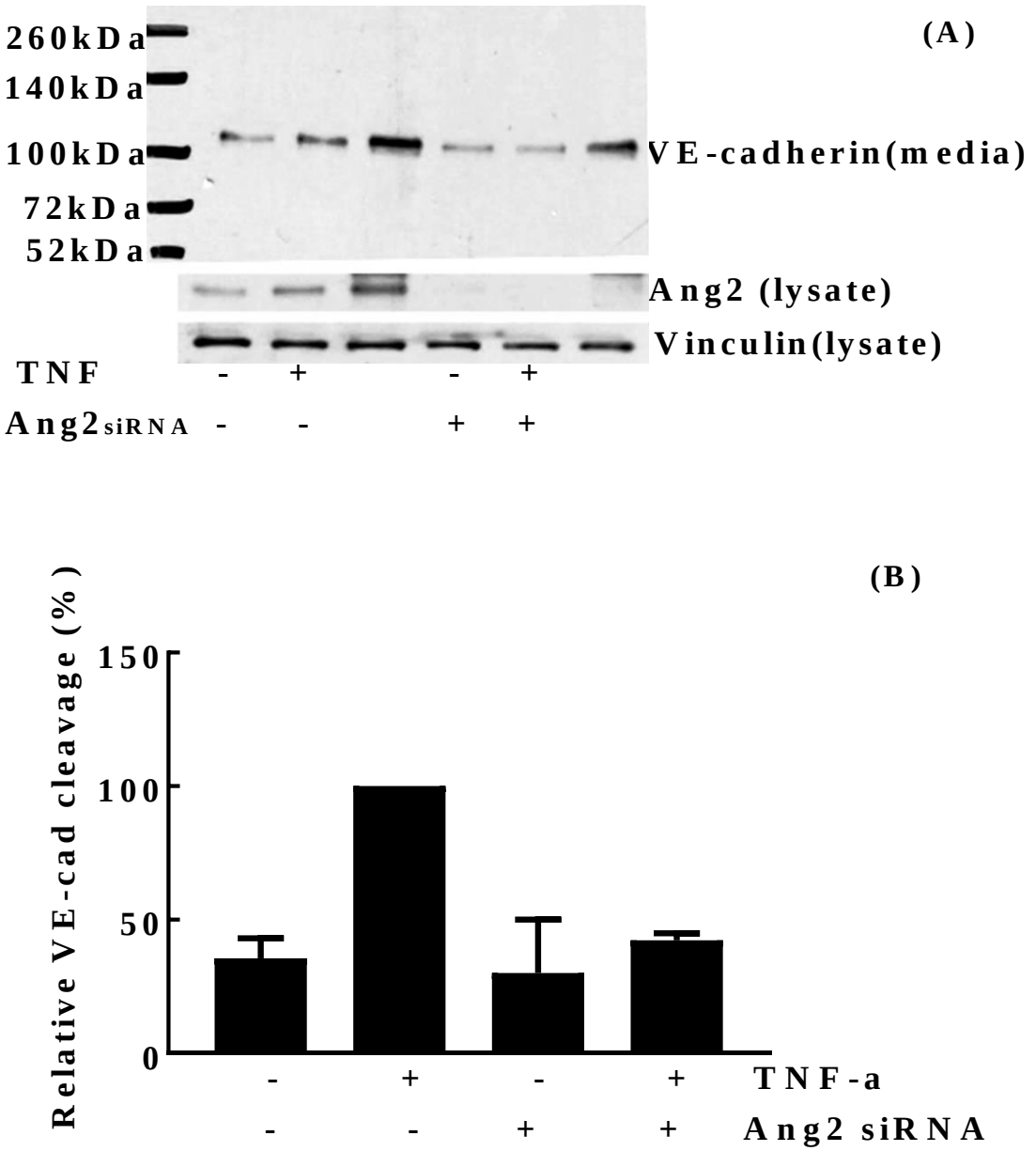


Figure 4.3: Western blot showing TNF-α induces VE-cadherin cleavage through Ang2 activation in HUVECs.

Cells were stimulated as follows: C: control. TNF-α: 20ng/ml TNF-α. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. Complete blot shown, only the relevant tracks are indicated (B): Blots were quantified and data shown as average ∓ SEM for two independent experiments. The average suppression of Ang2 expression was 64 ∓ 5 %(n=2, mean ∓SD).

In order to test whether or not Ang2 mediates TNF-α pro-cleavage effect in HCMECs. Cell were transfected with control siRNA or siRNA targeting Ang2, forty-eight hours post-transfection cells were activated with TNF-α and released VE-cadherin ectodomain was detected in media by immunoblotting. As shown in Figure 4.4, TNF-α stimulates release of VE-cadherin ectodomain in control transfected HCMECs. However, in HCMECs transfected with Ang2 siRNA, TNF-α was unable to stimulate release of VE-cadherin ectodomain. Probing cell lysates with anti-Ang2 confirmed suppression of Ang2 in the cell transfected with Ang2 siRNA. These data demonstrated that autocrine Ang2 is required for TNF-α induced VE-cadherin ectodomain release from HCMECs.

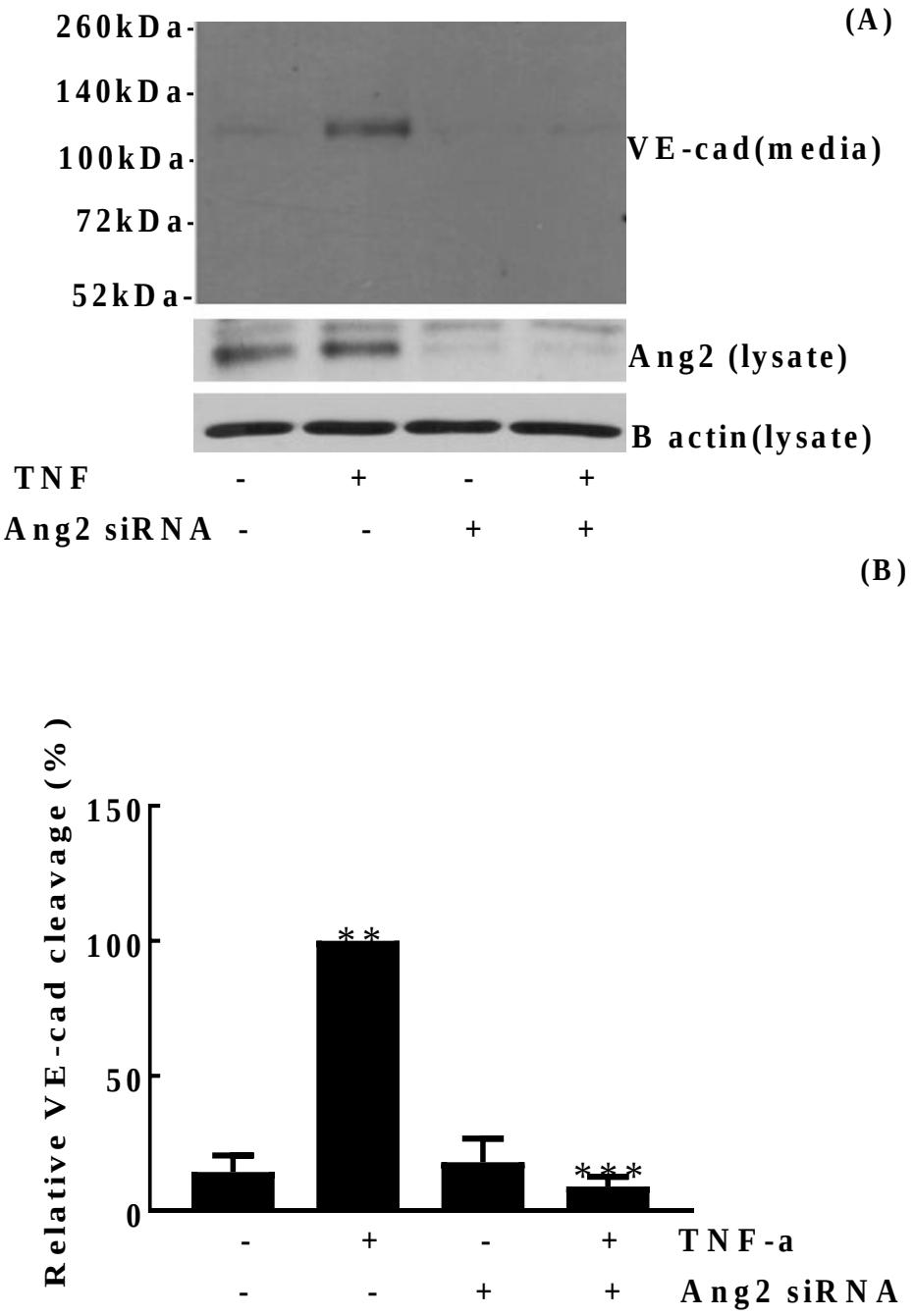


Figure 4.4: Western blot showing Ang2 knockdown attenuates TNF-α effect and Ang2 induces VE-cadherin cleavage in HCMECs.

Cells were stimulated as follows: C: control. TNF-α: 20ng/ml TNF-α. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. **p<0.01 for TNF-α compared with compared control. *** p < 0.001 for TNF-α + Ang2 knockdown compared with compared TNF-α, Tukey's post hoc test. The average suppression of Ang2 expression was 81 ∓ 3 % (n=3, mean ∓SEM).

4.3 ADAM10 and 17 regulates VE-cadherin cleavage

Previous data reported that TNF-α induces cleavage of VE-cadherin through ADAM10 activation (Flemming *et al*, 2015a). Several studies refer to the crucial contribution of ADAM10 and 17 in shedding a variety of transmembrane proteins (Sahin *et al*, 2004; Wetzel *et al*, 2017; Miller *et al*, 2017). Therefore the possibility of involvement ADAM10 and 17 in VE-cadherin cleavage in response to TNF-α plus Ang2 was investigated.

In order to perform this test, 20-30% confluent HUVECs were used. ADAM10 and ADAM17 were suppressed individually using siRNA treatment (Section: 2.11.3). Forty-eight hours of after siRNA transfection, the cells were incubated with TNF-α plus Ang2 for one hour and the media sample collected as described in (Section: 2.10 and 2.10.1) and loaded on to a SDS gel then blotted using western blot test (Section: 2.12.7).

As shown in Figure 4.5, ADAM10 and 17 silencing resulted in decreased expression of each metalloprotease, represented by the band seen in cell lysate for each of ADAM10 and ADAM17. The Figure 4.5, showed that VE-cadherin cleavage was induced in the presence of Ang2 and the cleavage was inhibited by ADAM10 (Figure 4.5). However, the ADAM10 inhibition was not significant because of the error bar, but the inhibition was clear. This indicates that ADAM10 are the proteases responsible VE-cadherin cleavage when Ang2 stimulate the cells.

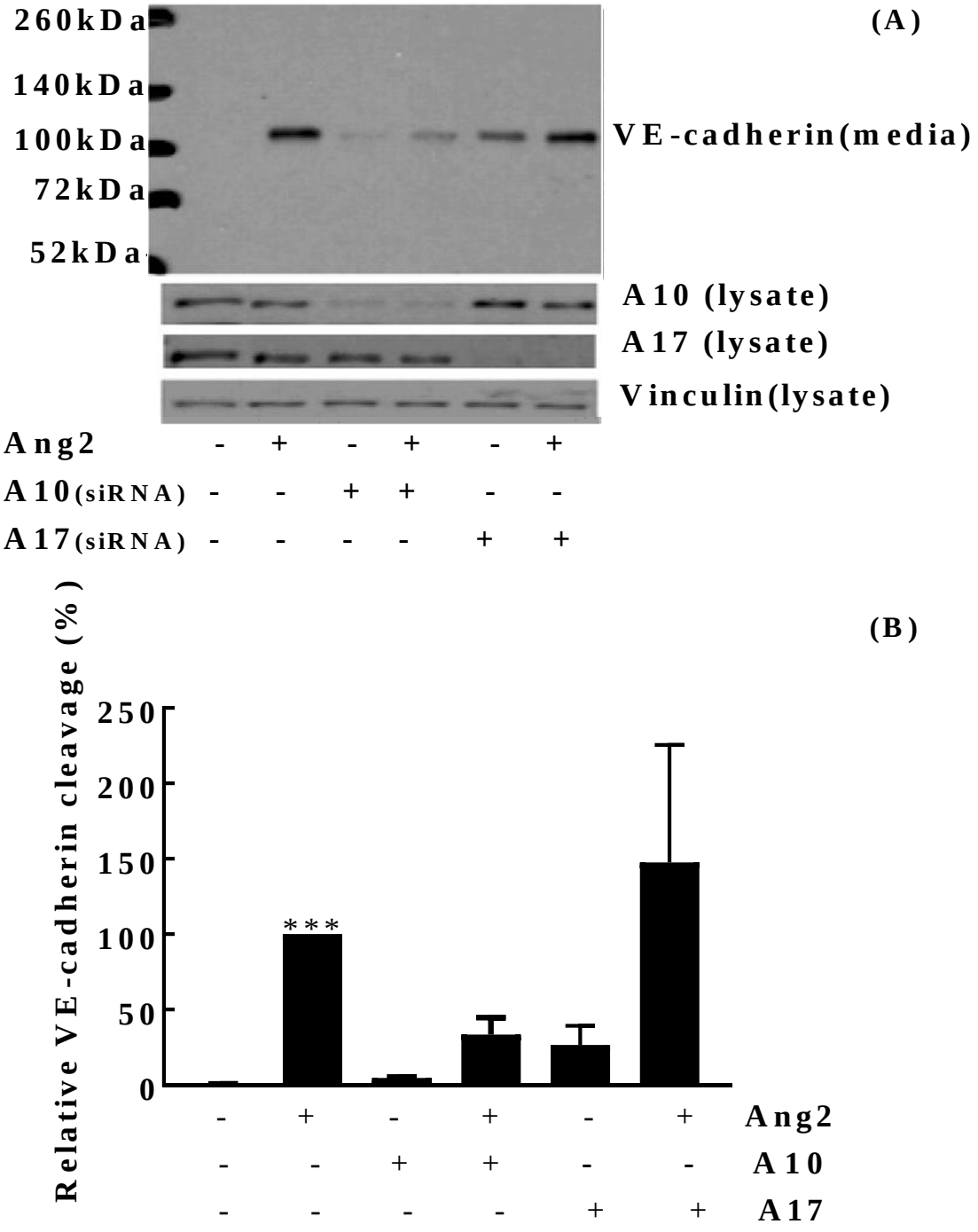


Figure 4.5: **Western blot showing Ang2 induces VE-cadherin cleavage by ADAM10 in HUVECs.**

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, ADAM10 and ADAM17 were detected in cell lysate, and cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three experiments. *** $p<0.001$ for Ang2 compared with control, Tukey's post hoc test. The average suppression of ADAM10 expression was 66 ∓ 16 %, ADAM17 53 ∓ 26 % (n=3, mean ∓SEM).

The role of ADAM10 and ADAM17 in Ang2 stimulated VE-cadherin cleavage was tested in HCMECs. In order to perform this test HCMECs were used. ADAM10 and 17 silencing were performed individually using siRNA (Section: 2.11.3). After forty-eight hours of siRNA transfection, the cells were stimulated for one hour with Ang2 as shown in (Section: 2.11.1) and media samples collected and loaded into SDS gel then probed with anti VE-cadherin antibody.

As shown in Figure 4.6 in HCMEC lysate, siRNA treatment for ADAM10 decreased its expression level, represented by the bands seen in cell lysate. Similarly ADAM17 expression was depressed by siRNA treatment represented by the bands seen in cell lysate. Probing for vinculin confirmed similar cell loading. Probing for released VE-cadherin ectodomain revealed stimulation by Ang2 as before. In cells in which ADAM10 was supressed, Ang2 failed to activate VE-cadherin release. In contrast, suppression of ADAM17 did not affect Ang2 stimulated VE-cadherin release. These data showed that ADAM10 has a role in Ang2-induced VE-cadherin release in HCMEC.

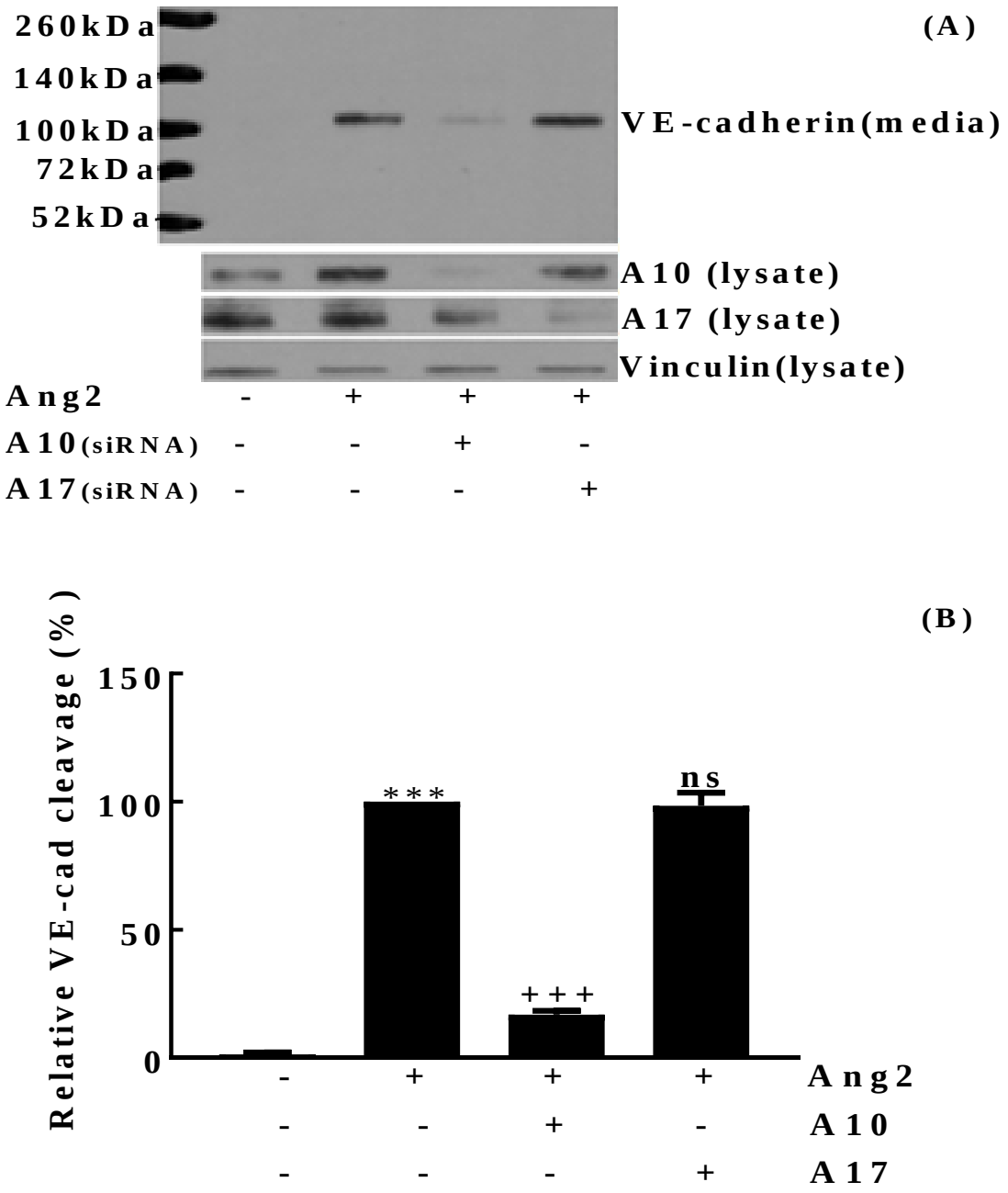


Figure 4.6: **Western blot showing Ang2 induces VE-cadherin cleavage by ADAM10 in HCMECs**

Cells were stimulated as follows: C: control. A2: 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, ADAM10 and ADAM17 were detected in cell lysate, and cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. *** indicates $p<0.001$ for Ang2 compared with control and $^{+++}$ indicates $p<0.001$ for Ang2 + A10compared with Ang2, ns: indicates $p= 0.9830$ for Ang2 compared with Ang2+ A17, Tukey's post hoc test. The average suppression of ADAM10 expression was 80 ∓ 6 %, ADAM17 77 ∓ 9 % (n=3, mean ∓SEM).

Further experiments were performed in order to test the role of ADAM10 and ADAM17 in Ang2 plus TNF-α stimulated VE-cadherin cleavage in HUVECs. ADAM10 and ADAM17 was suppressed each individually using siRNA treatment (Section: 2.11.3). Forty-eight hours of after siRNA transfection, the cells were incubated with TNF-α plus Ang2 for one hour and the media sample collected as described in (Section: 2.10 and 2.10.1) and loaded on to a SDS gel then blotted using western blot test (Section: 2.12.7).

As shown in Figure 4.7, ADAM10 and 17 silencing resulted in reduce expression each of the ADAM, represented by the band seen in cell lysate for each of ADAM10 and ADAM17. The Figure 4.7 showed that VE-cadherin cleavage was induced in the presence of TNF-α plus Ang2 and the cleavage was inhibited significantly by ADAM10 (Figure 4.7). This indicates that ADAM10 is the protease responsible for VE-cadherin cleavage when both Ang2 and TNF-α together stimulate the cells. Also, it was confirmed that TNF-α mediates its effect through Ang2.

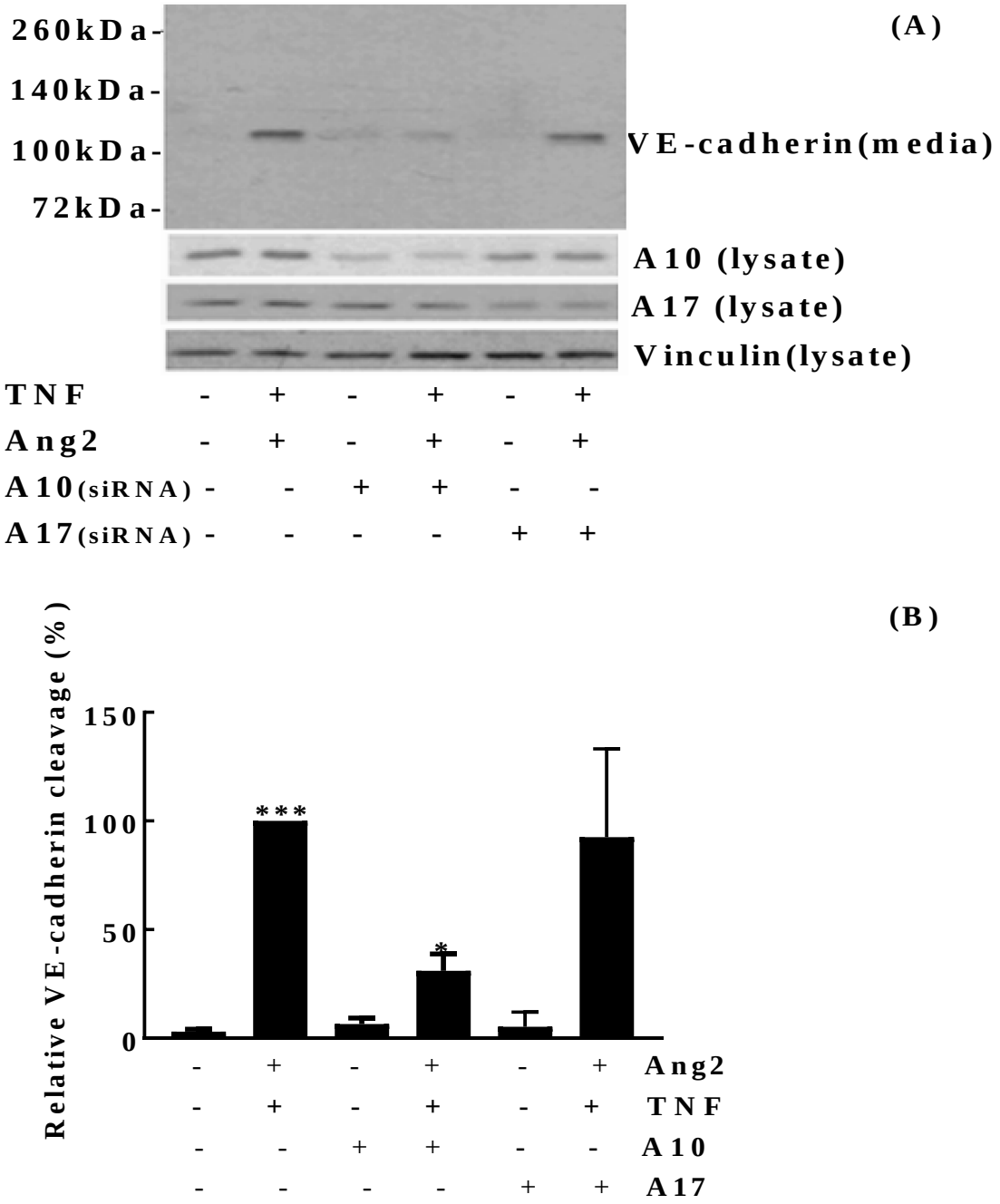


Figure 4.7: Western blot showing TNF-α induces VE-cadherin cleavage by ADAM10 and ADAM17 in HUVECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2 plus 20ng/ml TNF-α. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, ADAM10 and ADAM17 were detected in cell lysate, and cell were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. ***p<0.001 for TNF+Ang2 compared with control and * p<0.05 for TNF+Ang2+ A10 compared with TNF+Ang2 and ns indicates p>0.999 for TNF+Ang2+ A17 compared with TNF+Ang2, Tukey's post hoc test. The average suppression of ADAM10 expression was 67 ∓ 5 %, ADAM17 64 ∓ 10 % (n=3, mean ∓SEM).

4.4 Ang2 induces VE-cadherin cleavage through Tie2 independent pathway

Ang2 is known to act through Tie2 receptor. However, recently it has been shown that Ang2 acts in a Tie2-independent pathway to regulate actin re-orgnaization in endothelial cells (Hakanpaa *et al*, 2015). Therefore, an experiment was performed in order to test whether or not Tie2 is required for the pro-cleavage effect of Ang2 on VE-cadherin. To do this a siRNA approach was used for Tie2 knockdown (Section: 2.11.3).

HUVECs were transfected into siRNA targeting Tie2 or control siRNA. Forty-eight hours post-transfection cells were stimulated with Ang2 and released VE-cadherin ectodomain into the media was detected by immunoblotting.

As shown in Figure 4.8 , although Tie2 silencing resulted in a significant decrease in Tie2 expression, Ang2 still induced VE-cadherin cleavage in the absence of Tie2. These results show that Tie2 is not required for the pro-cleavage effect of Ang2 on VE-cadherin and suggest the presence of alternative pathway through which Ang2 mediates its VE-cadherin cleavage effect.

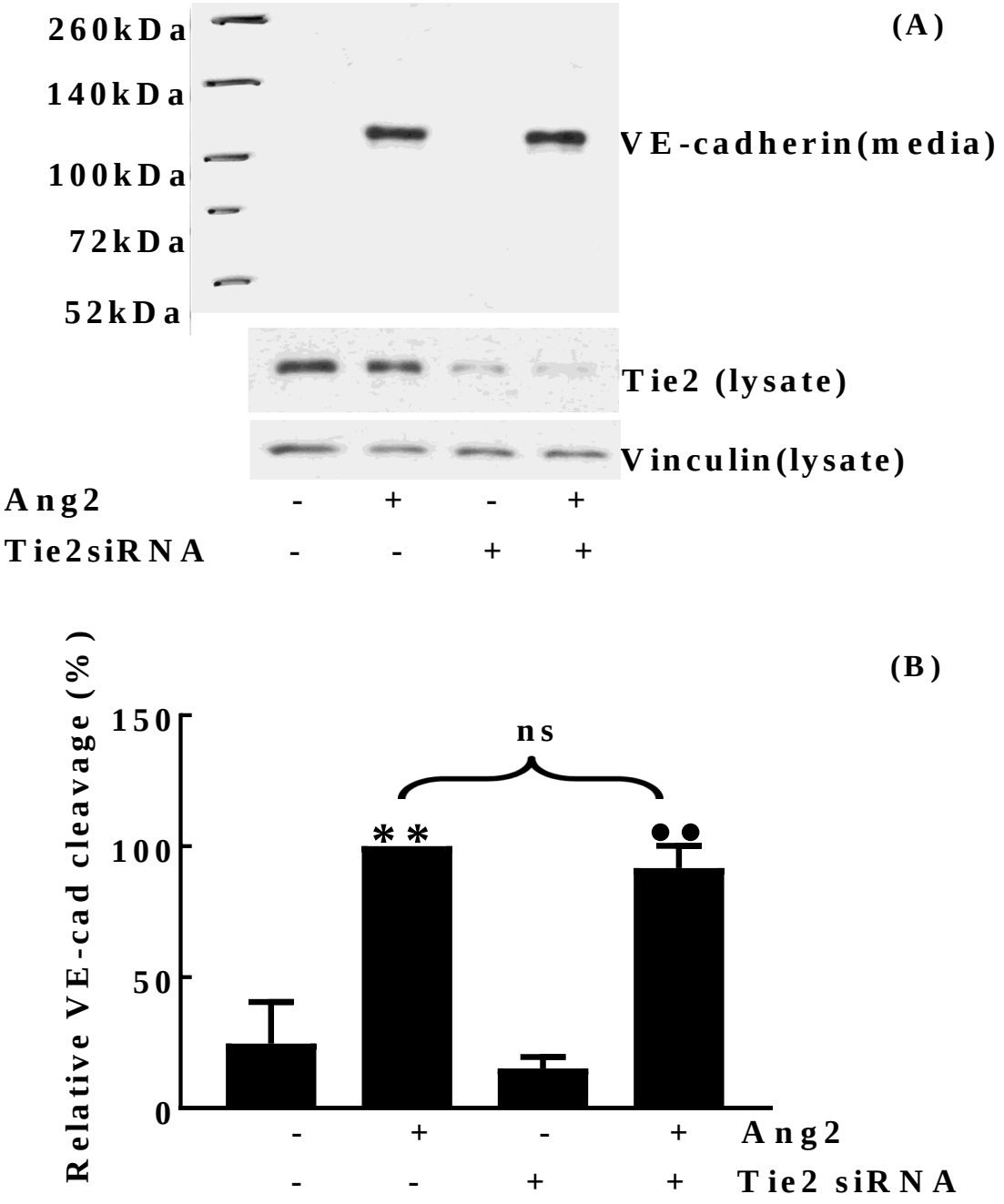
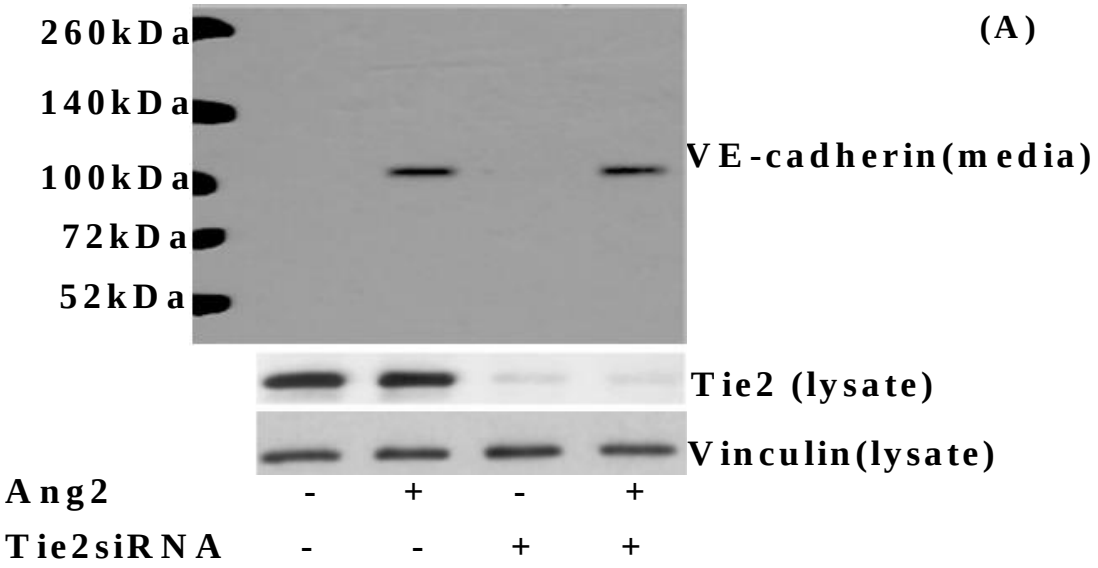


Figure 4.8: **Western blot showing Ang2 induces VE-cadherin cleavage through a Tie2 independent pathway in HUVECs**

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. **p<0.05 for Ang2 in comparison to control (control siRNA), •• p<0.05 for Ang2 (Tie2 siRNA) in comparison to control (control siRNA) and ns: indicates p= 0.9117 for Ang2 (Tie2 siRNA) in comparison to Ang2 (control siRNA), Tukey's post hoc test. The average suppression of Tie2 expression was 78 ∓ 11 % (n=3, mean ∓SEM).

A similar experiment was performed using HCMECs in order to test if Tie2 is required for the pro-cleavage effect of Ang2. HCMECs were transfected with siRNA targeting Tie2 or control siRNA. Forty-eight hours post-transfection cells were stimulated with Ang2 and released VE-cadherin ectodomain into the media was detected by immunoblotting. As shown in Figure 4.10, Tie2 silencing show significant decreases in the Tie2 expression in HCMECs.

In the presence of Tie2, Ang2-induced significant increases of VE-cadherin cleavage in comparison to its control. The Figure 4.9 showed that Ang2-induced significant increases of VE-cadherin cleavage in the absence of Tie2 receptor. Indicating that Tie2 is not required for the pro-cleavage effect of Ang2 in HCMECs.These results suggest that the Ang2 mediated VE-cadherin cleavage occurs through Tie2 independent pathway (Figure 4.9).



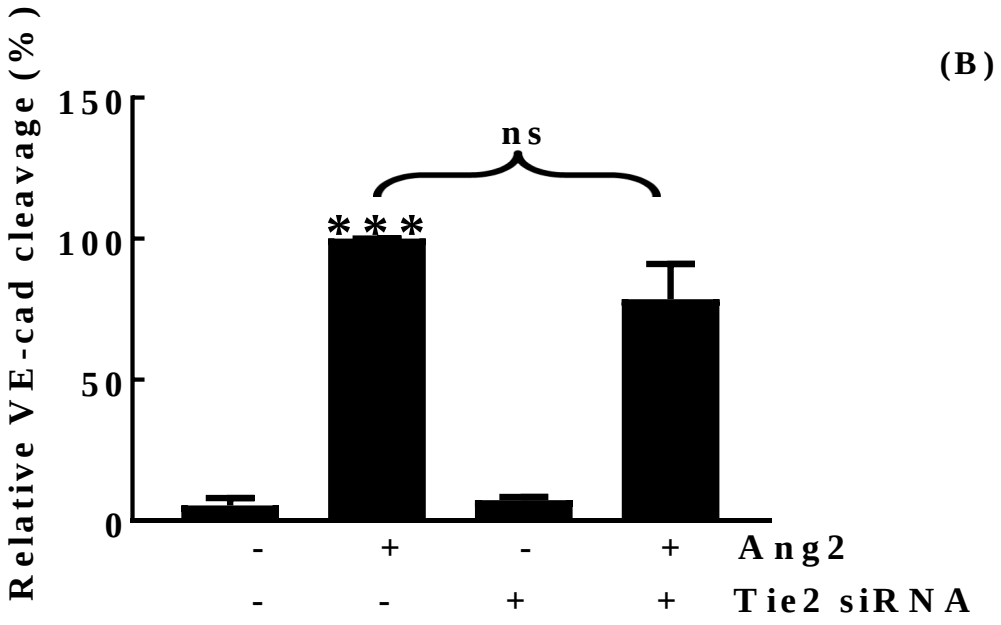


Figure 4.9: Western blot showing Ang2 induces VE-cadherin cleavage through a Tie2 independent pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. *** p= <0.001 for Ang2 compared control (control siRNA), ns: indicates p= 0.488 for Ang2 (control siRNA) in comparison to Ang2 (Tie2 siRNA), Tukey's post hoc test. The average suppression of Tie2 expression was 87 ∓ 10 % (n=3, mean ∓SEM).

4.5 Ang2 induces VE-cadherin cleavage through an integrin dependent pathway

The finding that Tie2 is not required for Ang2 effects on VE-cadherin suggests an alternative receptor is likely to be involved. Ang2 has been reported to bind integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_1$ (Yun *et al*, 2017; Imanishi *et al*, 2007; Felcht *et al*, 2012b). Therefore, the possibility that integrin might be involved in Ang2-induced VE-cadherin cleavage was investigated using integrin blocking antibodies. Previous data have already showed that using $\alpha_5\beta_1$ antibody will block binding to extracellular matrix and this also inhibits Ang2 binding (Hakanpaa *et al*, 2018).

Data reported that integrins are one of the cell surface proteins that are able to form a complex with Tie2 in response to Ang2 stimulation. An experiment proceeded to investigate the possibility of integrin involvement in the VE-cadherin cleavage pathway, specifically the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. In order to perform this test, integrin blocking antibodies were added 10-15 minutes before cell stimulation with Ang2 or TNF-α. The cells were stimulated for one hour with Ang2 and/or TNF-α and media sample collected and loaded into SDS gel then blot probed with anti VE-cadherin.

As shown in Figure 4.10, there is significant increases in VE-cadherin cleavage in the presence of Ang2, this was a significantly inhibited when incubated with α5β1 integrin blocker. This indicates that Ang2 requires α5β1 to mediate VE-cadherin cleavage effect (Tie2 independent pathway). Surprisingly in some experiment, the αvβ3 blocking antibody itself stimulated VE-cadherin release even in the absence of Ang2.

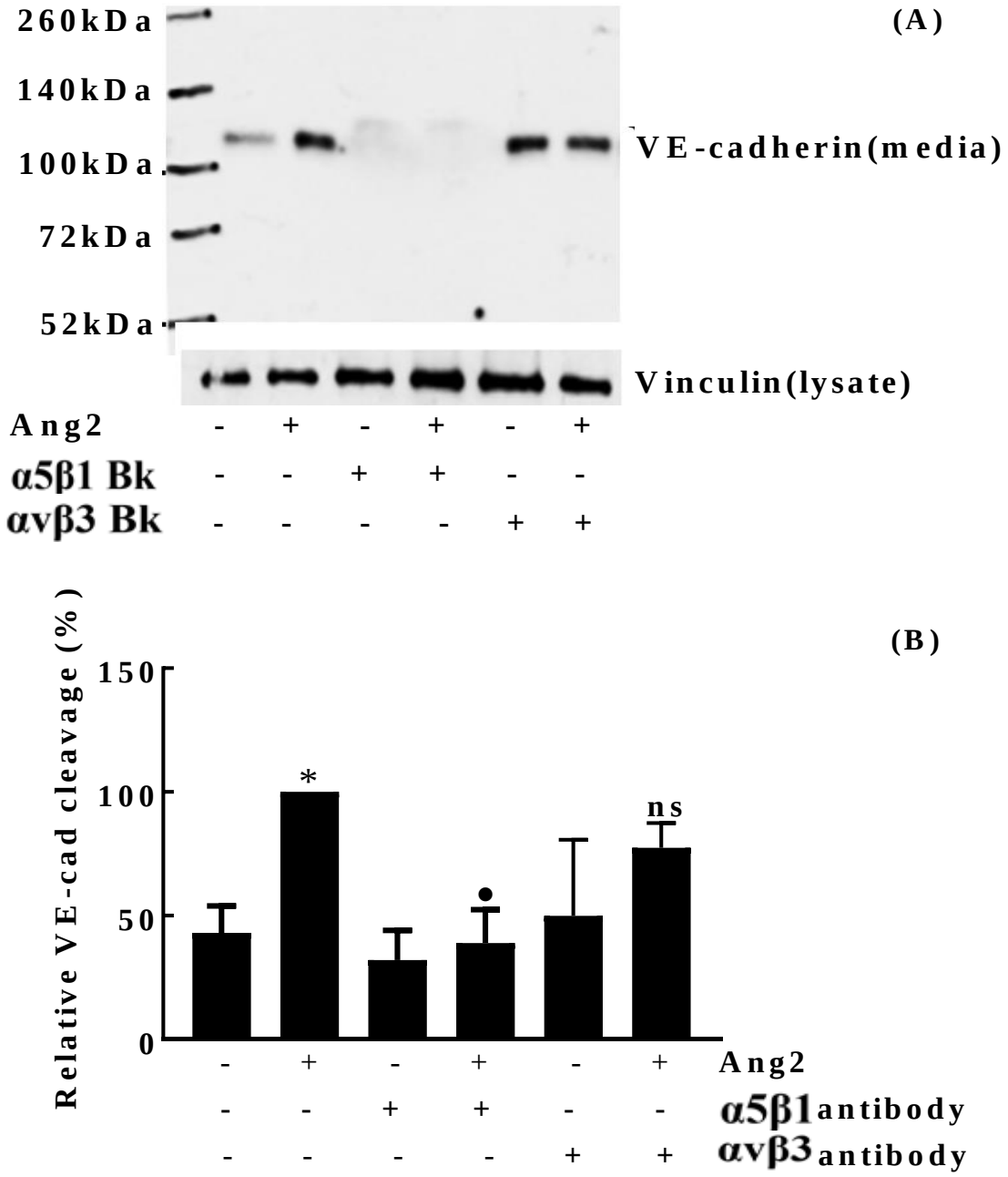


Figure 4.10: **Western blot showing Ang2 stimulates VE-cadherin cleavage through an integrin pathway in HUVECs.**

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker 10µg/ml cons (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. *p<0.05 for Ang2 in comparison to control, •p<0.05 for Ang2 compared Ang2 +α5β1-Bk and ns: indicates p= 0.2189 for Ang2 in comparison to Ang2 +αvβ3-Bk, Tukey's post hoc test.

In order to test whether Ang2-induced VE-cadherin cleavage requires integrin pathway. Experments were performed to test whether blocking $\alpha_5\beta_1$ or $\alpha_v\beta_3$ effects Ang2-induced VE-cadherin release in HCMECs. Integrin blocking antibodies were added 10-15 minutes before cell stimulation with Ang2. The cells were stimulated for one hour with Ang2 and media sample was collected and loaded into SDS gel then blot probed with anti VE-cadherin.

Figure 4.11, Ang2-induced significant increases in VE-cadherin cleavage. In the presence of $\alpha_5\beta_1$ integrin blocker but not $\alpha_v\beta_3$, the VE-cadherin cleavage effect induced by Ang2 was significantly inhibited. This suggests that Ang2 mediates VE-cadherin cleavage through an $\alpha_5\beta_1$ integrin pathway. This study also observed that in some of HCMECs experiment the VE-cadherin cleavage was stimulated when incubated with $\alpha_v\beta_3$.

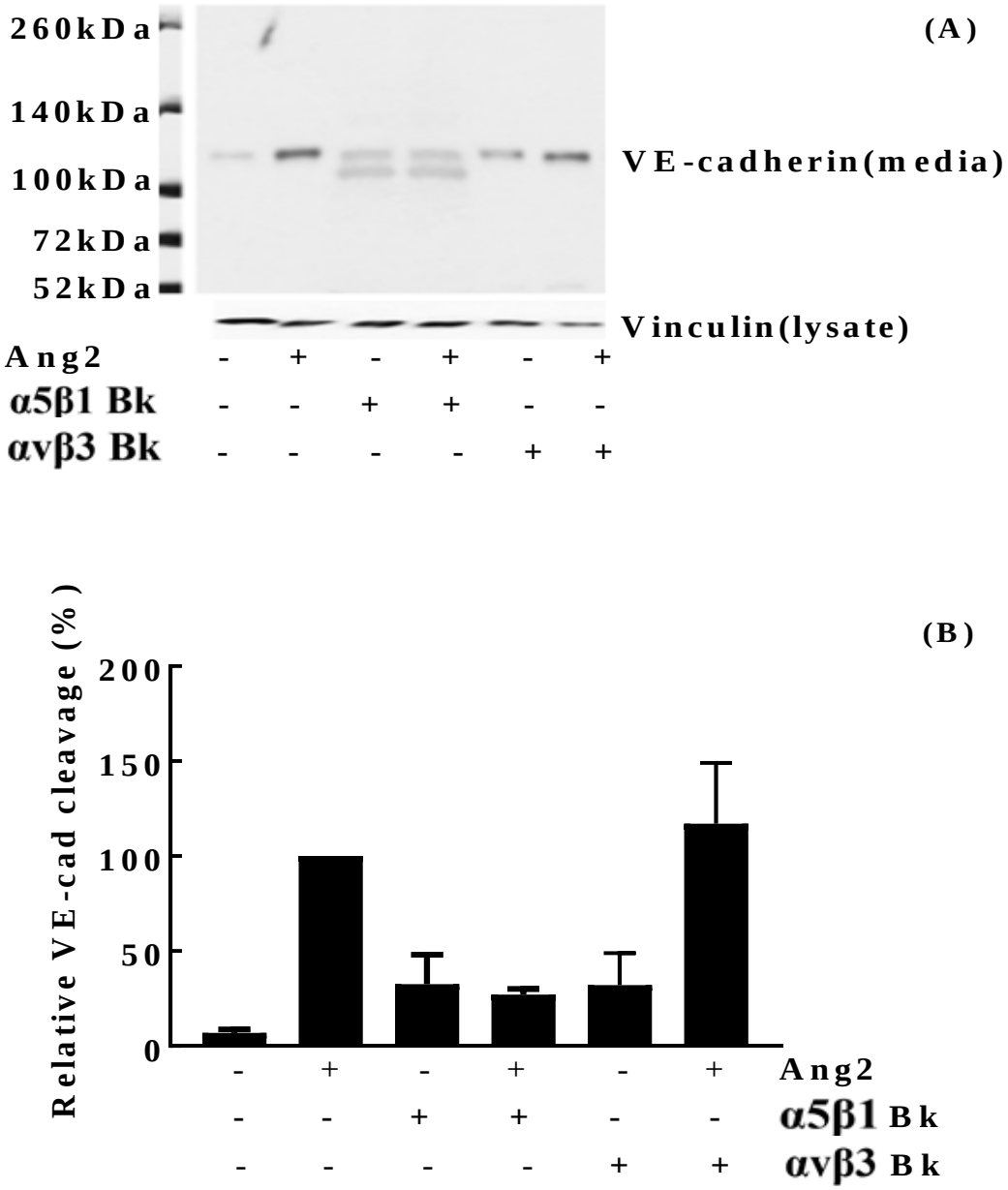


Figure 4.11: Western blot showing Ang2 stimulates VE-cadherin cleavage through an integrin pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker 10µg/ml cons (A): cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SD for two independent experiments.

Further experiments were performed in order to test the involvement of $\alpha_5\beta_1$ in the Ang2-induced VE-cadherin cleavage in HCMECs. A peptide inhibitor of $\alpha_5\beta_1$ called ATN161 was used for this purpose (Stoeltzing *et al*, 2003a). The cells were treated with ATN161 or control vehicle for 10 minutes then stimulated with Ang2 for an hour. Media samples were collected, concentrated and probed for VE-cadherin ectodomain.

As shown in Figure 4.12, Ang2 stimulated VE-cadherin cleavage and this was inhibited by ATN 161. These data suggests $\alpha_5\beta_1$ integrin may have a role in Ang2-induced VE-cadherin cleavage. Due to time limitation and unavalability of cells it was not possible to perform additional experiments with ATN161.

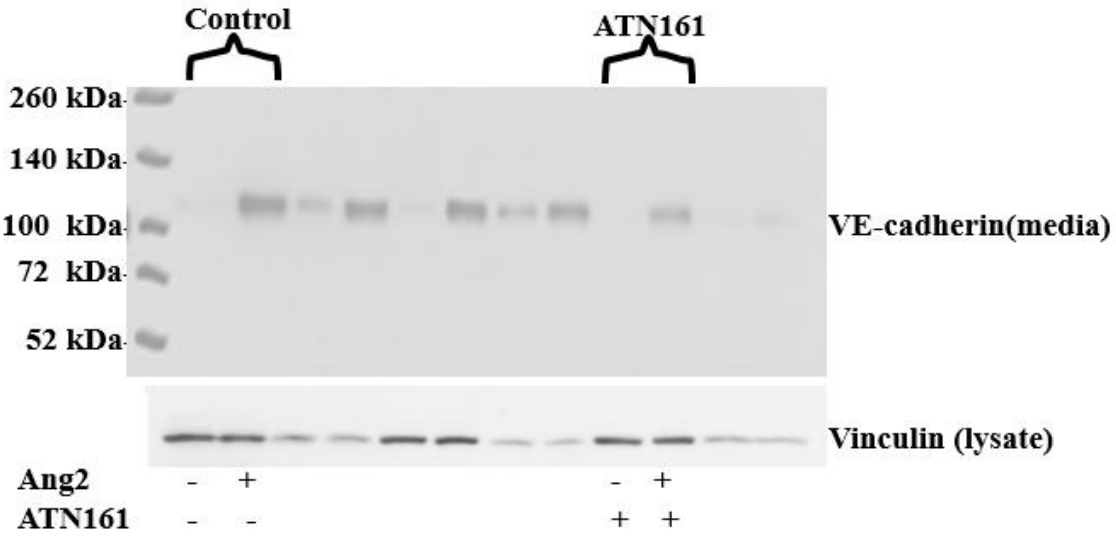


Figure 4.12: Western blot showing Ang2 induce VE-cadherin cleavage through integrin pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker (ATN161) 20µM. Cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. This experiment was performed twice. Complete blot shown, only the relevant tracks are indicated.

An experiment was performed in order to investigate if there are any differences in the size of cleaved ectodomain between HUVECs and HCMECs when the cells were stimulated with Ang2.

As shown in Figure 4.13, there is no differences in the size of the cleaved ectodomain between HUVECs and HCMECs when the cell were stimulated with Ang2. Indicating that the VE-cadherin cleavage occurs at a similar position in both endothelial cell types.

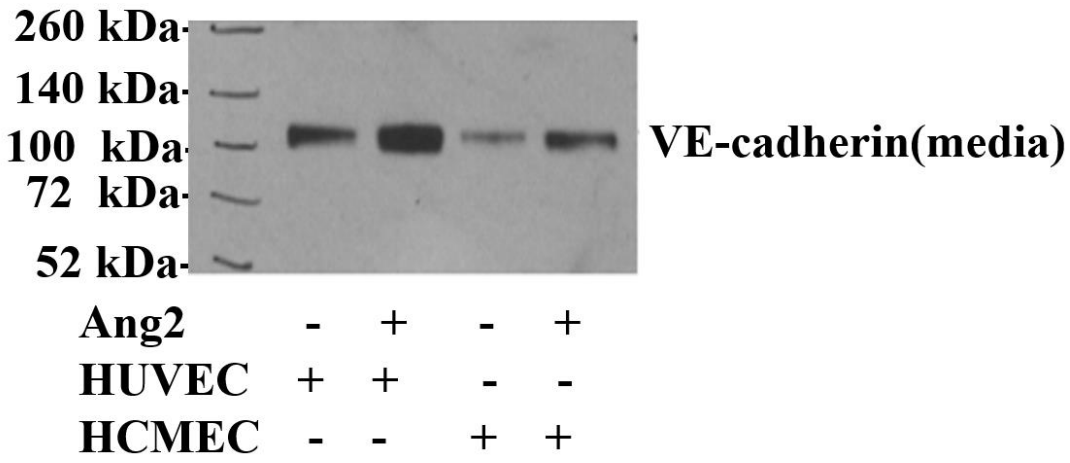


Figure 4.13: Western blot showing the differences in size between HUVECs and HCMECs when Ang2 induce VE-cadherin cleavage.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Cleaved VE-cadherin ectodomain was detected at about 100 kDa in concentrated media from cells. This experiment was performed once.

4.6 Discussion

The present study shows for the first time that: (1) Ang2 stimulates VE-cadherin cleavage in HUVECs and HCMECs; (2) Ang2 is required for TNF-α induced VE-cadherin cleavage; (3) ADAM10 is required for VE-cadherin cleavage; (4) Ang2 effects on VE-cadherin do not require Tie2; (5) Ang2 effects on VE-cadherin are inhibited by blocking $\alpha_5\beta_1$ integrin.

This study found that Ang2-induced release of the extracellular domain of VE-cadherin through a Tie2 independent pathway, specifically through $\alpha_5\beta_1$ pathway. This was confirmed by Tie2 silencing when the cells were stimulated with Ang2 and/or TNF-α to test the involvement of Tie2 in the Ang2 induce VE-cadherin cleavage effect.

Additional experments were performed in order to test the involvement of the integrin in Ang2-induced VE-cadherin cleavage, this was confirmed by using a $\alpha_5\beta_1$ blocker which resulted in reduced VE-cadherin cleavage up to 80% indicating the involvement of $\alpha_5\beta_1$ in the Ang2-induced VE-cadherin cleavage.

Furthermore, this study found TNF-α mediates VE-cadherin cleavage effects through Ang2. This was confirmed by Ang2 silencing when the cells were stimulated using TNF-α. This study also found that ADAM10 is the protease responsible for Ang2-induced VE-cadherin cleavage. This was confirmed by transfecting the cells with ADAM10 and 17 siRNA each individually and when the cells were stimulated with Ang2 or Ang2 plus TNF-α.

Cleavage of VE-cadherin is likely to increase endothelial cell permeability. Several studies show the crucial contribution of ADAM10 and ADAM17 in cleaving a variety of cell surface proteins (Dreymueller *et al*, 2012). Others have already shown ADAM10 mediates VE-cadherin ectodomain cleavage in response to TNF-α and thrombin (Schulz *et al*, 2008). In addition, inhibition of VE-cadherin cleavage by suppression of ADAM10 has been shown to decrease endothelial permiability (Schulz *et al*, 2008; Flemming *et al*, 2015). Although these data are consistent with VE-cadherin cleavage having a role in regulating endothelial permeability, they do not directly demonstrate cleavage of this adhesion molecule results in permeability. This is beacause ADAM10 also cleaves other junctional proteins sauch as claudin-5, and therefore inhibiting ADAM10 could be supressing permeability by blocking cleavage of these junctional proteins (Jiao *et al*, 2011; Argaw *et al*, 2009; Kondo *et al*, 2009).

However, the study of Fleming *et al*, (2015) directly demonstrated that VE-cadherin shedding increases vascular permeability of endothelial cell monolayers in response to inflammatory mediators such as TNF-α (Flemming *et al*, 2015). The study also demonstrated the crucial role of ADAM10 in mediating endothelial permeability through VE-cadherin cleavage. The increase in monolayer permeability induced by VE-cadherin cleavage may result from loss of VE-cadherin in cell junctions. In addition, Fleming 2010 demonstrated that the released VE-cadherin ectodomain acts as a competitive binder to disrupt the VE-cadherin homo typic junctions. Furthermore, (Kabacik and Raj, 2017) demonstrated the junctional disruption effect resulted from exposing endothelial cells to ionising radiation (IR). IR induces VE-cadherin cleavage through activating ADAM10 and this was associated with increased vascular permeability which can contribute in CVD through increasing formation of atherosclerotic plaques. The study confirmed this by using an ADAM10 blocker to block the permeability effect resulting from VE-cadherin shedding (Kabacik and Raj, 2017). The study showed the presence of an association between VE-cadherin cleavage and vascular permeability. Previous work on VE-cadherin showed that displacement of VE-cadherin from its binding site could result in increased vascular permeability. The study confirms this result by using recombinant VE-cadherin able to compete with the endogenous VE-cadherin on the same β-catenin binding site. Displacement of endogenous VE-cadherin from its binding site resulted in barrier disruption and increased the gap between endothelial cells and induced vascular

permeability (Sawant *et al*, 2011). The study showed the possible mechansim through which VE-cadherin might regulate the permeability. The microvascular blood retinal barrier in diabetes with retinopathy was disrupted due to VE-cadherin shedding and by the effect of metalloproteinases (Navaratna *et al*, 2007). The study suggests the possible mechanism through which blood retinal barrier in a diabetes was disrupted. Treating mice with an antibody directed against the VE-cadherin extracellular domain affects VE-cadherin adhesion, clustering and changes the endothelial permeability (Corada *et al*, 2002). This denotes the important role of VE-cadherin in maintaining the integrity of the endothelial cells.

High levels of Ang2 are associated with several cardiovascular diseases, such as hypertension, acute and chronic congestive heart failure and atherosclerosis, as well as cancer, including lung and breast cancer, and diabetes(Tsai *et al*, 2018; Lukasz *et al*, 2013; Fiedler *et al*, 2004; David *et al*, 2009; Holopainen *et al*, 2012; Imanishi *et al*, 2007). Previous studies reported that Ang2-Tie2 binding blocks the protective effect of Ang1 and mediates the vascular leakage effect (Eklund *et al*, 2017; Zonneveld *et al*, 2017; Scharpfenecker *et al*, 2005). Junctional proteins play an important role in maintaining the monolayer integrity of the endothelial cells. VE-cadherin is one of these junctional proteins that keeps the cells in a confluent state by forming a strong connection among adjacent cells (Harris and Nelson, 2010). Disrupting this system is associated with vascular permeability and may contribute to cardiovascular diseases (Weis, 2008).

Several diseases such as rheumatoid arthritis, sepsis and diabetic retinopathy are associated with increased endothelial permeability as a result of inducing VE-cadherin cleavage (Sidibé *et al*, 2014). Furthermore, diseases such as stroke, diabetic retinopathy and MI are associated with endothelial barrier disruption and hyper permeability (Weis, 2008). Increased endothelial permeability contributes to vascular dysfunction which is one of the main events in vascular related diseases (Chistiakov *et al*, 2015). Persistence of vascular permeability contributes in elongation of the intracerebral haemorrhage in stroke rate (Lee *et al*, 2007). Also vascular inflammation contributes to the progression of several cardiovascular diseases such as hypertension and atherosclerosis (Packard and

Libby, 2008). This is associated with vascular permeability and increased endothelial leukocyte infiltration (Ponnuchamy and Khalil, 2008).

On the other hand decreasing oedema in myocardial infarction or stroke via decreasing vascular leakage contributes to decreasing infarction size and improve survival and function of the organ (Weis, 2008; Weis *et al*, 2004). In addition to CVD, increased vascular permeability contributes to pathology in cancer. In cancer, high levels of TNF-α can disrupt the vascular system, increase permeability, cause VE-cadherin disruption and prevent access of drugs to tumors (Menon *et al*, 2006). Furthermore, VE-cadherin shedding could enhance migration of cancer cells, apoptosis and decrease T-cell infiltration to solid tumors (Zhao *et al*, 2017; Herren *et al*, 1998). Suppression of vascular permeability possibly by inhibiting VE-cadherin cleavage could decrease the pathological effects in cancer.

Increased levels of TNF-α was reported in cardiovascular diseases and cancers. Several diseases were associated with high levels of TNF-α such as diabetes, sepsis, chronic inflammatory disease, chronic obstructive pulmonary disease, (Vernooy *et al*, 2002; TSAO *et al*, 2001; Ritchie and Connell, 2007; Balkwill, 2006; Holopainen *et al*, 2012; Imanishi *et al*, 2007). Additional studies demonstrated that VE-cadherin loss is increased in response to inflammatory mediators such as TNF-α. The study of (Menon *et al*, 2006) showed that TNF-α increased permeability of tumour vasculature likely through disruption the junctional protein VE-cadherin. This could result from inducing VE-cadherin re-localization within the same cell surface but away from the endothelial cell-cell junctions leading to increased gaps between endothelial cells (Menon *et al*, 2006). This indicates the role of TNF-α in mediating permeability effect through VE-cadherin cleavage. Data reported that increased VE-cadherin shedding is one of the biomarkers associated with severe acute kidney injury in sepsis disease. The reported data also suggested that in this case shedding of VE-cadherin from the cell surface might exceed the production of VE-cadherin (Yu *et al*, 2019). As the severity of sepsis increased the VE-cadherin shedding also increases (Zhang *et al*, 2010). This suggests the role of inflammatory conditions on VE-cadherin cleavage. Another study showed that endothelial stimulation by bacterial toxin resulted in ADAM10 activation and VE-cadherin shedding. The study demonstrated also the mechanism through which ADAM10 was activated. In normal conditions, the pro-ADAM10 is attached to the calmodulin, and

this prevents ADAM10 maturation. Bacterial toxin increase intracellular Ca^{2+} influx and this resulting binding of Ca^{2+} to calmodulin releases ADAM10 which allows it to be activated and mediate VE-cadherin cleavage (Reboud *et al*, 2017). The current study found that Ang2 is required for the effect of TNF-α on VE-cadherin shedding. This suggests that the effects that have been reported by previous studies on TNF-α inducing the permeability effect may be due to Ang2.

It was believed that the pathogenic effects of Ang2 were mediated through binding to Tie2 and via blocking Ang1 protective effect. The current study showed that lack of Tie2 did not affect on the VE-cadherin cleavage effect induced by Ang2. This suggests an alternative pathway through which Ang2 induce VE-cadherin shedding.

Recent data showed the ability of Ang2 binding to $\alpha_5\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ (Yun *et al*, 2017; Imanishi *et al*, 2007; Felcht *et al*, 2012b). The study of (Hakanpaa *et al*, 2018) showed that Ang2 induce $\alpha_5\beta_1$ translocation resulting in junctional disruption and increased permeability. The study also showed that by using $\alpha_5\beta_1$ antibody they block the $\alpha_5\beta_1$ inducing permeability effect (Hakanpaa *et al*, 2018). The current study also showed that Ang2 requires $\alpha_5\beta_1$ but not $\alpha_v\beta_3$ in order to induce VE-cadherin cleavage through activating ADAM10 (Figure 4.14).

Maintaining monolayer integrity of the vascular endothelial barrier is critical to maintain vascular haemostasis. While disturbing this barrier through influencing adhesion protein VE-cadherin might increase vascular permeability and contributes to pathogenic effects in CVD and cancer (Kabacik and Raj, 2017). This study identifies Ang2-α5β1 binding as a novel regulator for VE-cadherin shedding through activating ADAM10. This may have a potential effect on vascular permeability. This insight may help identifcation of therapies required to block Ang2 pathogenic effects.

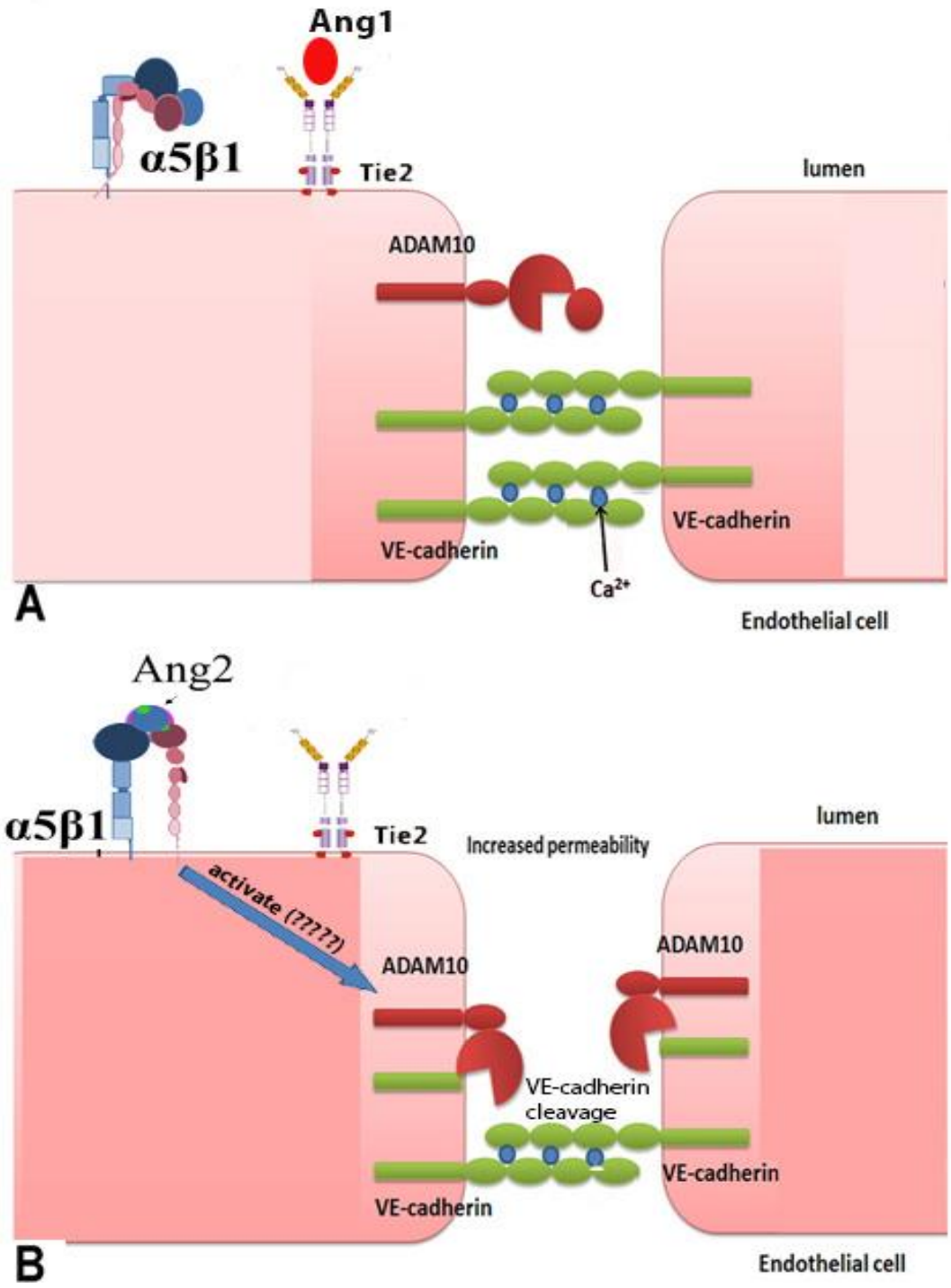


Figure 4.14: Summary of proposed effects of Ang2 on VE-cadherin cleavage through integrins

Scheme showing the cleavage of VE-cadherin by ADAM10 as a result of Ang2-α5β1 binding. A: Normal vascular endothelial cells with a tight VE-cadherin junction between cells due to Ang1-Tie2 binding. B: Ang2-α5β1 binding enhances VE-cadherin cleavage by ADAM10 through unknown mechansim and result in increased endothelial permeability. The image was adopted from (Kabacik and Raj, 2017).

Chapter 5: Angiopoietin-2 induce Tie1 cleavage through Tie2 independent pathway

Tie1 exists in a complex with Tie2 at the endothelial cell surface (Marron *et al*, 2000). Tie1 does not bind angiopoietin (Maisonpierre *et al*, 1997; Davis *et al*, 1996), and the interaction with Tie2 does not enable phosphorylation of Tie1 (Saharinen *et al*, 2005). In addtition, Tie1 interaction with Tie2 regulates signalling of Tie2 in response to angiopoietin. In subconfluent endothelial cells, Tie1 inhibits Ang1-induced Tie2 activation (Marron *et al*, 2007; Felcht *et al*, 2012; Hansen *et al*, 2010). In contrast, in quiescent endothelial cells, Tie1 enhances signalling by Ang1 through Tie2 and allows Ang2 to act as an agonist of Tie2 (Kim *et al*, 2016; Korhonen *et al*, 2016). Loss of Tie1 exerts antagonist effects in confluent endothelial cells (Kim *et al*, 2016b; Korhonen *et al*, 2016). Tie1 is subject to regulated ectodomain cleavage in which the ectodomain of the receptor is proteolytically cleavaed and released from the cell surface (Yabkowitz *et al*, 1997). This cleavage is stimulated by VEGF and inflammatory cytokines such as TNF-α (Yabkowitz *et al*, 1999; McCarthy *et al*, 1999; Tsiamis *et al*, 2002). Tie1 cleavage results in a decrease in cell surface Tie1 and would be expected to affect angiopoetin signalling through Tie2 in the same way as loss of Tie1 by decreased expression. In fact, inflammation induced Tie1 cleavage has been shown to be accompanied by a switch to Ang2 antagonistic action in mice *in vivo* and vascular destabilization (Kim *et al*, 2016; Korhonen *et al*, 2016). Regulation of Tie1 cleavage, therefore is potentially of great importance in controlling angiopoietin signalling. In this chapter the effects of Ang2 on Tie1 cleavage are investigated.

5.1 Ang2 induces Tie1 cleavage

In order to test the effect of Ang2 and TNF-α on the release of the extracellular domain of Tie1, HUVECs were incubated with Ang2 and /or TNF-α for a period of one hour. The media was recovered, concentrated, run on SDS gel then transferred by nitrocellulose member and probed with antibodies that recognise the extracellular domain of Tie1 (Section: 2.11.1).

As shown in the Figure 5.1, in the presence of Ang2 a band of approximately 100 kDa was seen in the media. The size of the released extracellular domain of Tie1 corresponds to the reported size of cleaved Tie1 ectodomain (Yabkowitz *et al*, 1997). Tie1 cleavage from the cell surface was significantly increase in the presence of Ang2 in comparison to control. Vinculin was quantified in the cell lysate to confirm the effects on released Tie1 ectodomain were not due to differences in cell numbers.

The Figure 5.1 also shows that TNF-α induced increases in Tie1 ectodomain cleavage in comparison to control as previously reported, although in these experiments this did not reach statistical significance. When both Ang2 and TNF-α were incubated together this resulted in synergistic effects producing 2-fold stimulation in the release of Tie1 ectodomain in comparison to the Ang2. These results show that Ang2 stimulates release of the Tie1 extracellular domain. Furthermore, Ang2 enhances Tie1 cleavage in the presence of TNF-α.

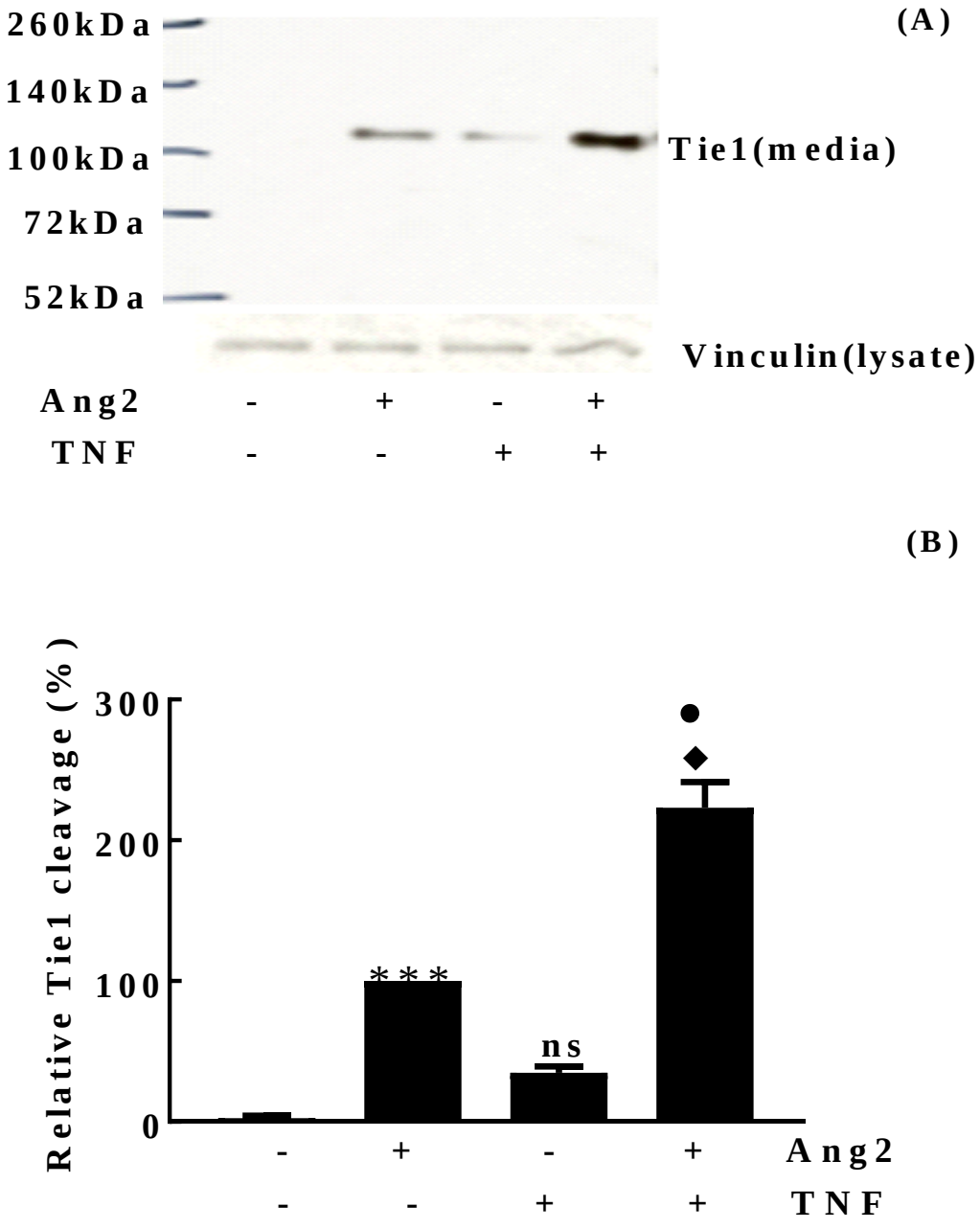


Figure 5.1: western blot showing Ang2 induce Tie1 cleavage in HUVECs.

Cells were stimulated as follows: C: control. A2: 0.5µg/ml Ang2. TNF-α: 20ng/ml TNF-α. TA: 20 ng/ml TNF-α plus 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. ***p <0.001 for Ang2 compared with control and •p<0.05 for TNF-α +Ang2 in comparison with Ang2, and ♦p<0.05 for TNF-α +Ang2 in comparison with control, and ns: indicates p= 0.0593 for TNF-α in comparison with control, Tukey's post hoc test.

In order to test whether the Ang2 inducing Tie1 cleavage effect was specific for HUVECs, the experiment was also performed in HCMECs. As shown in the Figure 5.2, when Ang2 was incubated with HCMECs, a Tie1 immunoreactive protein of approximately 100 kDa was seen in the media. Indicating that Ang2 significantly increased the release of the Tie1 ectodomain, as in comparison to control cell there is 4-fold increase in the Tie1 ectodomain released by Ang2 activation.

Similar experiments were performed to test the effect of TNF-α on Tie cleavage in HCMECs. In the presence of TNF-α there was a non-significant increase in the release of the Tie1 ectodomain in comparison to control (Figure 5.2). Nevertheless, when both Ang2 and TNF-α were used together they produce a significant synergistic effect, about 3-fold stimulation in the release of extracellular domain of Tie1 in comparison to Ang2.

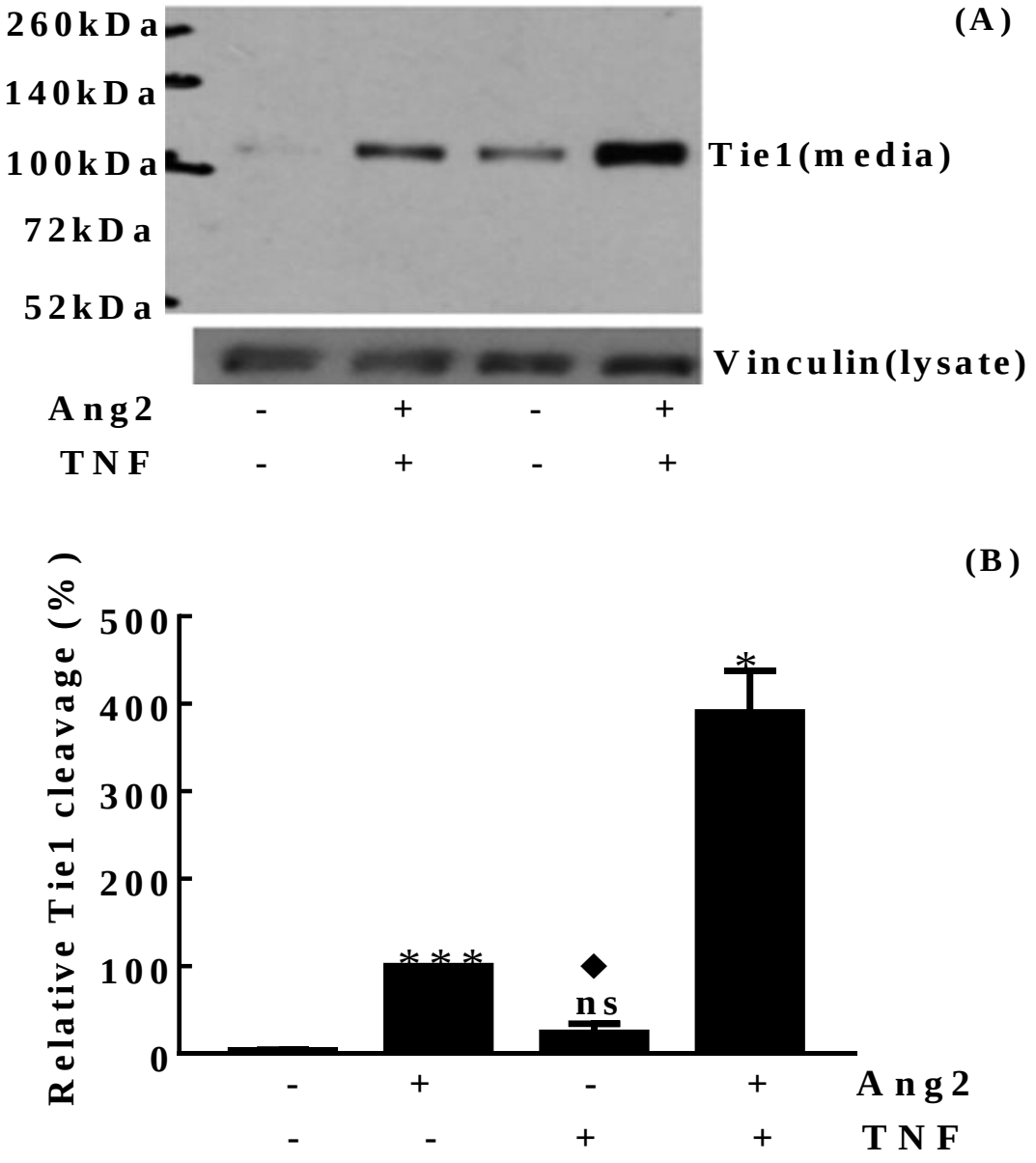


Figure 5.2: western blot showing Ang2 stimulates Tie1 cleavage in HCMECs.

Cells were stimulated as follows: C: control. A2: 0.5µg/ml Ang2. TNF-α: 20ng/ml TNF-α. TA: 20 ng/ml TNF-α plus 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. ***$p<0.001$ for Ang2 in comparison to control and ♦$p<0.05$ for TNF-α compared with Ang2 and ns: indicates $p= 0.399$ for TNF-α compared with Ang2. *$p<0.05$ for TNF-α +Ang2 compared with Ang2, Tukey's post hoc test.

5.2 TNF-α induced Tie1 cleavage requires Ang2

It is possible that TNF-α mediated Tie1 cleavage requires Ang2. In order to test this, the effect of silencing Ang2 expression on TNF-α induced Tie1 cleavage was examined. HUVECs were transfected with Ang2 siRNA and forty-eight hours later were stimulated with TNF-α for a period of one hour (Section: 2.11.3). The media and the lysate sample were collected and prepared as described in section: 2.10 and 2.10.1. The prepared samples were resolved by SDS gel then transferred to a nitrocellulose membrane and probed with antibodies against the extracellular domain of Tie1 (Section: 2.12.7).

As shown in the Figure 5.3, Ang2 silencing resulted in substantial decreases in the expression of Ang2 in HUVECs. The Figure 5.3 also showed that in the presence of TNF-α in cells expressing Ang2 a single band of approximately 100 kDa was seen in the media corresponding to Tie1 ectodomain. In contrast, Tie1 ectodomain cleavage was much lower in response to TNF-α in endothelial cells in which Ang2 expression was suppressed by siRNA. Data is shown from a single experiment. Other experments were performed however suppression of Ang2 by siRNA in the additional experments was poor.

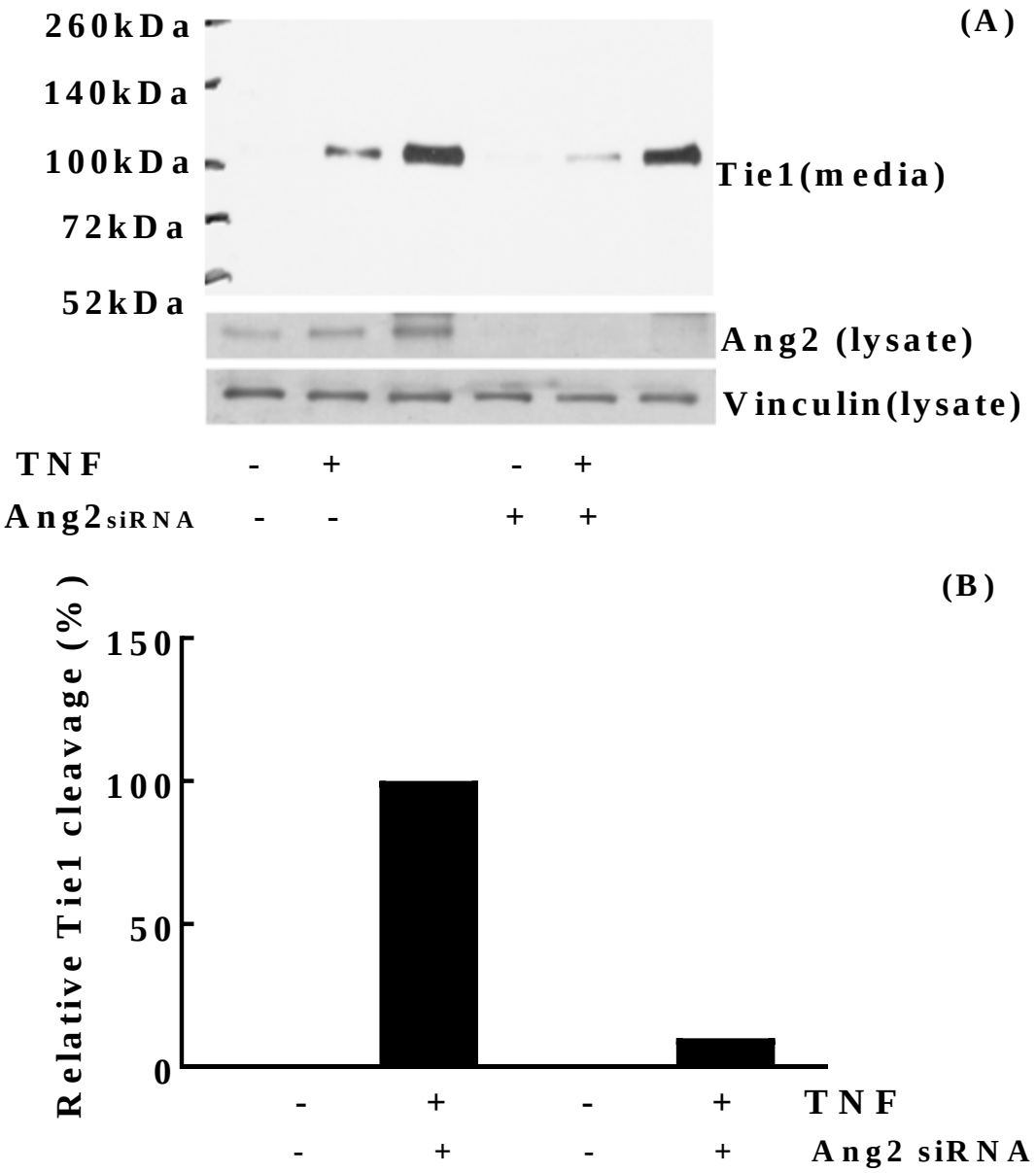


Figure 5.3: Western blot showing TNF-α mediate Tie1 cleavage through Ang2 activation in HUVECs.

Cells were stimulated as follows: C: control. TNF-α: 20ng/ml TNF-α. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. Complete blot shown, only the relevant tracks are indicated. (B): Blots were quantified and data is shown from the single experiment that was performed. The average suppression of Ang2 expression was 67% .

In order to investigate whether or not TNF-α inducing Tie1 cleavage through Ang2 is specific for HUVECs, a similar experiment were performed using HCMECs. Cells were transfected with control siRNA or siRNA targeting Ang2, forty-eight hours post-transfection cells were activated with TNF-α and released Tie1 ectodomain was detected in media by immunoblotting.

As shown in the Figure 5.4, Ang2 siRNA resulted in substantial decreases in Ang2 expression. The Figure 5.4 showed, in the presence of TNF-α, a band of approximately 100 kDa was detected in the media of control transfected HCMECs. However, in HCMECs transfected with Ang2 siRNA, Tie1 cleavage induced by TNF-α was significantly inhibited. These data show that Ang2 is required in order for TNF-α to activate Tie1 cleavage.

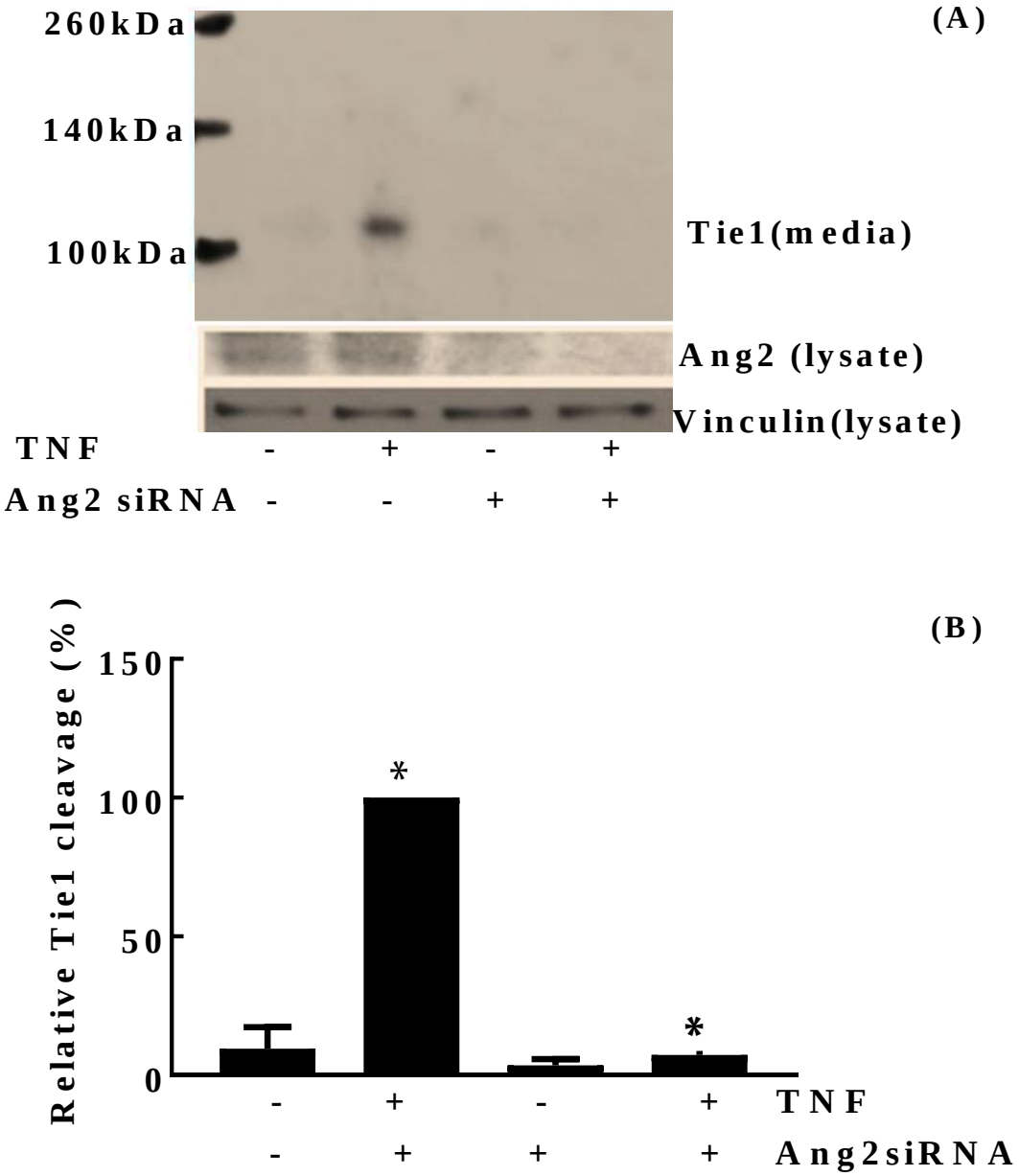


Figure 5.4: Western blot showing TNF-α mediate Tie1 cleavage through Ang2 activation in HCMECs.

Cells were stimulated as follows: C: control. TNF-α: 20ng/ml plus 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three experiments. *p< 0.05 for TNF-α compared with compared control. *p< 0.05for TNF-α + Ang2 knockdown compared with compared TNF-α, Tukey's post-hoc test.The average suppression of Ang2 expression was 80 ∓ 4 % (n=3, mean ∓ SEM).

5.3 ADAM17 regulates Tie1 cleavage

Previous studies showed that TNF-α induces Tie1 cleavage through metalloproteases (Yabkowitz *et al*, 1999c). ADAM10 and ADAM17 are two metalloproteases known to mediate cleavage of a number of cell surface receptors and ligand. Therefore, the possibility of involvement ADAM10 and 17 in Ang2 induce Tie cleavage was tested.

In order to investigate the role of ADAM10 and 17 in mediating Tie1 cleavage effects, suppression conditions for ADAM10 and 17 expression were established in HUVECs using siRNA approach. HUVECs were transfected with siRNA directed against ADAM10, ADAM17 or control siRNA. Forty-eight hours after siRNA transfection, the cells were incubated with Ang2 and TNF-α for one hour. Cells were lysed and media samples were collected post-stimulaton as described in sections: 2.10 and 2.10.1, and expression of the ADAMs in cell lysates were determined by immunoblotting.

As shown in Figure 5.5, ADAM10 and 17 siRNA resulted in decreased expression each of the metalloproteases in the cell lysates. Figure 5.5 showed that Tie1 cleavage was induced in the presence of Ang2 plus TNF-α and the cleavage was not inhibited by ADAM10 nor ADAM17 siRNA transfection. This suggests that Tie cleavage induced by Ang2 is not cleaved by ADAM10 nor ADAM17. However it should be noted that even after siRNA transfection there was still ADAM10 and ADAM17 expression detected to some extent in the cell. In these experments it was not possible to completely suppress ADAM10 and ADAM17 expression, therefore it can not be unequivocally concluded that these ADAMs are not included in Tie1cleavage in HUVECs.

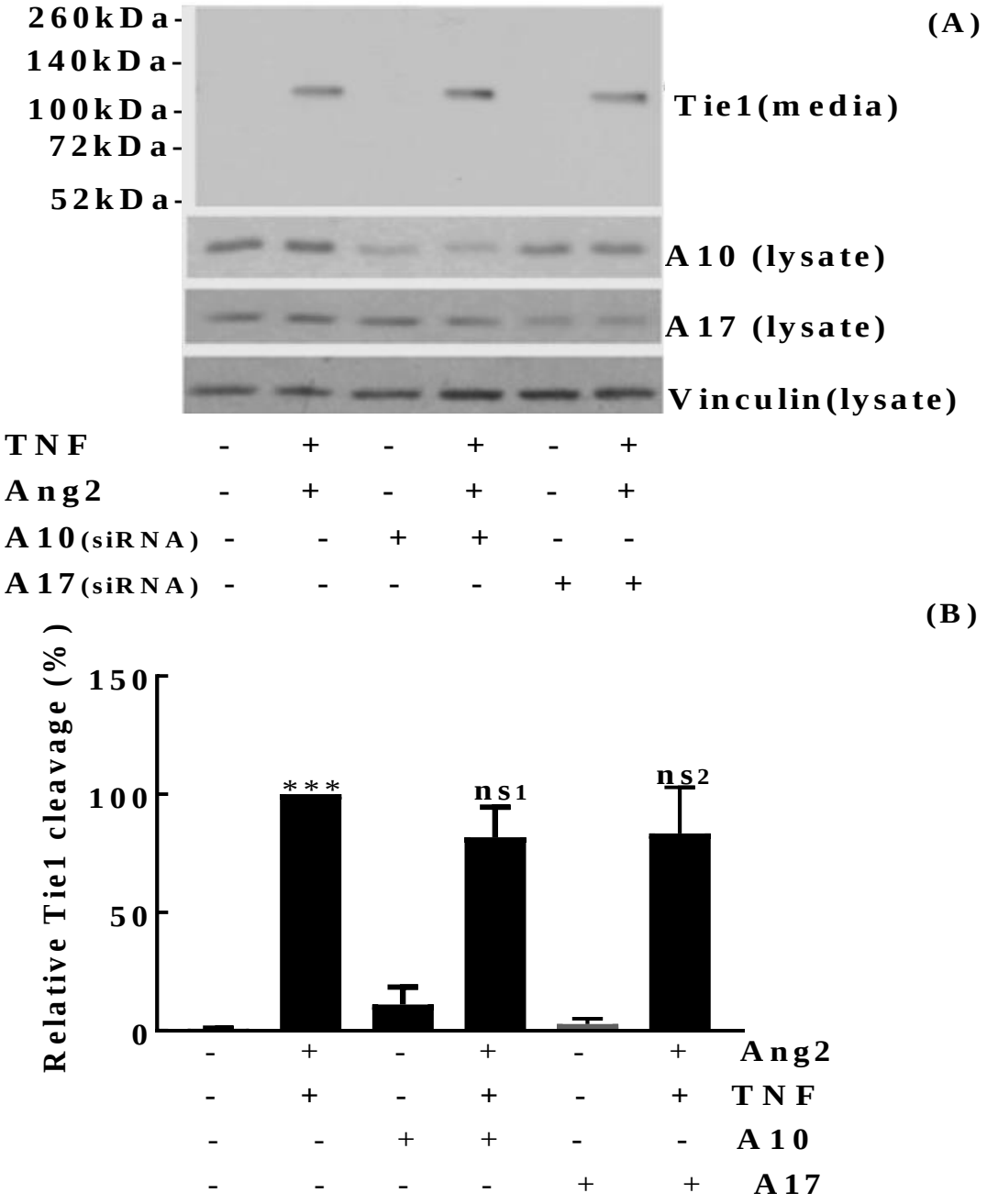


Figure 5.5: Western blot showing Ang2 induce Tie1 cleavage through activating ADAM10 and 17 in HUVECs.

Cells were stimulated as follows: C: control. 0.5µg/ml Ang2 plus TNF-α: 20ng/ml. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, ADAM10 and ADAM17 were detected in cell lysate, and cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. ***p< 0.001 for TNF-α+Ang2 compared with control and ns: indicates p= 0.5683 for TNF-α+Ang2+ A10 compared with TNF-α+Ang2 ns: indicates p= 0.803 for TNF-α +Ang2+ A17 compared with TNF-α +Ang2, Tukey's post hoc test. The average suppression of ADAM10 expression was 61 ∓ 11 %, ADAM17 44 ∓ 14 % (n=3, mean ∓ SEM).

Further experments were performed to test whether or not ADAM10 and 17 were involved in Tie1 cleavage in HCMECs. The HCMECs were transfected with control siRNA or siRNA targeting ADAM10 or ADAM17. Forty-eight hours post-transfection cells were stimulated with Ang2 and released Tie1 ectodomain in the media was detected by immunoblotting.

As shown in Figure 5.6, ADAM10 and 17 silencing resulted in decreased expression of each of the ADAMs. Figure 5.6 showed that Tie1 cleavage was induced in the presence of Ang2 and the cleavage was significantly inhibited by ADAM17 knockdown. This indicates that ADAM17 is the protease responsible for Ang2-induced Tie1 cleavage in HCMECs.

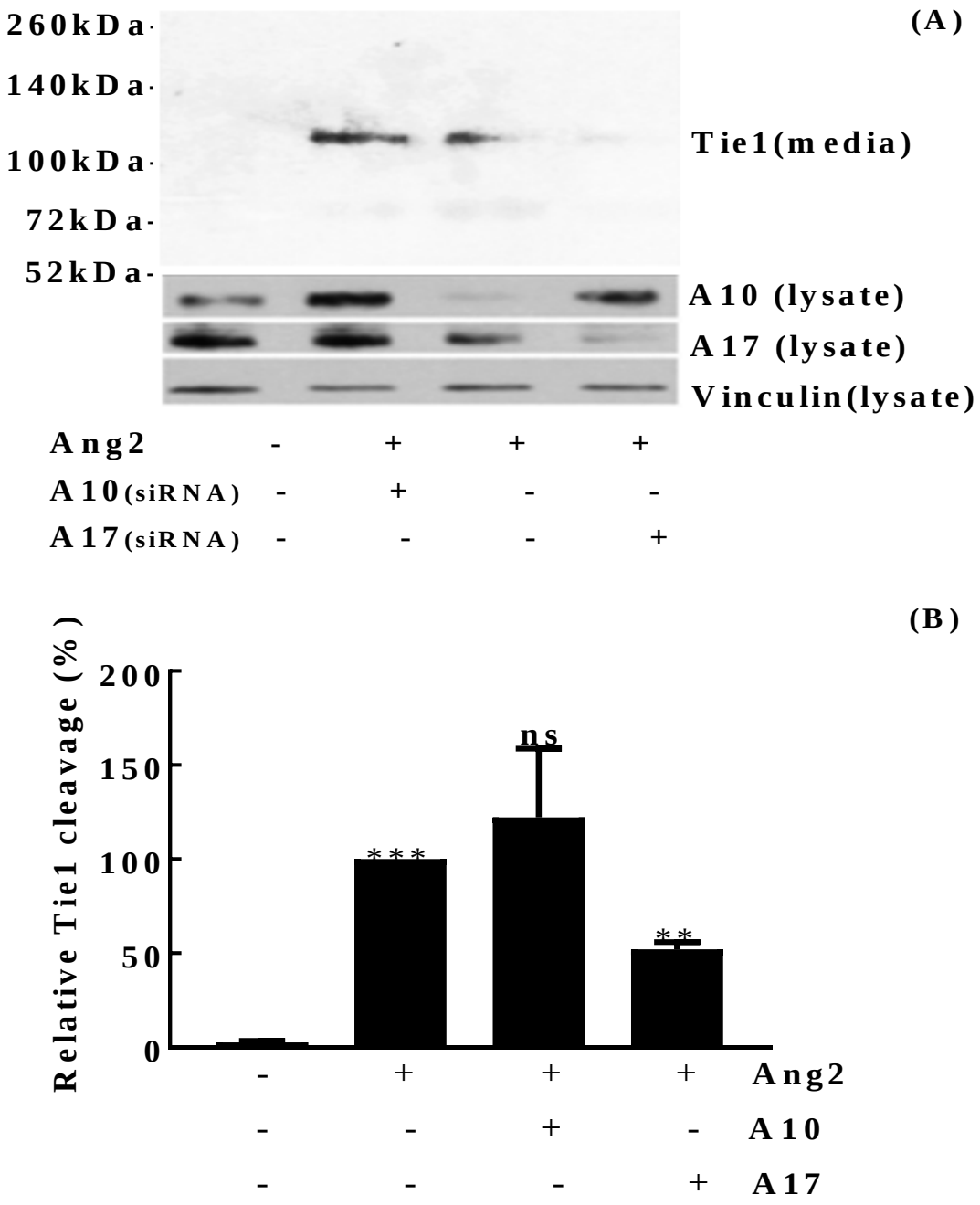


Figure 5.6: Western blot showing Ang2 activate ADAM17 and induce Tie1 in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, ADAM10 and ADAM17 were detected in cell lysate, and cell were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as an average ∓ SEM for three independent experiments. ***p <0.001 for Ang2 compared with control and ns: indicates = 0.873 for Ang2 compared with Ang2 + A10 and **p< 0.05 for Ang2 compared with Ang2+A17, Tukey's post hoc test. The average suppression of ADAM10 expression was 80 ∓ 8 %, ADAM17 76 ∓ 12 % (n=3, mean ∓ SEM).

A similar experiment performed in the presence of TNF-α only in HCMECs in order to identify the protease responsible for TNF-α inducing Tie1 cleavage. The cells were transfected with control siRNA or siRNA targeting ADAM10 or ADAM17. Forty-eight hours post-transfection cells were stimulated with TNF-α and released Tie1 ectodomain into the media was detected by immunoblotting.

As shown in Figure 5.7, TNF-α increases Tie1 cleavage. ADAM17 silencing resulted in significant inhibition of Tie1 cleavage induced by TNF-α. These data show ADAM17 is required for TNF-α induced Tie1 cleavage in HCMECs. The experiment was performed once due to a lack of time and availability of cells, it was difficult to perform additional experiments.

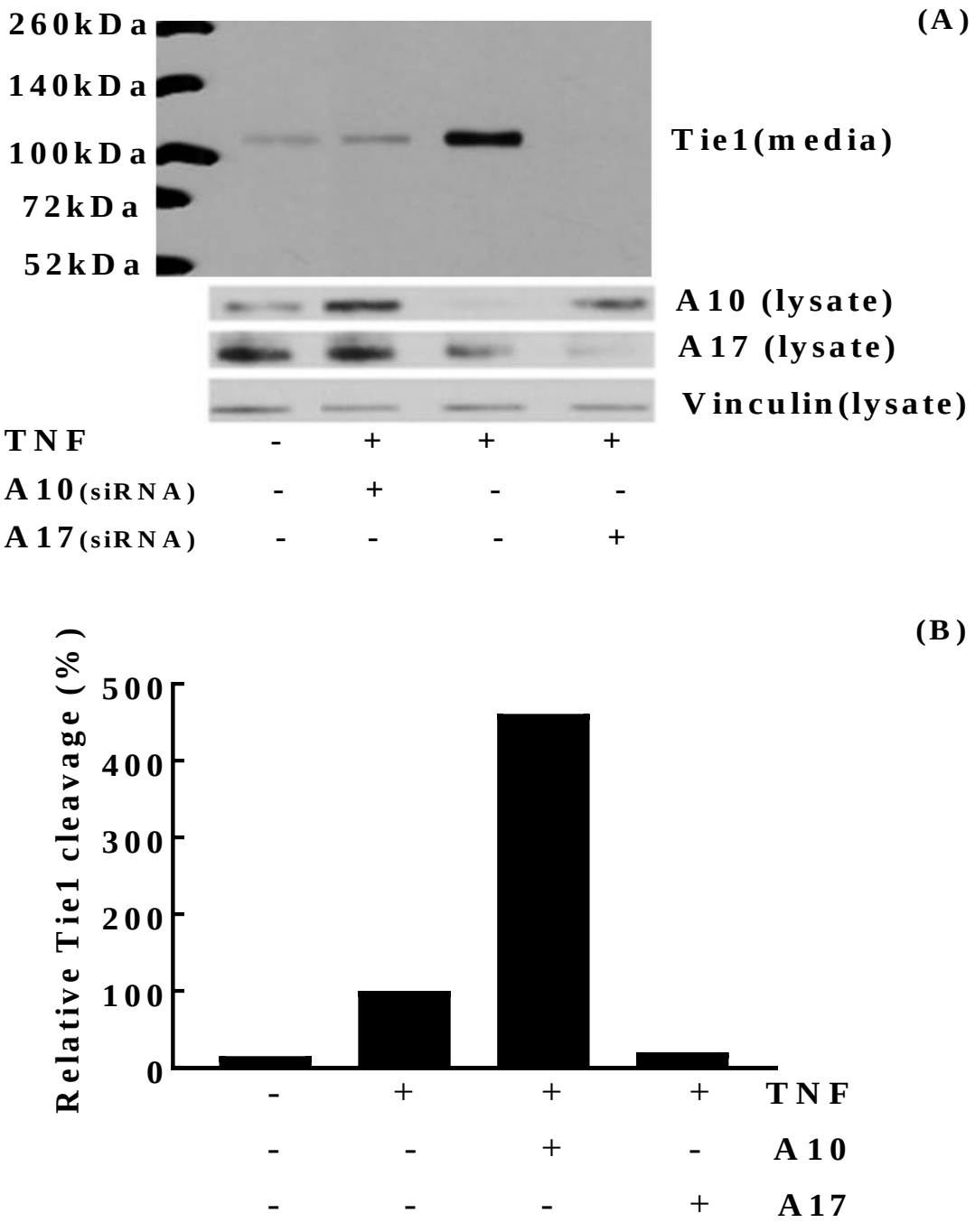


Figure 5.7: Western blot showing TNF-α activates ADAM17-mediated Tie1 cleavage in HCMECs.

Tie1 cleavage in HCMEC transfected with siRNA targeting ADAM10, ADAM17 or control siRNA, as indicated. HCMEC were stimulated as follows. C: control (no addition). TNF-α: 20/ml ng TNF-α. Figure A: Western blot showing the cleaved Tie1 domain which was detected at about 100 kDa and vinculin at 130kDa. ADAM10 and ADAM17 were detected in cell lysate. This experiment was performed once. B: Blots were quantified and data is shown from the single experiment. The blots shown from same film and same blots with intermediate trucks removed. The average suppression of ADAM10 was 92 %, ADAM17 89 % (n=1)

5.4 Ang2 induces Tie1 cleavage through Tie2 independent pathway

As previously discussed, Ang2 is known to have actions independent of Tie2. Therefore to test whether Tie2 is required for Ang2-induced Tie1 cleavage, HUVECs were transfected with control siRNA or Tie2 siRNA. Forty-eight hours post-transfection, the cells were incubated with Ang2 for one hour (as described in section: 2.11.1) and the media samples collected (as described in section: 2.10 and 2.10.1) and released Tie1 ectodomain in the media was detected by immunoblotting.

As shown in Figure 5.8, Tie2 silencing resulted in significant decreases in the Tie2 expression in HUVECs lysate. In the presence and absence of Tie2, Ang2 showed increases in Tie1 cleavage in comparison to control (Figure 5.8). Indicating that Tie2 is not required for pro-cleavage effect of Ang2. Two experiment are presented as additional experiments failed to show a good Tie2 knockdown.

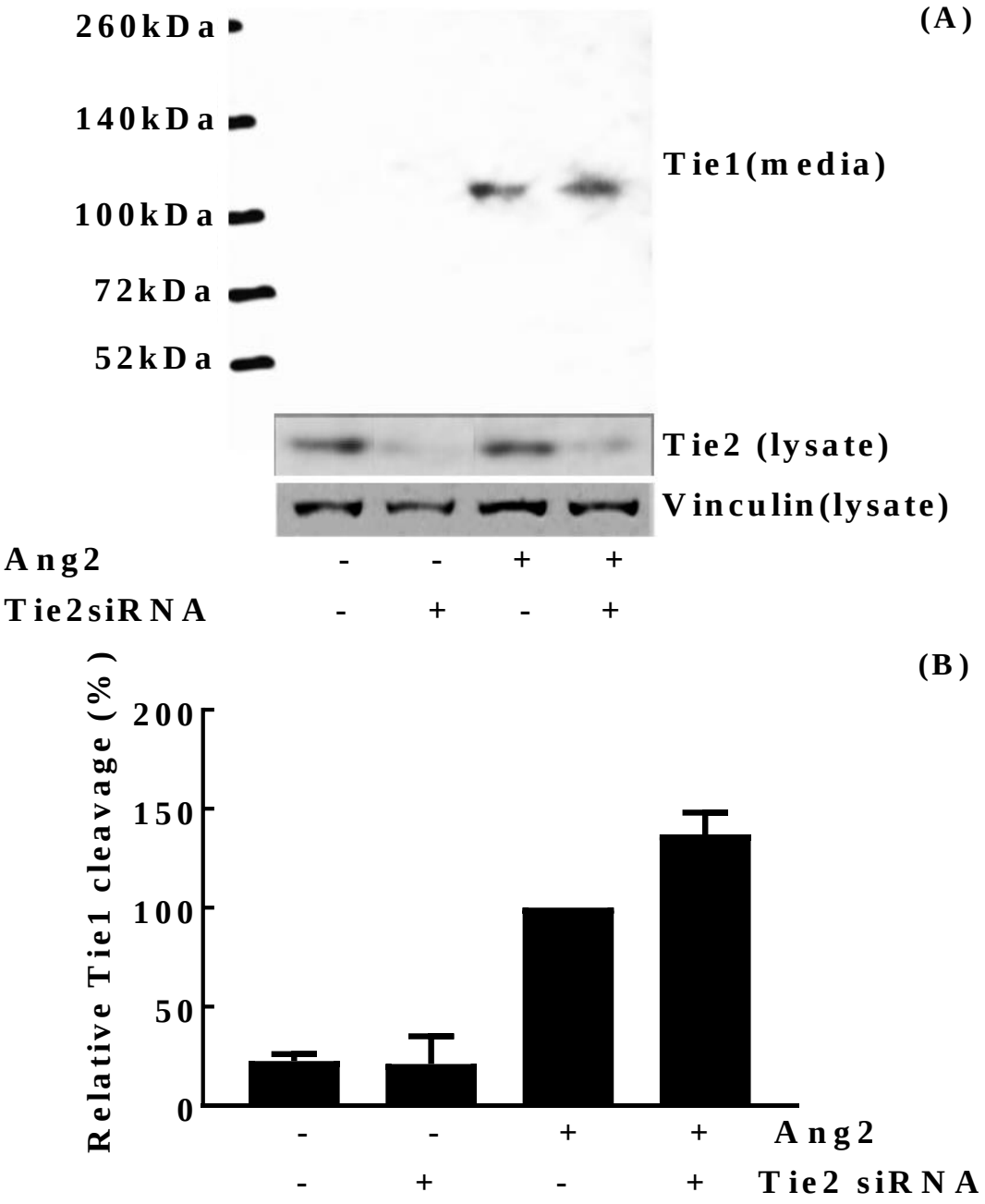


Figure 5.8: Western blot showing Ang2 stimulates Tie1 cleavage through Tie2-independent pathway in HUVECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for two independent experiments. The blots shown are from the same film with intermediate tracks removed. The average suppression of Tie2 was 76 ∓ 26 % (n=2, mean ∓ SD).

A similar experiment was performed using HCMECs in order to test whether or not Tie2 is required for the pro-cleavage effect of Ang2 in HCMECs. The cells were transfected into siRNA targeting Tie2 or control siRNA. Forty-eight hours post-transfection cells were stimulated with Ang2 and released Tie1 ectodomain into the media was detected by immunoblotting. As shown in Figure 5.9, cells transfected with Tie2 siRNA have significant decreases in Tie2 expression in HCM ECs.

In the presence of Tie2, Ang2-induced significant increases in Tie1 cleavage. In the absence of Tie2, Ang2 also induced significant increases in Tie1 cleavage indicating that Tie2 is not required for Ang2-induce Tie1 cleavage effect. This confirms the result obtained using the HUVECs and shows that Ang2-induced Tie1 cleavage in both HUVECs and HCM ECs does not require Tie2.

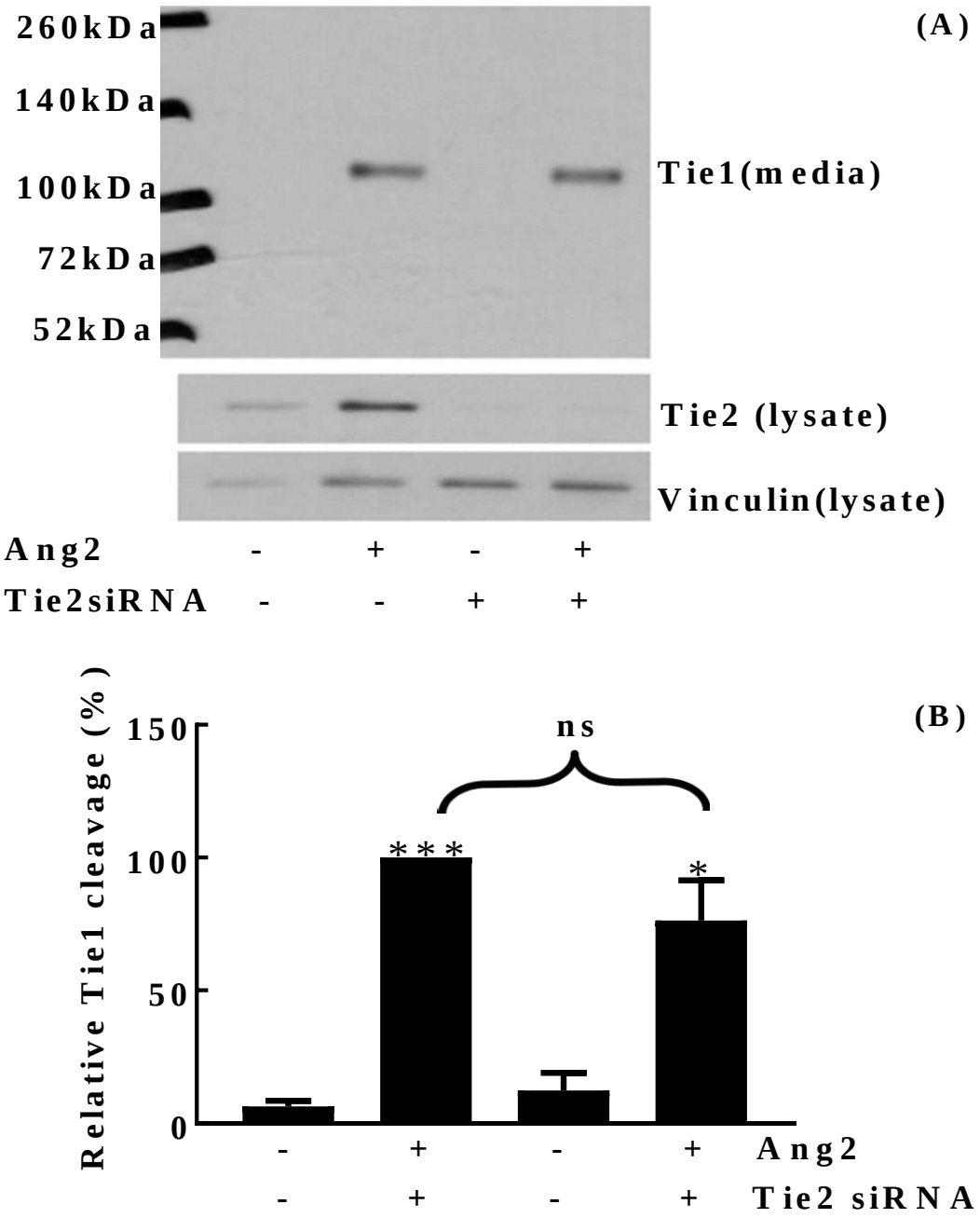


Figure 5.9: Western blot showing Ang2 induce Tie1 cleavage through a Tie2 independent pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. ***p< 0.001 for Ang2 (control siRNA) in comparison to control (control siRNA). *p< 0.05 for Ang2 (Tie2 siRNA) in comparison to control (Tie2 siRNA). ns: indicates p= 0.539 for Ang2 (control siRNA) in comparison to Ang2 (Tie2 siRNA), Tukey's post hoc test. The average suppression of Tie2 expression was 87 ∓ 9 % (n=3, mean ∓ SEM).

5.5 Roles of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in Ang2-induced Tie1 cleavage

Previous studies have shown Ang2 can act through $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to regulate some aspects of endothelilal cell function (Felcht *et al*, 2012a). As Ang2-induced Tie1 cleavage independent of Tie2, the possible roles of $\alpha_5\beta_1$and $\alpha_v\beta_3$ in Ang2 actions on Tie1 cleavage were tested.

In order to perform this test, HUVECs were used for this purpose. The cells were incubated with integrin blocking antibodies added 10-15 minutes before cell stimulation with Ang2. The cells were stimulated for one hour with Ang2 and/or TNF-α and media sample collected and loaded into SDS gel then blots probed with anti-Tie1. Vinculin showed no effect on Tie cleavage induced by Ang2, indicating releases of Tie1 ectodmain from the same number of cells.

As shown in Figure 5.10 the presence of $\alpha_v\beta_3$-integrin blocking antibody did not inhibit Ang2-induced Tie1 cleavage. Unfortuntely in the presence of $\alpha_5\beta_1$-blocking antibody Tie1 cleavage was increased even in the absence of Ang2. The reason for this is not known, it could be reflect a specific action of the antibody. However as Tie1 cleavage was increased in the presence of this antibody it was not possible to test whether blocking $\alpha_5\beta_1$ inhibited Ang2 action on Tie1 cleavage.

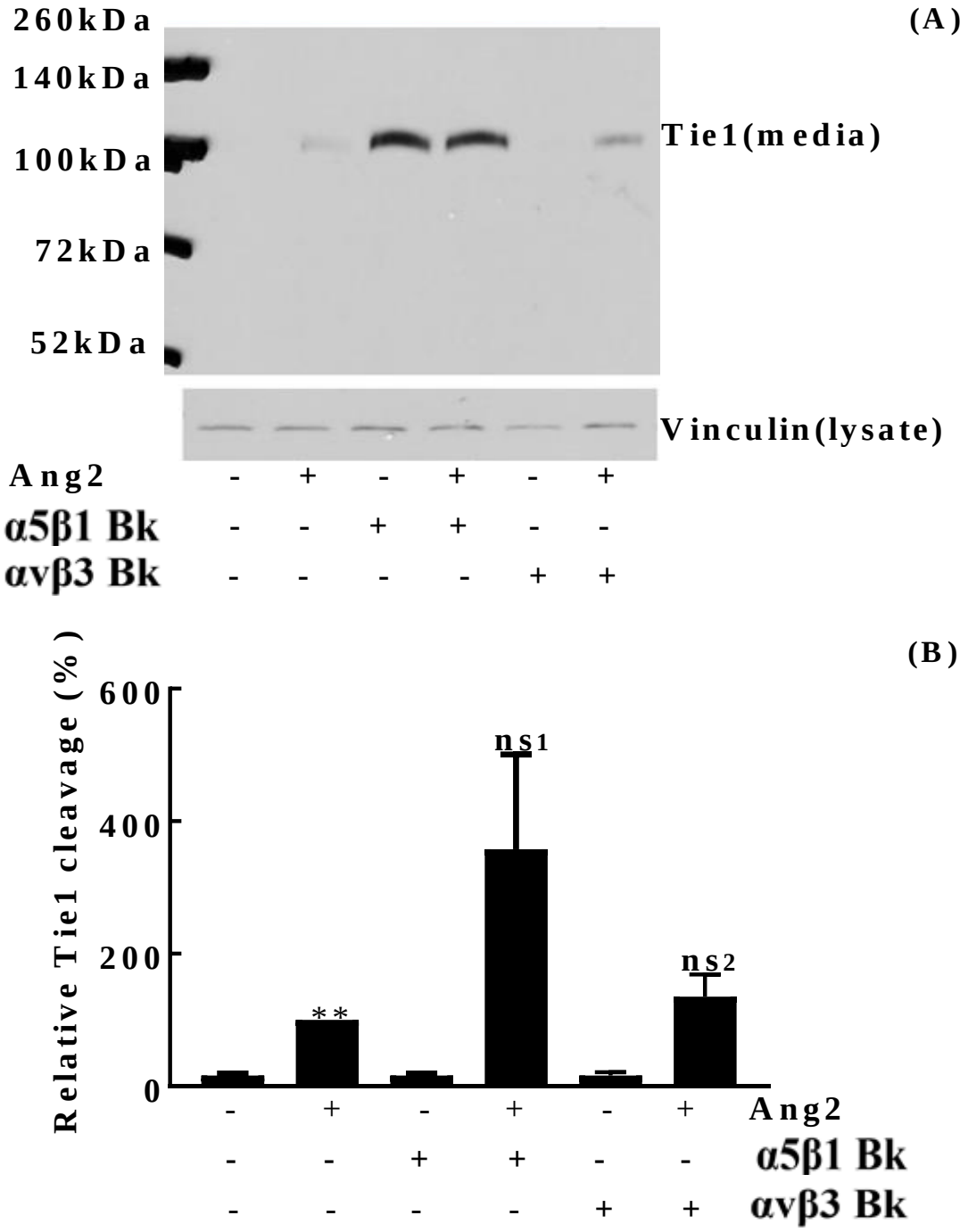


Figure 5.10: Western blot showing Ang2 induces Tie1 cleavage through integrin pathway in HUVECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker 10µg/ml cons (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. **p<0.05 for Ang2 compared control, ns1: indicates = 0.4571 for Ang2 compared Ang2 +$\alpha_5\beta_1$-Bk and ns2: indicates p= 0.751 for Ang2 compared Ang2 +$\alpha_v\beta_3$-Bk, Tukey's post hoc test.

Further experiments were performed with HCMECs to examine the effects of blocking antibodies to $\alpha_5\beta_1$and $\alpha_v\beta_3$ on Ang2–induced Tie1 cleavage. As before, cells were pre-incubated with blocking antibodies for 10-15 minutes before addition of Ang2. Tie1 cleavage was determined by immunoblotting of concentrated media taken one hour after Ang2 addition.

As showing in (Figure 5.11), there is no effects of $\alpha_v\beta_3$ antibody but with $\alpha_5\beta_1$ antibody Tie1 cleavage is increased in the cells not stimulated with Ang2. Therefore as with HUVECs it was not possible to test the role of integrin $\alpha_5\beta_1$ in Ang2-induced Tie1 cleavage using this antibody.

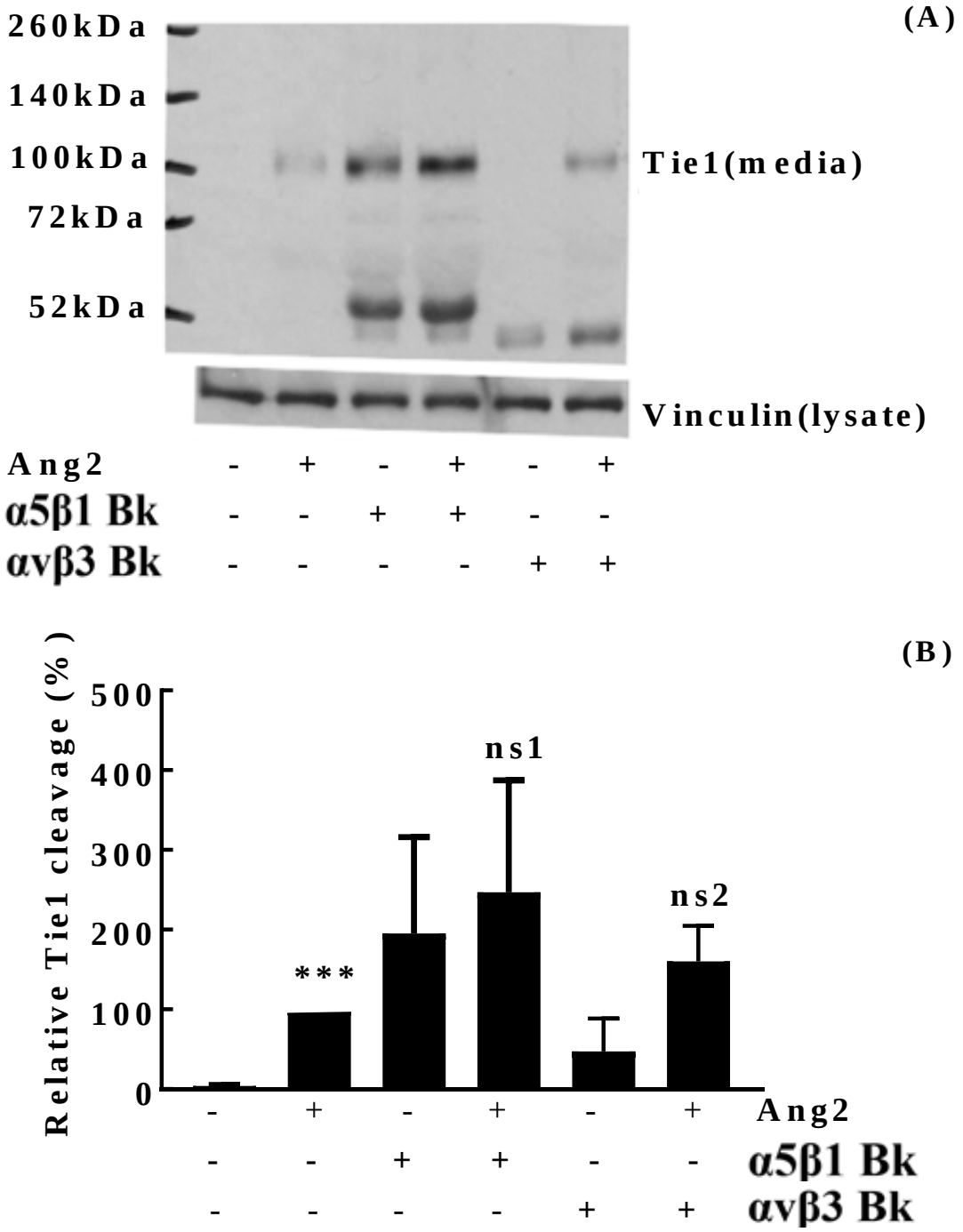


Figure 5.11: Western blot showing Ang2 induce Tie1 cleavage through integrin pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker 10µg/ml cons (A): cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysate were probed for vinculin to confirm agonists did not affect cell numbers. (B): Blots were quantified and data shown as average ∓ SEM for three independent experiments. *** $p<0.001$ for Ang2 compared control, ns1: indicates = 0.680 for Ang2 compared Ang2 +α5β1-Bk and ns2: indicates p= 0.533 for Ang2 compared Ang2 +αvβ3-Bk, Tukey's post hoc test.

As the antibody against $\alpha_5\beta_1$ integrin appeared to activate Tie1 cleavage it was not possible to use this reagent to assess the role of this integrin in Tie1 cleavage in my experments. A peptide inhibitor of $\alpha_5\beta_1$ has been described, called ATN161 (Stoeltzing *et al*, 2003a). This was tested for effects of Ang2-induced Tie1 cleavage in HCMECs. The cells were treated with ATN161 or control vehicle for 10 minutes then stimulated with Ang2 for hour. Media samples were collected and concentrated and probed for Tie1 ectodomain.

As shown in Figure 5.12, Ang2 stimulated Tie1 cleavage and this was inhibited by ATN 161. These data suggests $\alpha_5\beta_1$ integrin may have a role in Ang2-induced Tie1 cleavage. Due to a lack of time and unavailability of cells it was not possible to perform additional experiments with ATN161.

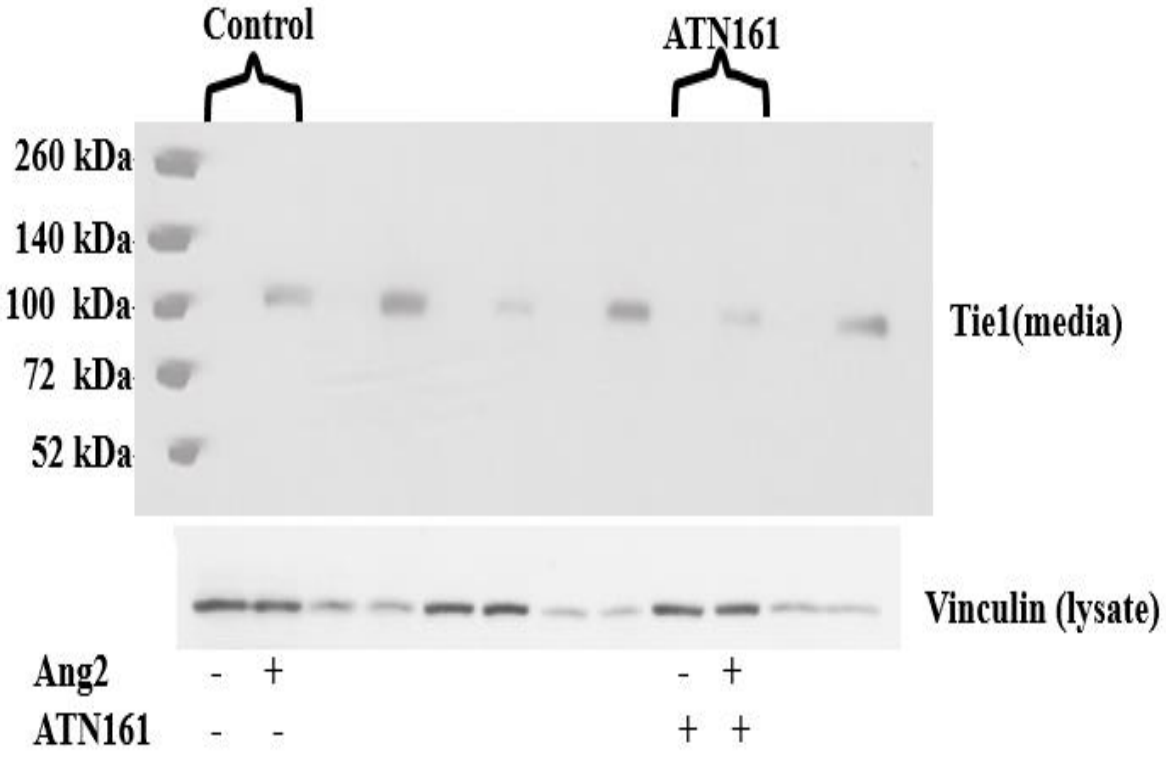


Figure 5.12: Western blot showing Ang2 induce Tie1 cleavage through integrin pathway in HCMECs.

Cells were stimulated as follows: C: control. Ang2: 0.5µg/ml Ang2. Integrin blocker (ATN161) 20µM. Cleaved Tie1 ectodomain was detected at about 100 kDa in concentrated media from cells, cell lysates were probed for vinculin to confirm agonists did not affect cell numbers. This experiment was performed twice. Complete blot shown, only the relevant tracks are indicated.

5.6 Discussion

This study found that: (1) Ang2 is a potent activator of vascular Tie1 cleavage in HUVECs and HCMECs. (2) Ang2 is required for TNF-α induced Tie1 cleavage. (3) ADAM17 is required for Tie1 cleavage. (4) Ang2 effects on Tie1 do not required Tie2. (5) Ang2 effects on Tie1 may involve integrin.

This study found for the first time that Ang2 stimulates Tie1 cleavage and that Ang2 is required for the pro-cleavage effect of TNF-α shedding of the extracellular domain of Tie1. The role of autocrine Ang2 was confirmed by Ang2 silencing to investigate whether or not TNF-α required Ang2 to mediate its effects on the cells.

The study also found that Tie2 is not required for Ang2-induced Tie1 cleavage effects, this was confirmed by Tie2 siRNA transfection when the cells was stimulated with Ang2 and/or TNF-α. Furthermore, this study found that ADAM17 regulated Tie1 shedding when the cells were induced with Ang2. This was confirmed by using ADAM10 and 17 siRNA when the cells were stimulated using Ang2 and/or TNF-α. ADAM17 silencing resulted in significant decreases in Tie1 cleavage.

Further confirmation performed by using β-Integrin blocker to test the involvement of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in the Ang2 inducing Tie1 cleavage pathway. Using the $\alpha_v\beta_3$-integrin blocker confirmed that $\alpha_v\beta_3$ is not involved in the Ang2-induced Tie1 cleavage. On the other hand using $\alpha_5\beta_1$-integrin blocker did not confirm nor exclude the involvement of $\alpha_5\beta_1$ in Ang2-induced Tie1 cleavage. However, additional experiments using ATN161 showed that the $\alpha_5\beta_1$ may be invoved in the Ang2-induced Tie1 cleavage, suggesting further investigation is required in order to confirm this.

The Tie/Ang system plays a crucial role in regulating vascular stability during normal and pathological conditions. Several studies refer to the crucial role of Tie1 as a part of this system that contribute in maintaining vascular stability (Jones *et al*, 2001). In confluent quiescent cells Tie1 enhances the agonistic effects of Ang1 and Ang2 (Kim *et al*, 2016; Korhonen *et al*, 2016). While in subconfluent (growing cells), Tie1 inhibits actions of Ang1 (Marron *et al*, 2007; Felcht *et al*, 2012; Hansen *et al*, 2010). Tie1 plays a crucial role in regulating Tie2 action allowing maintenance or regression of vascular endothelial cells (Savant *et al*, 2015; Loos, 2013). The study of (Loos, 2013) demonstrated that Tie1 may interfere with EC apoptosis, migration and proliferation signalling through an unknown mechanism and suggested Tie2 activation as a possible mechanism (Loos, 2013). Previous data demonstrated the importance of Tie1 as a part of the Tie-Ang system. The study showed clearly that the extracellular domain of Tie1 has the ability to regulate the Ang-Tie2 binding and activation (Marron *et al*, 2007). Tie1 cleavage converts the action of Ang2 from weak agonist to an antagonist, this induces vascular regression effects by antagonising the protective effect resulting from Ang1-Tie2 binding (Kim *et al*, 2016; Marron *et al*, 2007).

Tie1 cleavage might potentially influence vascular stability by changing the weak agonist effect of Ang2 into an antagonist in quiescent endothelium. Several groups show that TNF-α induces Tie1 ecctodomain cleavage. During normal quiescent conditions, Ang2 provides agonistic effects similar to Ang1. This maintains the vascular endothelial cells in homeostasis and when TNF-α induces Tie1 cleavage this promotes the Ang2 antagonist effects by stimulating Tie1 cleavage resulting in blocking of Ang1-Tie2 binding (Kim *et al*, 2016). Furthermore, (Korhonen *et al*, 2016) study demonstrated that during inflammatory conditions, the Tie1 ectodomain regulates the interaction with Tie2. Tie1 ectodomain shedding resulted in decreased Tie2 phosphorylation, Tie2 and Ang1 expression, switches Ang2 from weak agonist to an antagonists and also increased Ang2 expression which promotes vascular instability (Korhonen *et al*, 2016). (Ogura *et al*, 2017) refer to the effects of TNF-α induced Tie1 cleavage in a diabetic retinopathy animal model with pericyte-free endothelial cells. The study demonstrated that Tie1 shedding allow changing the agonistic effect of Ang2 to an antagonist when binding to Tie2. This can induce vascular endothelial regression characterised by increased permeability, hypoperfusion and neoangiogenesis (Ogura *et al*, 2017). The current study found that

Ang2 is required for the effect of TNF-α on Tie1 cleavage. Suggesting that the TNF-α effects reported by previous studies are likely to require autocrine Ang2.

Both ADAM10 and 17 play an important role in mediating cancer and cardiovascular diseases (Caiazza *et al*, 2015; Donners *et al*, 2010; Hartmann *et al*, 2002; Wu *et al*, 1997). Extracellular domain cleavage of many tyrosine kinase receptors is mediated by ADAM10 and/or ADAM17 (Kreitman *et al*, 2018). Although several studies refer to the possibility of metalloproteases involvement in Tie1 cleavage, none specify the ADAMs responsible for Tie1 cleavage. The study of (Yabkowitz *et al*, 1999) demonstrated that in the presence of TNF-α, Tie1 cleavage was induced as a result of endothelial metalloprotease activation (Yabkowitz *et al*, 1999). The study of (McCarthy *et al*, 1999) demonstrated that endothelial activation of protein kinase C triggers Tie1 ectodomain shedding as a result of Tie1 endoproteolytic cleavage (McCarthy *et al*, 1999). Other data reported that increased endothelial shear stress induced Tie1 ectodomain shedding via increased activity of metalloprotease (Chen-Konak *et al*, 2003). The data of the current study showed that Tie1 cleavage requires ADAM17 when the cells are stimulated by Ang2 to induce Tie1 cleavage in HCMECs. Experiments in HUVECs were inconclusive as ADAM10 and ADAM17 expression was unable to be completely suppressed. Additional work will be required to test the roles of these ADAMs in HUVECs.

In the present study, using siRNA that targets Tie2, it was found that Tie2 is not required for the action of Ang2 on Tie1 cleavage. Recent studies reported that Ang2 can also bind to integrins such as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Yun *et al*, 2017; Imanishi *et al*, 2007; Felcht *et al*, 2012). Therefore this study investigated the possibility of integrin involvement in Ang2-induced Tie1 cleavage. The current study found that using $\alpha_5\beta_1$ blocking reagents increased stimulation of Tie1 cleavage even in the absence of Ang2, this makes it difficult to asses the involvement of $\alpha_5\beta_1$ in Ang2-induced Tie1 cleavage. One possible reason for that was using this blocking antibody clusters $\alpha_5\beta_1$ receptor and causes signalling by the integrin, as these integrins start signalling when they come together. On the other hand, using another blocker called the ATN161 integrin blocker in the current study suggested that, $\alpha_5\beta_1$ may be involved in the Ang2-induced Tie1 cleavage. However, additional experiments are required to confirm these results.

Although this study found Ang2-induced release of Tie1 ectodomain through activating ADAM17 in HCMECs, further investigation is required to determine the alternative receptor through which Ang2 meditates Tie1 cleavage. This will help in identifying the mechanism by which Ang2 mediates its vascular regression effect. Also, it may facilitate the identification of therapeutic agents to inhibit the pathogenic effects of Ang2 mediated through different receptors, Tie2 and integrins.

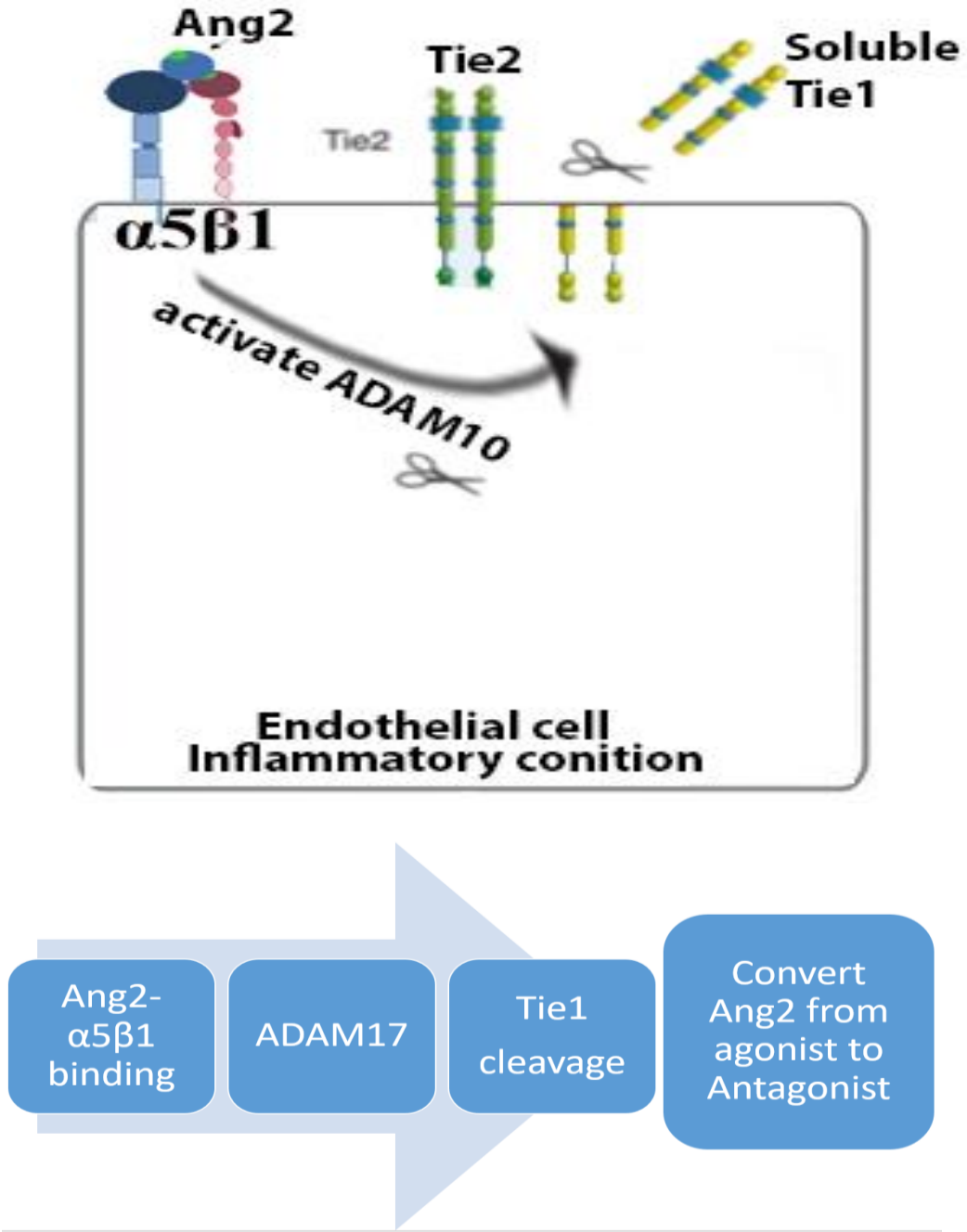


Figure 5.13: Summary of proposed effects of Ang2 on Tie1 cleavage through integrins

Ang2-α5β1 binding during endothelial inflammatory condition enhances Tie1 cleavage by ADAM17, through unknown mechansims and results in changing Ang2 agonist effect to antagonist.

Chapter 6: Discussion

Ang/Tie2 signalling plays a crucial role in regulating vasculature system during homeostasis and diseases. Angiopoietin-2 is a vascular growth factor that binds to the Tie2 receptor and is expressed mainly in endothelial cells. Several studies demonstrate the destabilizing effects of Ang2 in the vascular system of the human body. On the other hand, Ang1 has a stabilizing effect necessary for maintaining the integrity of the vessels. Increased levels of Ang2 can block the binding of the Ang1 to the Tie2 receptor and stimulate pathogenic signalling through a β-integrin pathway. These effects can promote vessel destabilization through the induction of apoptosis, inflammation, and leakage.

The aim of the work described in chapter three was to improve the affinity of an Ang2 ligand-trap that could be used as a therapy to reduce the adverse effects resulting from Ang2 acting through Tie2. This was approached by: (1) Introducing additional changes into the ectodomain by performing further mutagenesis. Additional mutant forms of Tie2 were identified. They demonstrated improved binding to Ang2 whilst mantiaining selectivity. These mutants showed 2-4 fold improved affinity toword Ang2 and remained selective as it did not bind to Ang1. This suggests higher Ang2 affinity than R3 which could produce better therapeutic in the future. (2) Engineering a pentameric molecule able to trap Ang2 and reduce its adverse effects when its levels are high.

The pentameric version of R3 increased affinity 18-fold for Ang2 but also produced some binding to Ang1. While still selective to Ang2, the level of Ang1 binding would not be desired for therapeutics as it could interfere with the protective effects of Ang1. It is intresting that the pentameric form of R3 is able to bind Ang1 though at a much lower level than it binds to Ang2. This suggests that R3 has a low binding activity for Ang1 that only becomes evident when binding ability of R3 is uniformily increased by pentamerization.

The ability of R3 and the newly identified mutant to selectively bind and block Ang2 actions through Tie2 is likely to have therapeutic benefit in blocking the effects of Ang2 that inhibit Ang1 protection via Tie2. However, Ang2 has recently been found to also

signal through integrins. Therfore in chapter 4 and 5 the possible effects of Ang2 acting independently of Tie2 to regulate VE-cadherin cleavage and Tie1 cleavage were investigated.

This study successfully improved the affinity of the R3-Fc through engineering the penatmeric R3 which resulted in 18-fold improvement in the relative affinity of Ang2. Although the oligomeric R3 can not be used for therapeutic purposes, as the COMP design increases the relative binding affinity of R3 to Ang2 as well as Ang1, this study shows a successful new approach to improve binding affinity.

VE-cadherin is one of the junctional proteins that controls the permeability of endothelial monolayers. Several studies demonstrated the crucial role of VE-cadherin in maintaining the endothelial junction in a tight state. VE-cadherin ectodomain shedding could result in junctional disruption and increase vascular permeability (Flemming *et al*, 2015; Sawant *et al*, 2011; Karki *et al*, 2018; Navaratna *et al*, 2007). Increased vascular permeability could contribute to cardiovascular diseases (Weis, 2008). Previous data demonstrated that high levels of TNF-α were associated with vascular permeability partly through inducing VE-cadherin cleavage (Flemming *et al*, 2015). Therefore, this study investigated the possibility of Ang2-induced pro-cleavage effect mediated through Tie2 independent pathways.

The data showed that Ang2-induced VE-cadherin cleavage through a Tie2 independent pathway, specifically through $\alpha_5\beta_1$ integrin and by regulating ADAM10. The data also demonstrated that Ang2 mediates the effects of TNF-α on VE-cadherin cleavage. This might have a potential therapeutic implications as it gives new insight about the mechanism by which TNF-α stimulation causes VE-cadherin cleavage and also suggests that the TNF-α regression effects reported by previous studies may be mediated by Ang2. The current study also found that Ang2-induced VE-cadherin cleavage in HUVECs and HCMECs. This indicates that Ang2 induces the same pro-cleavage effect in primary endothelial cells. This also indicates that the Ang2 pro-cleavage is regulated in the same way in different endothelial cell types.

The finding that Ang2 stimulation of VE-cadherin cleavage is inhibited by blocking $\alpha_5\beta_1$ integrin suggests targeting this integrin may be of therapeutic value to inhibit pro-permiability effects of TNF-α and Ang2. Indeed Hakanpaa has recently shown that blocking $\alpha_5\beta_1$ decreases vascular leakage (Hakanpaa *et al*, 2018).

Chapter 5 examines the possibility of Ang2 inducing Tie1 cleavage. Tie1 is an orphan receptor that regulates the Ang/Tie2 signalling pathway. Ang1 provides the endothelial cells with protective effects required for cell survival. Ang2 has context dependant effects, it has a weak agonist effect which supports Ang1 effects during normal conditions. This agonist effect is changed to an antagonist effects during inflammatory conditions as a result of Tie1 ectodomain cleavage (Mueller and Kontos, 2016). Tie1 shedding during inflammatory conditions could result in vascular regression through reducing Ang1 protective effects and by supporting Ang2 antagonist effects which can leads to several vascular pathogenesis (Korhonen *et al*, 2016).

The present study found that Ang2-induced Tie1 cleavage. Thus it is possible that in diseases in which Ang2 is increased, Tie1 cleavage is stimulated causing Ang2 to act as an antagonist thus contributing to vessel destabilization. The data also shows that TNF-α induced Tie1 cleavage required Ang2. This suggests that the TNF-α effects on Tie1 reported by earlier studies may require Ang2. Using Tie2 siRNA it was found that the pro-cleavage effects of Ang2 on Tie1 were due to Ang2 acting through a Tie2 independent pathway.

Attempts to examine the role of integrin in Ang2 effects using blocking antibodies were inconclusive as the $\alpha_5\beta_1$ blocking antibody itself apparently increased Tie1 cleavage. The reason for this is not known. However it is possible that as this antibody is bivalent and it binds $\alpha_5\beta_1$ integrin, it could cause some clustering of this integrin. This clustering could itself activate integrin signalling and this may stimulate Tie1 cleavage. Additional experiments will need to be performed to test this hypothesis. For example using monomeric Fab fragment of the $\alpha_5\beta_1$ antibody and testing whether dimerization of these targets leads to Tie1 cleavage. Towards the end of this work, the $\alpha_5\beta_1$ inhibitor ATN161 was tested for its ability to block Ang2-induced Tie1 cleavage. This inhibitor did block

Ang2-induced Tie1 cleavage, however more experiments will be needed to confirm this. Such experiments would include testing different concentrations of the peptide inhibitor.

The work reported in this thesis describes high affinity forms of a selective inhibitors of Ang2 binding to cellular Tie2. In addition, the ability of integrin $\alpha_5\beta_1$ blockade to inhibit Ang2 action on VE-cadherin cleavge and possibly Tie1 cleavage. Taken together these data suggests Ang2 action could be inhibited by combined use of the R3 ligand and its derivative, to block antagonistic effects of Ang2 through Tie2, together with $\alpha_5\beta_1$ inhibitors to block effects of Ang2 through integrins. It is possible that blocking Ang2 antagonism of Tie2 alone could be sufficient to also block Ang2 actions through integrins as other have reported Ang2 action through integrin can be blocked by Ang1 in mice (Korhonen *et al*, 2016a; Kim *et al*, 2016). Thus prevention of high Ang2 in antagonising Ang1 action at Tie2 by using an Ang2 ligand-trap may also prevent Ang2 actions through integrins. This should be tested in future work.

6.1 Future work

Additional work will be required to confirm some aspects of the present study, as well as to extend the work to develop strategies to suppress Ang2 pathogenic effects *in vivo*. Specifically, the $\alpha_5\beta_1$ inhibitor ATN161 was tested for its ability to block Ang2-induced Tie1 cleavage, however additional experiments will need to confirm the inhibitory effect of ATN161 using different concentrations of this peptide inhibitor., Furthermore, in the present study the effects of VE-cadherin cleavage on permeability have not been directly examined. Therefore, additional experiments are required to test whether or not there is a relationship between VE-cadherin cleavage and changes in endothelial permeability. Also, experiments could be performed to test whether additional junctional proteins such as claudin-5 or JAM protein are affected by Ang2 and cleaved by ADAM10.

Ultimately, the aim of this work is to develop strategies to inhibit pathogenic Ang2 effects in vivo. It is possible that binding of the Ang2 ligand-trap may be sufficient to inhibit Ang2 effects via both Tie2 and integrins, for example if the trap sterically hinders Ang2 binding to integrin. This will need to be examined in future work. If the trap is only able to block Tie2-mediated effects of Ang2 it is likely that combined use of the trap and an integrin blocker such as ATN161 will be required to suppress all pathogenic actions of Ang2. Once these experiments have been completed it will be necessary to examine the ability of the ligand-trap, with and without ATN161, on Ang2 pathogenic effects *in vivo*, using animal disease models.

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Appendix

Appendix (1)

DNA sequence - Tie2 receptor – obtained from PubMed (Accession NM_000459)

```
   1 agtttcccgc ctatgagagg ataccctat tgtttctgaa aatgctgacc gggacccaca
  61 cttccaacaa aaattcctct gcccctacag cagcagcaaa agcagcagca gaagcaacag
 121 caacagataa gtgttttgat gaattgcgag atggataggg cttgagtgcc cccagccctg
 181 ctgataccaa atgcctttaa gatacagcct ttcccatcct aatctacaaa ggaaacagga
 241 aaaaggaact taaaactccc tgtgctcaga cagaaatgag actgttacag cctgcttctg
 301 tgctgttcct tcttgcctct aacttgtaaa caagacgtag taggacgatg ctaatggaaa
 361 gtcacaaacc gctgggtttt tgaaaggatc cttgggacct catgcacatt tgtggaaact
 421 ggatggagag atttggggaa gcatggactc tttagccagc ttagttctct gtggagtcag
 481 cttgctcctt tctggaactg tggaaggtgc catggacttg atcttgatca attccctacc
 541 tcttgtatct gatgctgaaa catctctcac ctgcattgcc tctgggtggc gcccccatga
 601 gcccatcacc ataggaaggg actttgaagc cttaatgaac cagcaccagg atccgctgga
 661 agttactcaa gatgtgacca gagaatgggc taaaaaagtt gtttggaaga gagaaaaggc
 721 tagtaagatc aatggtgctt atttctgtga agggcgagtt cgaggagagg caatcaggat
 781 acgaaccatg aagatgcgtc aacaagcttc cttcctacca gctactttaa ctatgactgt
 841 ggacaaggga gataacgtga acatatcttt caaaaaggta ttgattaaag aagaagatgc
 901 agtgatttac aaaaatggtt ccttcatcca ttcagtgccc cggcatgaag tacctgatat
 961 tctagaagta cacctgcctc atgctcagcc ccaggatgct ggagtgtact cggccaggta
1021 tataggagga aacctcttca cctcggcctt caccaggctg atagtccgga gatgtgaagc
1081 ccagaagtgg ggacctgaat gcaaccatct ctgtactgct tgtatgaaca atggtgtctg
1141 ccatgaagat actggagaat gcatttgccc tcctgggttt atgggaagga cgtgtgagaa
1201 ggcttgtgaa ctgcacacgt ttggcagaac ttgtaaagaa aggtgcagtg gacaagaggg
1261 atgcaagtct tatgtgttct gtctccctga cccctatggg tgttcctgtg ccacaggctg
1321 gaagggtctg cagtgcaatg aagcatgcca ccctggtttt tacgggccag attgtaagct
1381 taggtgcagc tgcaacaatg gggagatgtg tgatcgcttc caaggatgtc tctgctctcc
1441 aggatggcag gggctccagt gtgagagaga aggcataccg aggatgaccc caaagatagt
```

1501 ggatttgcca gatcatatag aagtaaacag tggtaaattt aatcccattt gcaaagcttc

1561 tggctggccg ctacctacta atgaagaaat gaccctggtg aagccggatg ggacagtgct

1621 ccatccaaaa gactttaacc atacggatca tttctcagta gccatattca ccatccaccg

1681 gatcctcccc cctgactcag gagtttgggt ctgcagtgtg aacacagtgg ctgggatggt

1741 ggaaaagccc ttcaacattt ctgttaaagt tcttccaaag cccctgaatg ccccaaacgt

1801 gattgacact ggacataact ttgctgtcat caacatcagc tctgagcctt actttgggga

1861 tggaccaatc aaatccaaga agcttctata caaacccgtt aatcactatg aggcttggca

1921 acatattcaa gtgacaaatg agattgttac actcaactat ttggaacctc ggacagaata

1981 tgaactctgt gtgcaactgg tccgtcgtgg agagggtggg gaagggcatc ctggacctgt

2041 gagacgcttc acaacagctt ctatcggact ccctcctcca agaggtctaa atctcctgcc

2101 taaaagtcag accactctaa atttgacctg gcaaccaata tttccaagct cggaagatga

2161 cttttatgtt gaagtggaga gaaggtctgt gcaaaaaagt gatcagcaga atattaaagt

2221 tccaggcaac ttgacttcgg tgctacttaa caacttacat cccagggagc agtacgtggt

2281 ccgagctaga gtcaacacca aggcccaggg ggaatggagt gaagatctca ctgcttggac

2341 ccttagtgac attcttcctc ctcaaccaga aaacatcaag atttccaaca ttacacactc

2401 ctcagctgtg atttcttgga caatattgga tggctattct atttcttcta ttactatccg

2461 ttacaaggtt caaggcaaga atgaagacca gcacgttgat gtgaagataa agaatgccac

2521 catcactcag tatcagctca agggcctaga gcctgaaaca gcataccagg tggacatttt

2581 tgcagagaac aacatagggt caagcaaccc agccttttct catgaactgg tgaccctccc

2641 agaatctcaa gcaccagcgg acctcggagg ggggaagatg ctgcttatag ccatccttgg

2701 ctctgctgga atgacctgcc tgactgtgct gttggccttt ctgatcatat tgcaattgaa

2761 gagggcaaat gtgcaaagga gaatggccca agccttccaa aacgtgaggg aagaaccagc

2821 tgtgcagttc aactcaggga ctctggccct aaacaggaag gtcaaaaaca acccagatcc

2881 tacaatttat ccagtgcttg actggaatga catcaaattt caagatgtga ttggggaggg

2941 caattttggc caagttctta aggcgcgcat caagaaggat gggttacgga tggatgctgc

3001 catcaaaaga atgaaagaat atgcctccaa agatgatcac agggactttg caggagaact

3061 ggaagttctt tgtaaacttg gacaccatcc aaacatcatc aatctcttag gagcatgtga

3121 acatcgaggc tacttgtacc tggccattga gtacgcgccc catggaaacc ttctggactt

3181 ccttcgcaag agccgtgtgc tggagacgga cccagcattt gccattgcca atagcaccgc

3241 gtccacactg tcctcccagc agctccttca cttcgctgcc gacgtggccc ggggcatgga

3301 ctacttgagc caaaaacagt ttatccacag ggatctggct gccagaaaca ttttagttgg

3361 tgaaaactat gtggcaaaaa tagcagattt tggattgtcc cgaggtcaag aggtgtatgt

3421 gaaaaagaca atgggaaggc tcccagtgcg ctggatggcc atcgagtcac tgaattacag

3481 tgtgtacaca accaacagtg atgtatggtc ctatggtgtg ttactatggg agattgttag

3541 cttaggaggc acaccctact gcgggatgac ttgtgcagaa ctctacgaga agctgcccca

3601 gggctacaga ctggagaagc ccctgaactg tgatgatgag gtgtatgatc taatgagaca

3661 atgctggcgg gagaagcctt atgagaggcc atcatttgcc cagatattgg tgtccttaaa

3721 cagaatgtta gaggagcgaa agacctacgt gaataccacg ctttatgaga agtttactta

3781 tgcaggaatt gactgttctg ctgaagaagc ggcctaggac agaacatctg tataccctct

3841 gtttcccttt cactggcatg ggagaccctt gacacctgct gagaaaacat gcctctgcca

3901 aaggatgtga tatataagtg tacatatgtg ctgtacacct gggaccttca ccactgtaga

3961 tcccatgcat ggatctatgt agtatgctct gactctaata ggactgtata tactgtttta

4021 agaatgggct gaaatcagaa tgcctgtttg tggtttcata tgcaataata tatttttta

4081 aaaatgtgga cttcatagga aggcgtgagt acaattagta taatgcataa ctcattgttg

4141 tcctagatat tttgatattt acctttatgt tgaatgctat taaatgtttt cctgtgtcaa

4201 agtaaaatat tgttaataaa cctaacaatg accctgatag tacaggttaa gtgagagaac

4261 tatatgaatt ctaacaagtc ataggttaat atttaagaca ctgaaaaatc taagtgatat

4321 aaatcagatt cttctctctc aattttatcc ctcacctgta gcagccagtc ccgtttcatt

4381 tagtcatgtg accactctgt cttgtgtttc cacagcctgc aagtcagtcc aggatgctaa

4441 catctaaaaa tagacttaaa tctcattgct tacaagccta agaatcttta gagaagtata

4501 cataagttta ggataaaata atgggatttt cttttctttt ctctggtaat attgacttgt

4561 atattttaag aaataacaga aagcctgggt gacatttggg agacatgtga catttatata

4621 ttgaattaat atccctacat gtattgcaca ttgtaaaaag ttttagtttt gatgagttgt

4681 gagtttacct tgtatactgt aggcacactt tgcactgata tatcatgagt gaataaatgt

4741 cttgcctact cacgtctcaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa

4801 aaaaaaaaaa aaaaaaa

Appendix (2)

DNA sequence - extracellular domain – (residues 1-442) – obtained from PubMed

```
                atggactc tttagccagc ttagttctct gtggagtcag

 481 cttgctcctt tctggaactg tggaaggtgc catggacttg atcttgatca attccctacc

 541 tcttgtatct gatgctgaaa catctctcac ctgcattgcc tctgggtggc gcccccatga

 601 gcccatcacc ataggaaggg actttgaagc cttaatgaac cagcaccagg atccgctgga

 661 agttactcaa gatgtgacca gagaatgggc taaaaaagtt gtttggaaga gagaaaaggc

 721 tagtaagatc aatggtgctt atttctgtga agggcgagtt cgaggagagg caatcaggat

 781 acgaaccatg aagatgcgtc aacaagcttc cttcctacca gctactttaa ctatgactgt

 841 ggacaaggga gataacgtga acatatcttt caaaaaggta ttgattaaag aagaagatgc

 901 agtgatttac aaaaatggtt ccttcatcca ttcagtgccc cggcatgaag tacctgatat

 961 tctagaagta cacctgcctc atgctcagcc ccaggatgct ggagtgtact cggccaggta

1021 tataggagga aacctcttca cctcggcctt caccaggctg atagtccgga gatgtgaagc

1081 ccagaagtgg ggacctgaat gcaaccatct ctgtactgct tgtatgaaca atggtgtctg

1141 ccatgaagat actggagaat gcatttgccc tcctgggttt atgggaagga cgtgtgagaa

1201 ggcttgtgaa ctgcacacgt ttggcagaac ttgtaaagaa aggtgcagtg gacaagaggg

1261 atgcaagtct tatgtgttct gtctccctga cccctatggg tgttcctgtg ccacaggctg

1321 gaagggtctg cagtgcaatg aagcatgcca ccctggtttt tacgggccag attgtaagct

1381 taggtgcagc tgcaacaatg gggagatgtg tgatcgcttc caaggatgtc tctgctctcc

1441 aggatggcag gggctccagt gtgagagaga aggcataccg aggatgaccc caaagatagt

1501 ggatttgcca gatcatatag aagtaaacag tggtaaattt aatcccattt gcaaagcttc

1561 tggctggccg ctacctacta atgaagaaat gaccctggtg aagccggatg ggacagtgct

1621 ccatccaaaa gactttaacc atacggatca tttctcagta gccatattca ccatccaccg

1681 gatcctcccc cctgactcag gagtttgggt ctgcagtgtg aacacagtgg ctgggatggt

1741 ggaaaagccc ttcaacattt ctgttaaagt
```

Translation of DNA sequence - extracellular domain – residues 1-442 – obtained from PubMed

MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPI
TIGRDFEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRV
RGEAIRIRTMKMRQQASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNG
SFIHSVPRHEVPDILEVHLPHAQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEA
QKWGPECNHLCTACMNNGVCHEDTGECICPPGFMGRTCEKACELHTFGRTC
KERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNEACHPGFYGPDCKLRCS
CNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIEVNSGKFNPI
CKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSGVW
VCSVNTVAGMVEKPFNISVK

Number of amino acids: 442
Molecular weight: 48962.3

Appendix (3)

Wild type ectodomain (Tie2 Ig Fc-His-tag)

ATGGACTCTTTAGCCAGCTTAGTTCTCTGTGGAGTCAGCTTGCTCCTTTCTGGAACTGTGGAAG
GTGCCATGGACTTGATCTTGATCAATTCCCTACCTCTTGTATCTGATGCTGAAACATCTCTCAC
CTGCATTGCCTCTGGGTGGCGCCCCCATGAGCCCATCACCATAGGAAGGGACTTTGAAGCCTT
AATGAACCAGCACCAGGATCCGCTGGAAGTTACTCAAGATGTGACCAGAGAATGGGCTAAAA
AAGTTGTTTGGAAGAGAGAAAAGGCTAGTAAGATCAATGGTGCTTATTTCTGTGAAGGGCGA
GTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAAGCTTCCTTCCTACC
AGCTACTTTAACTATGACTGTGGACAAGGGAGATAACGTGAACATATCTTTCAAAAAGGTATT
GATTAAAGAAGAAGATGCAGTGATTTACAAAAATGGTTCCTTCATCCATTCAGTGCCCCGGCA
TGAAGTACCTGATATTCTAGAAGTACACCTGCCTCATGCTCAGCCCCAGGATGCTGGAGTGTA
CTCGGCCAGGTATATAGGAGGAAACCTCTTCACCTCGGCCTTCACCAGGCTGATAGTCCGGAG
ATGTGAAGCCCAGAAGTGGGGACCTGAATGCAACCATCTCTGTACTGCTTGTATGAACAATGG
TGTCTGCCATGAAGATACTGGAGAATGCATTTGCCCTCCTGGGTTTATGGGAAGGACGTGTGA
GAAGGCTTGTGAACTGCACACGTTTGGCAGAACTTGTAAAGAAAGGTGCAGTGGACAAGAGG
GATGCAAGTCTTATGTGTTCTGTCTCCCTGACCCCTATGGGTGTTCCTGTGCCACAGGCTGGAA
GGGTCTGCAGTGCAATGAAGCATGCCACCCTGGTTTTTACGGGCCAGATTGTAAGCTTAGGTG
CAGCTGCAACAATGGGGAGATGTGTGATCGCTTCCAAGGATGTCTCTGCTCTCCAGGATGGCA
GGGGCTCCAGTGTGAGAGAGAAGGCATACCGAGGATGACCCCAAAGATAGTGGATTTGCCAG
ATCATATAGAAGTAAACAGTGGTAAATTTAATCCCATTTGCAAAGCTTCTGGCTGGCCGCTAC
CTACTAATGAAGAAATGACCCTGGTGAAGCCGGATGGGACAGTGCTCCATCCAAAAGACTTT
AACCATACGGATCATTTCTCAGTAGCCATATTCACCATCCACCGGATCCTCCCCCCTGACTCAG
GAGTTTGGGTCTGCAGTGTGAACACAGTGGCTGGGATGGTGGAAAAGCCCTTCAACATTTCTG
TTAAGAATTCTGGTACCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGG
GGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCC
CTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGG
TACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACA
GCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGT
ACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAA
GAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTG
GGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACG
GCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCT
TCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGT
CTCCGGGTAAAGGCAGCGGCAGCGGCCACCATCACCATCACCATTGA

Translated Wild type ectodomain (Tie2 Ig Fc-His-tag)

MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPI
TIGRDFEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRV
RGEAIRIRTMKMRQQASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNG
SFIHSVPRHEVPDILEVHLPHAQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEA
QKWGPECNHLCTACMNNGVCHEDTGECICPPGFMGRTCEKACELHTFGRTC
KERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNEACHPGFYGPDCKLRCS
CNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIEVNSGKFNPI
CKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSGVW
VCSVNTVAGMVEKPFNISVKNSGTDKTHTCPPCPAPELLGGPSVFLFPPKPKDT
LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGSGSGHHHHHH

Number of amino acids: 684

Molecular weight: 76019.77

Theoretical pI: 6.73

Appendix (4)

Wild-type ectodomain: There is no differences between the Tie2 ectodomain and the wild-type sequence.

unnamed protein product

Sequence ID: Query_218193 **Length:** 442 **Number of Matches:** 1

Range 1: 1 to 442 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
940 bits(2429)	0.0	Compositional matrix adjust.	442/442(100%)	442/442(100%)	0/442(0%)

```
Query  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
           MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD
Sbjct  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60

Query  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
           FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ
Sbjct  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120

Query  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180
           QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH
Sbjct  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180

Query  181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240
           AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC
Sbjct  181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240

Query  241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300
           ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE
Sbjct  241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300

Query  301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360
           ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE
Sbjct  301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360

Query  361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420
           VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG
Sbjct  361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420

Query  421  VWVCSVNTVAGMVEKPFNISVK  442
           VWVCSVNTVAGMVEKPFNISVK
Sbjct  421  VWVCSVNTVAGMVEKPFNISVK  442
```

Appendix (5)

RH ectodomain sequence compared to the wild-type, there is a deletion of Arg and His amino acids

Wt: QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH

RH: QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVP -- EVPDILEVHLPH

unnamed protein product

Sequence ID: Query_176979 **Length:** 440 **Number of Matches:** 1

Range 1: 1 to 440 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
910 bits(2353)	0.0	Compositional matrix adjust.	440/442(99%)	440/442(99%)	2/442(0%)

```
Query  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
            MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD
Sbjct  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60

Query  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
            FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ
Sbjct  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120

Query  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180
            QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVP  EVPDILEVHLPH
Sbjct  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVP--EVPDILEVHLPH  178

Query  181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240
            AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC
Sbjct  179  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  238

Query  241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300
            ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE
Sbjct  239  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  298

Query  301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360
            ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE
Sbjct  299  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  358

Query  361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420
            VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG
Sbjct  359  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  418

Query  421  VWVCSVNTVAGMVEKPFNISVK  442
            VWVCSVNTVAGMVEKPFNISVK
Sbjct  419  VWVCSVNTVAGMVEKPFNISVK  440
```

Appendix (6)

R3 mutant ectodomain (Tie2 Ig Fc-His-tag)

ATGGACTCTTTAGCCAGCTTAGTTCTCTGTGGAGTCAGCTTGCTCCTTTCTGGAACTGTGGAAG
GTGCCATGGACTTGATCTTGATCAATTCCCTACCTCTTGTATCTGATGCTGAAACATCTCTCAC
CTGCATTGCCTCTGGGTGGCGCCCCCATGAGCCCATCACCATAGGAAGGGACTTTGAAGCCTT
AATGAACCAGCACCAGGATCCGCTGGAAGTTACTCAAGATGTGACCAGAGAATGGGCTAAAA
AAGTTGTTTGGAAGAGAGAAAAGGCTAGTAAGATCAATGGTGCTTATTTCTGTGAAGGGCGA
GTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAAGCTTCCTTCCTACC
AGCTACTTTAACTATGACTGTGGACAAGGGAGATAACGTGAACATATCTTTCAAAAAGGTATT
GATTAAAGAAGAAGATGCAGTGATTTACAAAAATGGTTCCATCATCCATTCAGTGCCTGAAGT
ACCTGATATTCTAGAAGTACACCTGCCTCATGCTCAGCCCCAGGATGCTGGAGTGTACTCGGC
CAGGTATATAGGAGGAAACCTCTTCACCTCGGCCTTCACCAGGCTGATAGTCCGGAGATGTGA
AGCCCAGAAGTGGGGACCTGAATGCAACCATCTCTGTACTGCTTGTATGAACAATGGTGTCTG
CCATGAAGATACTGGAGAATGCATTTGCCCTCCTGGGTTTATGGGAAGGACGTGTGAGAAGG
CTTGTGAACTGCACACGTTTGGCAGAACTTGTAAAGAAAGGTGCAGTGGACAAGAGGGATGC
AAGTCTTATGTGTTCTGTCTCCCTGACCCCTATGGGTGTTCCTGTGCCACAGGCTGGAAGGGTC
TGCAGTGCAATGAAGCATGCCACCCTGGTTTTTACGGGCCAGATTGTAAGCTTAGGTGCAGCT
GCAACAATGGGGAGATGTGTGATCGCTTCCAAGGATGTCTCTGCTCTCCAGGATGGCAGGGG
CTCCAGTGTGAGAGAGAAGGCATACCGAGGATGACCCCAAAGATAGTGGATTTGCCAGATCA
TATAGAAGTAAACAGTGGTAAATTTAATCCCATTTGCAAAGCTTCTGGCTGGCCGCTACCTAC
TAATGAAGAAATGACCCTGGTGAAGCCGGATGGGACAGTGCTCCATCCAAAAGACTTTAACC
ATACGGATCATTTCTCAGTAGCCATATTCACCATCCACCGGATCCTCCCCCCTGACTCAGGAG
TTTGGGTCTGCAGTGTGAACACAGTGGCTGGGATGGTGGAAAAGCCCTTCAACATTTCTGTTA
AGAATTCTGGTACCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGG
GGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCT
GAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTA
CGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGC
ACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAA
AGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGA
ACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGG
AGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC
TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCT
CCGGGTAAAGGCAGCGGCAGCGGCCACCATCACCATCACCATTGA

Translated R3 mutant ectodomain (Tie2 Ig Fc-His-tag)

MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRDFE
ALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMR
QQASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSIIHSVPEVPDILEVHLPHAQP
QDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGECIC
PPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNEA
CHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIEV
NSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSGVW
VCSVNTVAGMVEKPFNISVKNSGTDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL
NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGKGSGSGHHHHHH

Tie2-Ig FC His-tag

Number of amino acids: 678

Molecular weight: 75333.0

Appendix (7)

Blast n between Wt (Tie2 Ig Fc-His-tag) and R3 mutant ectodomain (Tie2 Ig Fc-His-tag)

There is a deletion of Arg and His amino acids with replacement of F with I

```
Query        1        ATGGACTCTTTAGCCAGCTTAGTTCTCTGTGGAGTCAGCTTGCTCCTTTCTGGAACTGTG  60
Query_169791 1        ............................................................  60

Query        61       GAAGGTGCCATGGACTTGATCTTGATCAATTCCCTACCTCTTGTATCTGATGCTGAAACA  120
Query_169791 61       ............................................................  120

Query        121      TCTCTCACCTGCATTGCCTCTGGGTGGCGCCCCCATGAGCCCATCACCATAGGAAGGGAC  180
Query_169791 121      ............................................................  180

Query        181      TTTGAAGCCTTAATGAACCAGCACCAGGATCCGCTGGAAGTTACTCAAGATGTGACCAGA  240
Query_169791 181      ............................................................  240

Query        241      GAATGGGCTAAAAAAGTTGTTTGGAAGAGAGAAAAGGCTAGTAAGATCAATGGTGCTTAT  300
Query_169791 241      ............................................................  300

Query        301      TTCTGTGAAGGGCGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAA  360
Query_169791 301      ............................................................  360

Query        361      CAAGCTTCCTTCCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATAACGTGAAC  420
Query_169791 361      ............................................................  420

Query        421      ATATCTTTCAAAAAGGTATTGATTAAAGAAGAAGATGCAGTGATTTACAAAAATGGTTCC  480
Query_169791 421      ............................................................  480

Query        481      TTCATCCATTCAGTGCCCCGGCATGAAGTACCTGATATTCTAGAAGTACACCTGCCTCAT  540
Query_169791 481      A..............------.....................................  534

Query        541      GCTCAGCCCCAGGATGCTGGAGTGTACTCGGCCAGGTATATAGGAGGAAACCTCTTCACC  600
Query_169791 535      ............................................................  594

Query        601      TCGGCCTTCACCAGGCTGATAGTCCGGAGATGTGAAGCCCAGAAGTGGGGACCTGAATGC  660
Query_169791 595      ............................................................  654

Query        661      AACCATCTCTGTACTGCTTGTATGAACAATGGTGTCTGCCATGAAGATACTGGAGAATGC  720
Query_169791 655      ............................................................  714
```

```
Query         721   ATTTGCCCTCCTGGGTTTATGGGAAGGACGTGTGAGAAGGCTTGTGAACTGCACACGTTT  780
Query_169791  715   ............................................................  774

Query         781   GGCAGAACTTGTAAAGAAAGGTGCAGTGGACAAGAGGGATGCAAGTCTTATGTGTTCTGT  840
Query_169791  775   ............................................................  834

Query         841   CTCCCTGACCCCTATGGGTGTTCCTGTGCCACAGGCTGGAAGGGTCTGCAGTGCAATGAA  900
Query_169791  835   ............................................................  894

Query         901   GCATGCCACCCTGGTTTTTACGGGCCAGATTGTAAGCTTAGGTGCAGCTGCAACAATGGG  960
Query_169791  895   ............................................................  954

Query         961   GAGATGTGTGATCGCTTCCAAGGATGTCTCTGCTCTCCAGGATGGCAGGGGCTCCAGTGT  1020
Query_169791  955   ............................................................  1014

Query         1021  GAGAGAGAAGGCATACCGAGGATGACCCCAAAGATAGTGGATTTGCCAGATCATATAGAA  1080
Query_169791  1015  ............................................................  1074

Query         1081  GTAAACAGTGGTAAATTTAATCCCATTTGCAAAGCTTCTGGCTGGCCGCTACCTACTAAT  1140
Query_169791  1075  ............................................................  1134

Query         1141  GAAGAAATGACCCTGGTGAAGCCGGATGGGACAGTGCTCCATCCAAAAGACTTTAACCAT  1200
Query_169791  1135  ............................................................  1194

Query         1201  ACGGATCATTTCTCAGTAGCCATATTCACCATCCACCGGATCCTCCCCCCTGACTCAGGA  1260
Query_169791  1195  ............................................................  1254

Query         1261  GTTTGGGTCTGCAGTGTGAACACAGTGGCTGGGATGGTGGAAAAGCCCTTCAACATTTCT  1320
Query_169791  1255  ............................................................  1314

Query         1321  GTTAAGAATTCTGGTACCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC  1380
Query_169791  1315  ............................................................  1374

Query         1381  CTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCC  1440
Query_169791  1375  ............................................................  1434

Query         1441  CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAG  1500
Query_169791  1435  ............................................................  1494

Query         1501  TTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAG  1560
Query_169791  1495  ............................................................  1554

Query         1561  CAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTG  1620
```

```
Query_169791  1555  ..........................................................  1614

Query         1621  AATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAA  1680
Query_169791  1615  ..........................................................  1674

Query         1681  ACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCC  1740
Query_169791  1675  ..........................................................  1734

Query         1741  CGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCC  1800
Query_169791  1735  ..........................................................  1794

Query         1801  AGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACG  1860
Query_169791  1795  ..........................................................  1854

Query         1861  CCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAG  1920
Query_ 169791  1855  ..........................................................  1914

Query         1921  AGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAAC  1980
Query_ 169791  1915  ..........................................................  1974

 Query        1981  CACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGCAGCGGCAGCGGCCACCAT   2040
 Query_  169791 1975  ..................................................  2034
 Query         2041  CACCATCACCATTGA  2055
 Query_169791  2035  ..............  2049
```

Translated R3 sequence compared to the wild-type, there is a deletion of Arg and His amino acids with replacement of F with I (before optimization)

Wt: QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH

R3: QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSI IHSVP -- EVPDILEVHLPH

unnamed protein product

Sequence ID: Query_25267 **Length:** 442 **Number of Matches:** 1

Range 1: 1 to 442 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
909 bits(2348)	0.0	Compositional matrix adjust.	439/442(99%)	439/442(99%)	2/442(0%)

```
Query  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
            MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD
Sbjct  1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60

Query  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
            FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ
Sbjct  61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120

Query  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSIIHSVP--EVPDILEVHLPH  178
            QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGS IHSVP  EVPDILEVHLPH
Sbjct  121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180

Query  179  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  238
            AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC
Sbjct  181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240

Query  239  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  298
            ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE
Sbjct  241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300

Query  299  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  358
            ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE
Sbjct  301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360

Query  359  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  418
            VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG
Sbjct  361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420

Query  419  VWVCSVNTVAGMVEKPFNISVK  440
            VWVCSVNTVAGMVEKPFNISVK
Sbjct  421  VWVCSVNTVAGMVEKPFNISVK  442
```

Appendix (8)

Gene sequence of R3-COMP ectodomain ordered from life technology with aminocid transulation

Oligomeric R3 mutant ectodomain (COMP-Tie2 Ig-His-tag)

CGCTAGCACCatggacagtctggcttccctggtcctctgcggggtaagcctcctcctgtcaggcaccgtggaaggcgcaatggacctg
atactcattaactctctgccactggtcagcgatgctgagacatccctgacatgcattgcatcagggtggaggcctcatgagcccattaccataggg
agggactttgaggcccttatgaaccagcaccaggatcctctggaagttacacaggacgtgaccagagagtgggccaagaaggtggtgtggaa
aagggaaaaggcctccaagatcaatggcgcgtatttctgtgaggggagagtacgcggggaagcaatccgcatcaggacgatgaaaatgcga
caacaggcctcattcctgcctgctaccttgactatgacggtggacaaaggtgacaacgtcaacatcagcttcaagaaggttctgatcaaagaaga
ggatgcagtgatatacaagaatggaagcatcattcactccgtgccagaagttccagacattcttgaagtgcatcttccccatgctcaaccccagga
tgctggcgtgtatagcgcacggtacataggcggcaatttgttcacatctgcgtttacccggttgatcgtgaggcgatgtgaggcgcagaaatggg
gacccgaatgtaatcacctgtgcactgcctgcatgaataacggcgtctgccatgaggacaccggtgagtgtatttgtcctccagggtttatgggg
cgcacttgcgagaaagcctgcgaactccacactttcgggagaacatgcaaagaacggtgcagtggtcaggaaggttgcaagagctacgtgtttt
gtctgcccgatccatacgggtgttcttgtgccacagggtggaaagggctgcaatgcaacgaggcttgtcaccctggcttttatggtccggattgta
agctgcgttgctcttgcaataatggcgagatgtgcgacagatttcagggttgtctgtgtagccctggatggcaaggcctccagtgcgaacgtgag
ggcattcctcggatgaccccaaagatcgttgacttgcccgatcacatagaagtcaactctggaaagttcaaccccatttgcaaggcctcaggctg
gcccctgcccacaaacgaggagatgaccctggttaagcccgatggaaccgtgctgcatccgaaagacttcaaccacactgaccacttttccgta
gccatctttaccatccacagaatcctgccaccggatagtggggtgtgggtctgttccgtgaatacagtcgccggtatggtggagaaaccattcaa
catcagcgtgaagaattctggaggcggcggatcaggcggaggaggttctggcggaggtgggagcgaccttgctcctcagatgctccgcgaa
ctgcaggagactaatgctgcacttcaggatgttcgagagttgctgcggcaacaggtaaaagagattacgttcctcaagaatactgtcatggagtg
cgatgcctgtggaggaggaggcggctcccatcatcaccaccaccat**TGA**AGCGGCCGC

blue = NheI and *Not* I sites

green= Kozak sequence (also uses final C in *Nhe-I* site)

Black bold underline - stop codon

*Eco*R-I site

Translated Oligomeric R3 ectodomain (ordered from life technology.)

MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITI

GRDFEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTM

KMRQQASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSIIHSVPEVPDILEVHLPH

AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGE

CICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCN

EACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE

VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSGVW

VCSVNTVAGMVEKPFNISVKNSGGGGSGGGGSGGGGSDLAPQMLRELQETNAALQDVRE

LLRQQVKEITFLKNTVMECDACGGGGGSHHHHHH

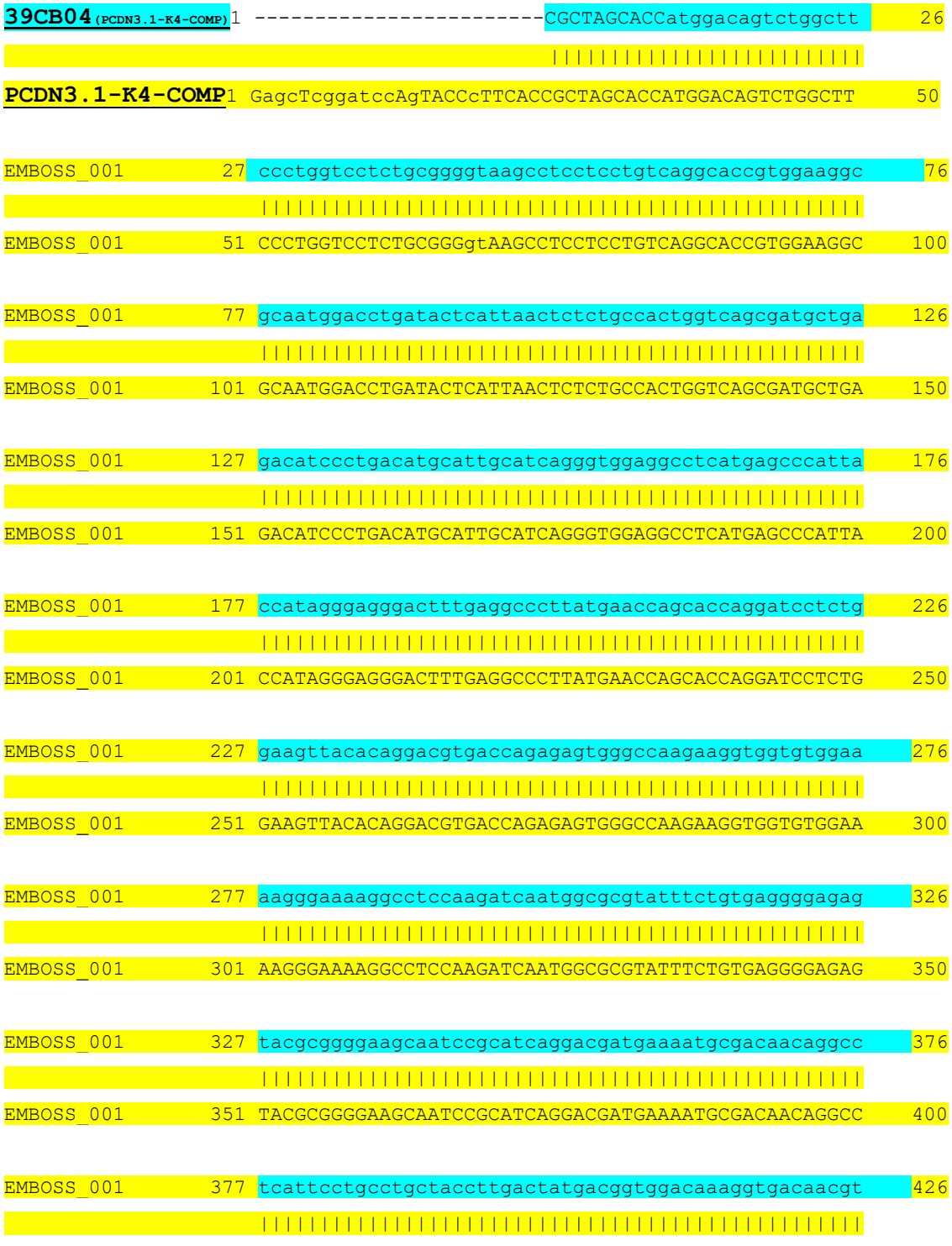
R3 ectodomain(Ig-1 ,Ig2, Ig-3) *Eco*R-I Linker COMP His-tag

Number of amino acids: 513

Molecular weight: 56065.07

Appendix (9)

Sequencing of the pcDNA3.1-R3-COMP after ligation to compare the sequence of the insert (R3-COMP) for the ligated PCDN3.1-R3-COMP with the insert (R3-COMP) of the synthesized pEX-K4-R3-COMP. The blue line represents the gene sequence of the R3-COMP of the ligated PCDN3.1-K4-COMP. While the yellow line represents the gene sequence of the R3-COMP of the synthesized PEX-K4-R3-COMP



```
EMBOSS_001        401 TCATTCCTGCCTGCTACCTTGACTATGACGGTGGACAAAGGTGACAACGT     450

EMBOSS_001        427 caacatcagcttcaagaaggttctgatcaaagaagaggatgcagtgatat     476
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        451 CAACATCAGCTTCAAGAAGGTTCTGATCAAAGAAGAGGATGCAGTGATAT     500

EMBOSS_001        477 acaagaatggaagcatcattcactccgtgccagaagttccagacattctt     526
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        501 ACAAGAATGGAAGCATCATTCACTCCGTGCCAGAAGTTCCAGACATTCTT     550

EMBOSS_001        527 gaagtgcatcttccccatgctcaaccccaggatgctggcgtgtatagcgc     576
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        551 GAAGTGCATCTTCCCCATGCTCAACCCCAGGATGCTGGCGTGTATAGCGC     600

EMBOSS_001        577 acggtacataggcggcaatttgttcacatctgcgtttacccggttgatcg     626
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        601 ACGGTACATAGGCGGCAATTTGTTCACATCTGCGTTTACCCGGTTGATCG     650

EMBOSS_001        627 tgaggcgatgtgaggcgcagaaatggggacccgaatgtaatcacctgtgc     676
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        651 TGAGGCGATGTGAGGCGCAGAAATGGGGACCCGAATGTAATCACCTGTGC     700

EMBOSS_001        677 actgcctgcatgaataacggcgtctgccatgaggacaccggtgagtgtat     726
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        701 ACTGCCTGCATGAATAACGGCGTCTGCCATGAGGACACCGGTGAGTGTAT     750

EMBOSS_001        727 ttgtcctccagggtttatggggcgcacttgcgagaaagcctgcgaactcc     776
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        751 TTGTCCTCCAGGGTTTATGGGgCGCACTTGCGAGAAAGCCTGCGAACTCC     800

EMBOSS_001        777 acactttcgggagaacatgcaaagaacggtgcagtggtcaggaaggttgc     826
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        801 ACACTTTCGGGAGAACATGCAAAGAACGGTGCAGTGGTCAGGAAGGTTGC     850

EMBOSS_001        827 aagagctacgtgttttgtctgcccgatccatacgggtgttcttgtgccac     876
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        851 AAGAGCTACGTGTTTTGTCTGCCCGATCCATACGGGTGTTCtTtGTGCCAC     900

EMBOSS_001        877 agggtggaaagggctgcaatgcaacgaggcttgtcaccctggcttttatg     926
                      ||||
EMBOSS_001        901 AGGg
```

Appendix (10)

Oligomeric R3 mutant ectodomain (CD5-COMP-Tie2 Ig-His-tag)

GCTAGCATGCCCATGGGGTCTCTGCAACCGCTGGCCACCTTGTACCTGCTGGGGATGCTGGTCGC
TTCCTGCCTCGGAATGGACCTGATACTCATTAACTCTCTGCCACTGGTCAGCGATGCTG
AGACATCCCTGACATGCATTGCATCAGGGTGGAGGCCTCATGAGCCCATTACCATAG
GGAGGGACTTTGAGGCCCTTATGAACCAGCACCAGGATCCTCTGGAAGTTACACAGG
ACGTGACCAGAGAGTGGGCCAAGAAGGTGGTGTGGAAAAGGGAAAAGGCCTCCAAG
ATCAATGGCGCGTATTTCTGTGAGGGGAGAGTACGCGGGGAAGCAATCCGCATCAGG
ACGATGAAAATGCGACAACAGGCCTCATTCCTGCCTGCTACCTTGACTATGACGGTGG
ACAAAGGTGACAACGTCAACATCAGCTTCAAGAAGGTTCTGATCAAAGAAGAGGATG
CAGTGATATACAAGAATGGAAGCATCATTCACTCCGTGCCAGAAGTTCCAGACATTCT
TGAAGTGCATCTTCCCCATGCTCAACCCCAGGATGCTGGCGTGTATAGCGCACGGTAC
ATAGGCGGCAATTTGTTCACATCTGCGTTTACCCGGTTGATCGTGAGGCGATGTGAGG
CGCAGAAATGGGGACCCGAATGTAATCACCTGTGCACTGCCTGCATGAATAACGGCG
TCTGCCATGAGGACACCGGTGAGTGTATTTGTCCTCCAGGGTTTATGGGGCGCACTTG
CGAGAAAGCCTGCGAACTCCACACTTTCGGGAGAACATGCAAAGAACGGTGCAGTGG
TCAGGAAGGTTGCAAGAGCTACGTGTTTTGTCTGCCCGATCCATACGGGTGTTCTTGT
GCCACAGGGTGGAAAGGGCTGCAATGCAACGAGGCTTGTCACCCTGGCTTTTATGGT
CCGGATTGTAAGCTGCGTTGCTCTTGCAATAATGGCGAGATGTGCGACAGATTTCAGG
GTTGTCTGTGTAGCCCTGGATGGCAAGGCCTCCAGTGCGAACGTGAGGGCATTCCTCG
GATGACCCCAAAGATCGTTGACTTGCCCGATCACATAGAAGTCAACTCTGGAAAGTT
CAACCCCATTTGCAAGGCCTCAGGCTGGCCCCTGCCCACAAACGAGGAGATGACCCT
GGTTAAGCCCGATGGAACCGTGCTGCATCCGAAAGACTTCAACCACACTGACCACTTT
TCCGTAGCCATCTTTACCATCCACAGAATCCTGCCACCGGATAGTGGGGTGTGGGTCT
GTTCCGTGAATACAGTCGCCGGTATGGTGGAGAAACCATTCAACATCAGCGTGAAGA
ATTCTGGAGGCGGCGGATCAGGCGGAGGAGGTTCTGGCGGAGGTGGGAGCGACCTTG
CTCCTCAGATGCTCCGCGAACTGCAGGAGACTAATGCTGCACTTCAGGATGTTCGAGA
GTTGCTGCGGCAACAGGTAAAAGAGATTACGTTCCTCAAGAATACTGTCATGGAGTG
CGATGCCTGTGGAGGAGGAGGCGGCTCCCATCATCACCACCACCATTGAGCGGCCG
CGTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTA

***Nhe* I , CD5, R3COMP, *Eco*R I , stop codon, *Not* I**

Translated Oligomeric R3 ectodomain (CD5-COMP-Tie2 Ig-His-tag)

MPMGSLQPLATLYLLGMLVASCLGMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRDFE
ALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ
QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSIIHSVPEVPDILEVHLPHAQPQ
DAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGECICPP
GFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNEACH
PGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIEVNSG
KFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSGVWVCSV
NTVAGMVEKPFNISVKNSGGGGSGGGGSGGGGSDLAPQMLRELQETNAALQDVRELLRQ
QVKEITFLKNTVMECDACGGGGGSHHHHHH

CD5, R3 ectodomain(Ig-1 ,Ig2, Ig-3) *Eco*R-I Linker COMP His-tag

Number of amino acids: 513

Molecular weight: 56065.07

Appendix (11)

pTIE2-14 -Ig Fc-His-tag

```
   1 ATGGACTCTT TAGCCAGCTT AGTTCTCTGT GGAGTCAGCT TGCTCCTTTC TGGAACTGTG
  61 GAAGGTGCCA TGGACTTGAT CTTGATCAAT TCCCTACCTC TTGTATCTGA TGCTGAAACA
 121 TCTCTCACCT GCATTGCCTC TGGGTGGCGC CCCCATGAGC CCATCACCAT AGGAAGGGAC
 181 TTTGAAGCCT TAATGAACCA GCACCAGGAT CCGCTGGAAG TTACTCAAGA TGTGACCAGA
 241 GAATGGGCTA AAAAAGTTGT TTGGAAGAGA GAAAAGGCTA GTAAGATCAA TGGTGCTTAT
 301 TTCTGTGAAG GGCGAGTTCG AGGAGAGGCA ATCAGGATAC GAACCATGAA GATGCGTCAA
 361 CAAGCTTCCT TCCTACCAGC TACTTTAACT ATGACTGTGG ACAAGGGAGA TAACGTGAAC
 421 ATATCTTTCA AAAAGGTATT GATTAAAGAA GAAGATGCAC TGATTTACAA AAATGGTTCC
 481 ATCATCCATT CAGTGCCTGA AGTAGCCGAT ATTCTAGAAG TACACCTGCC TCATGCTCAG
 541 CCCCAGGATG CTGGAGTGTA CTCGGCCAGG TATATAGGAG GAAACCTCTT CACCTCGGCC
 601 TTCACCAGGC TGATAGTCCG GAGATGTGAA GCCCAGAAGT GGGGACCTGA ATGCAACCAT
 661 CTCTGTACTG CTTGTATGAA CAATGGTGTC TGCCATGAAG ATACTGGAGA ATGCATTTGC
 721 CCTCCTGGGT TTATGGGAAG GACGTGTGAG AAGGCTTGTG AACTGCACAC GTTTGGCAGA
 781 ACTTGTAAAG AAAGGTGCAG TGGACAAGAG GGATGCAAGT CTTATGTGTT CTGTCTCCCT
 841 GACCCCTATG GGTGTTCCTG TGCCACAGGC TGGAAGGGTC TGCAGTGCAA TGAAGCATGC
 901 CACCCTGGTT TTTACGGGCC AGATTGTAAG CTTAGGTGCA GCTGCAACAA TGGGGAGATG
 961 TGTGATCGCT TCCAAGGATG TCTCTGCTCT CCAGGATGGC AGGGGCTCCA GTGTGAGAGA
1021 GAAGGCATAC CGAGGATGAC CCCAAAGATA GTGGATTTGC CAGATCATAT AGAAGTAAAC
1081 AGTGGTAAAT TTAATCCCAT TTGCAAAGCT TCTGGCTGGC CGCTACCTAC TAATGAAGAA
1141 ATGACCCTGG TGAAGCCGGA TGGGACAGTG CTCCATCCAA AAGACTTTAA CCATACGGAT
1201 CATTTCTCAG TAGCCATATT CACCATCCAC CGGATCCTCC CCCCTGACTC AGGAGTTTGG
1261 GTCTGCAGTG TGAACACAGT GGCTGGGATG GTGGAAAAGC CCTTCAACAT TTCTGTTAAG
1321 AATTCTGGTA CCGACAAAAC TCACACATGC CCACCGTGCC CAGCACCTGA ACTCCTGGGG
1381 GGACCGTCAG TCTTCCTCTT CCCCCCAAAA CCCAAGGACA CCCTCATGAT CTCCCGGACC
1441 CCTGAGGTCA CATGCGTGGT GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC
1501 TGGTACGTGG ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA GGAGCAGTAC
1561 AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC ACCAGGACTG GCTGAATGGC
1621 AAGGAGTACA AGTGCAAGGT CTCCAACAAA GCCCTCCCAG CCCCCATCGA GAAAACCATC
```

```
1681 TCCAAAGCCA AAGGGCAGCC CCGAGAACCA CAGGTGTACA CCCTGCCCCC ATCCCGGGAT
1741 GAGCTGACCA AGAACCAGGT CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC
1801 ATCGCCGTGG AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC CACGCCTCCC
1861 GTGCTGGACT CCGACGGCTC CTTCTTCCTC TACAGCAAGC TCACCGTGGA CAAGAGCAGG
1921 TGGCAGCAGG GGAACGTCTT CTCATGCTCC GTGATGCATG AGGCTCTGCA CAACCACTAC
1981 ACGCAGAAGA GCCTCTCCCT GTCTCCGGGT AAAGGCAGCG GCAGCGGCCA CCATCACCAT
2041 CACCATTGA
```

Amino acid translation of pTIE2-14 -Ig Fc-His-tag

```
   1 MDSLASLVLC GVSLLLSGTV EGAMDLILIN SLPLVSDAET SLTCIASGWR PHEPITIGRD
  61 FEALMNQHQD PLEVTQDVTR EWAKKVVWKR EKASKINGAY FCEGRVRGEA IRIRTMKMRQ
 121 QASFLPATLT MTVDKGDNVN ISFKKVLIKE EDALIYKNGS IIHSVPEVAD ILEVHLPHAQ
 181 PQDAGVYSAR YIGGNLFTSA FTRLIVRRCE AQKWGPECNH LCTACMNNGV CHEDTGECIC
 241 PPGFMGRTCE KACELHTFGR TCKERCSGQE GCKSYVFCLP DPYGCSCATG WKGLQCNEAC
 301 HPGFYGPDCK LRCSCNNGEM CDRFQGCLCS PGWQGLQCER EGIPRMTPKI VDLPDHIEVN
 361 SGKFNPICKA SGWPLPTNEE MTLVKPDGTV LHPKDFNHTD HFSVAIFTIH RILPPDSGVW
 421 VCSVNTVAGM VEKPFNISVK NSGTDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT
 481 PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG
 541 KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRD ELTKNQVSLT CLVKGFYPSD
 601 IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY
 661 TQKSLSLSPG KGSGSGHHHH HH
```

Appendix (12)

pTIE2-15 -Ig Fc-His-tag

```
   1 ATGGACTCTT TAGCCAGCTT AGTTCTCTGT GGAGTCAGCT TGCTCCTTTC TGGAACTGTG
  61 GAAGGTGCCA TGGACTTGAT CTTGATCAAT TCCCTACCTC TTGTATCTGA TGCTGAAACA
 121 TCTCTCACCT GCATTGCCTC TGGGTGGCGC CCCCATGAGC CCATCACCAT AGGAAGGGAC
 181 TTTGAAGCCT TAATGAACCA GCACCAGGAT CCGCTGGAAG TTACTCAAGA TGTGACCAGA
 241 GAATGGGCTA AAAAAGTTGT TTGGAAGAGA GAAAAGGCTA GTAAGATCAA TGGTGCTTAT
 301 TTCTGTGAAG GGCGAGTTCG AGGAGAGGCA ATCAGGATAC GAACCATGAA GATGCGTCAA
 361 CAAGCTTCCT TCCTACCAGC TACTTTAACT ATGACTGTGG ACAAGGGAGA TAACGTGAAC
 421 ATATCTTTCA AAAAGGTATT GATTAAAGAA GAAGATGCAG TGATTTACAA AAATGGTTCC
 481 ATCATCCATT CAGTGCCTGA CATCCCTGAT ATTCTAGAAG TACACCTGCC TCATGCTCAG
 541 CCCCAGGATG CTGGAGTGTA CTCGGCCAGG TATATAGGAG GAAACCTCTT CACCTCGGCC
 601 TTCACCAGGC TGATAGTCCG GAGATGTGAA GCCCAGAAGT GGGGACCTGA ATGCAACCAT
 661 CTCTGTACTG CTTGTATGAA CAATGGTGTC TGCCATGAAG ATACTGGAGA ATGCATTTGC
 721 CCTCCTGGGT TTATGGGAAG GACGTGTGAG AAGGCTTGTG AACTGCACAC GTTTGGCAGA
 781 ACTTGTAAAG AAAGGTGCAG TGGACAAGAG GGATGCAAGT CTTATGTGTT CTGTCTCCCT
 841 GACCCCTATG GGTGTTCCTG TGCCACAGGC TGGAAGGGTC TGCAGTGCAA TGAAGCATGC
 901 CACCCTGGTT TTTACGGGCC AGATTGTAAG CTTAGGTGCA GCTGCAACAA TGGGGAGATG
 961 TGTGATCGCT TCCAAGGATG TCTCTGCTCT CCAGGATGGC AGGGGCTCCA GTGTGAGAGA
1021 GAAGGCATAC CGAGGATGAC CCCAAAGATA GTGGATTTGC CAGATCATAT AGAAGTAAAC
1081 AGTGGTAAAT TTAATCCCAT TTGCAAAGCT TCTGGCTGGC CGCTACCTAC TAATGAAGAA
1141 ATGACCCTGG TGAAGCCGGA TGGGACAGTG CTCCATCCAA AAGACTTTAA CCATACGGAT
1201 CATTTCTCAG TAGCCATATT CACCATCCAC CGGATCCTCC CCCCTGACTC AGGAGTTTGG
1261 GTCTGCAGTG TGAACACAGT GGCTGGGATG GTGGAAAAGC CCTTCAACAT TTCTGTTAAG
1321 AATTCTGGTA CCGACAAAAC TCACACATGC CCACCGTGCC CAGCACCTGA ACTCCTGGGG
1381 GGACCGTCAG TCTTCCTCTT CCCCCCAAAA CCCAAGGACA CCCTCATGAT CTCCCGGACC
1441 CCTGAGGTCA CATGCGTGGT GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC
1501 TGGTACGTGG ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA GGAGCAGTAC
1561 AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC ACCAGGACTG GCTGAATGGC
1621 AAGGAGTACA AGTGCAAGGT CTCCAACAAA GCCCTCCCAG CCCCCATCGA GAAAACCATC
```

```
1681 TCCAAAGCCA AAGGGCAGCC CCGAGAACCA CAGGTGTACA CCCTGCCCCC ATCCCGGGAT
1741 GAGCTGACCA AGAACCAGGT CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC
1801 ATCGCCGTGG AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC CACGCCTCCC
1861 GTGCTGGACT CCGACGGCTC CTTCTTCCTC TACAGCAAGC TCACCGTGGA CAAGAGCAGG
1921 TGGCAGCAGG GGAACGTCTT CTCATGCTCC GTGATGCATG AGGCTCTGCA CAACCACTAC
1981 ACGCAGAAGA GCCTCTCCCT GTCTCCGGGT AAAGGCAGCG GCAGCGGCCA CCATCACCAT
2041 CACCATTGA
```

Amino acid translation ofp pTIE2-15 -Ig Fc-His-tag

```
  1 MDSLASLVLC GVSLLLSGTV EGAMDLILIN SLPLVSDAET SLTCIASGWR PHEPITIGRD
 61 FEALMNQHQD PLEVTQDVTR EWAKKVVWKR EKASKINGAY FCEGRVRGEA IRIRTMKMRQ
121 QASFLPATLT MTVDKGDNVN ISFKKVLIKE EDAVIYKNGS IIHSVPDIPD ILEVHLPHAQ
181 PQDAGVYSAR YIGGNLFTSA FTRLIVRRCE AQKWGPECNH LCTACMNNGV CHEDTGECIC
241 PPGFMGRTCE KACELHTFGR TCKERCSGQE GCKSYVFCLP DPYGCSCATG WKGLQCNEAC
301 HPGFYGPDCK LRCSCNNGEM CDRFQGCLCS PGWQGLQCER EGIPRMTPKI VDLPDHIEVN
361 SGKFNPICKA SGWPLPTNEE MTLVKPDGTV LHPKDFNHTD HFSVAIFTIH RILPPDSGVW
421 VCSVNTVAGM VEKPFNISVK NSGTDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT
```

Appendix (13)

pTIE2-16 -Ig Fc-His-tag(native signal peptide)

```
   1 ATGGACTCTT TAGCCAGCTT AGTTCTCTGT GGAGTCAGCT TGCTCCTTTC TGGAACTGTG
  61 GAAGGTGCCA TGGACTTGAT CTTGATCAAT TCCCTACCTC TTGTATCTGA TGCTGAAACA
 121 TCTCTCACCT GCATTGCCTC TGGGTGGCGC CCCCATGAGC CCATCACCAT AGGAAGGGAC
 181 TTTGAAGCCT TAATGAACCA GCACCAGGAT CCGCTGGAAG TTACTCAAGA TGTGACCAGA
 241 GAATGGGCTA AAAAAGTTGT TTGGAAGAGA GAAAAGGCTA GTAAGATCAA TGGTGCTTAT
 301 TTCTGTGAAG GGCGAGTTCG AGGAGAGGCA ATCAGGATAC GAACCATGAA GATGCGTCAA
 361 CAAGCTTCCT TCCTACCAGC TACTTTAACT ATGACTGTGG ACAAGGGAGA TAACGTGAAC
 421 ATATCTTTCA AAAAGGTATT GATTAAAGAA GAAGATGCAC TGATTTACAA AAATGGTTCC
 481 ATCATCCATT CAGTGCCTGA CATCGCCGAT ATTCTAGAAG TACACCTGCC TCATGCTCAG
 541 CCCCAGGATG CTGGAGTGTA CTCGGCCAGG TATATAGGAG GAAACCTCTT CACCTCGGCC
 601 TTCACCAGGC TGATAGTCCG GAGATGTGAA GCCCAGAAGT GGGGACCTGA ATGCAACCAT
 661 CTCTGTACTG CTTGTATGAA CAATGGTGTC TGCCATGAAG ATACTGGAGA ATGCATTTGC
 721 CCTCCTGGGT TTATGGGAAG GACGTGTGAG AAGGCTTGTG AACTGCACAC GTTTGGCAGA
 781 ACTTGTAAAG AAAGGTGCAG TGGACAAGAG GGATGCAAGT CTTATGTGTT CTGTCTCCCT
 841 GACCCCTATG GGTGTTCCTG TGCCACAGGC TGGAAGGGTC TGCAGTGCAA TGAAGCATGC
 901 CACCCTGGTT TTTACGGGCC AGATTGTAAG CTTAGGTGCA GCTGCAACAA TGGGGAGATG
 961 TGTGATCGCT TCCAAGGATG TCTCTGCTCT CCAGGATGGC AGGGGCTCCA GTGTGAGAGA
1021 GAAGGCATAC CGAGGATGAC CCCAAAGATA GTGGATTTGC CAGATCATAT AGAAGTAAAC
1081 AGTGGTAAAT TTAATCCCAT TTGCAAAGCT TCTGGCTGGC CGCTACCTAC TAATGAAGAA
1141 ATGACCCTGG TGAAGCCGGA TGGGACAGTG CTCCATCCAA AAGACTTTAA CCATACGGAT
1201 CATTTCTCAG TAGCCATATT CACCATCCAC CGGATCCTCC CCCCTGACTC AGGAGTTTGG
1261 GTCTGCAGTG TGAACACAGT GGCTGGGATG GTGGAAAAGC CCTTCAACAT TTCTGTTAAG
1321 AATTCTGGTA CCGACAAAAC TCACACATGC CCACCGTGCC CAGCACCTGA ACTCCTGGGG
1381 GGACCGTCAG TCTTCCTCTT CCCCCCAAAA CCCAAGGACA CCCTCATGAT CTCCCGGACC
1441 CCTGAGGTCA CATGCGTGGT GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC
1501 TGGTACGTGG ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA GGAGCAGTAC
1561 AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC ACCAGGACTG GCTGAATGGC
1621 AAGGAGTACA AGTGCAAGGT CTCCAACAAA GCCCTCCCAG CCCCCATCGA GAAAACCATC
1681 TCCAAAGCCA AAGGGCAGCC CCGAGAACCA CAGGTGTACA CCCTGCCCCC ATCCCGGGAT
```

```
1741 GAGCTGACCA AGAACCAGGT CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC
1801 ATCGCCGTGG AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC CACGCCTCCC
1861 GTGCTGGACT CCGACGGCTC CTTCTTCCTC TACAGCAAGC TCACCGTGGA CAAGAGCAGG
1921 TGGCAGCAGG GGAACGTCTT CTCATGCTCC GTGATGCATG AGGCTCTGCA CAACCACTAC
1981 ACGCAGAAGA GCCTCTCCCT GTCTCCGGGT AAAGGCAGCG GCAGCGGCCA CCATCACCAT
2041 CACCATTGA
```

Amino acid translation of pTIE2-16 -Ig Fc-His-tag (native signal peptide)

```
   1 MDSLASLVLC GVSLLLSGTV EGAMDLILIN SLPLVSDAET SLTCIASGWR PHEPITIGRD
  61 FEALMNQHQD PLEVTQDVTR EWAKKVVWKR EKASKINGAY FCEGRVRGEA IRIRTMKMRQ
 121 QASFLPATLT MTVDKGDNVN ISFKKVLIKE EDALIYKNGS IIHSVPDIAD ILEVHLPHAQ
 181 PQDAGVYSAR YIGGNLFTSA FTRLIVRRCE AQKWGPECNH LCTACMNNGV CHEDTGECIC
 241 PPGFMGRTCE KACELHTFGR TCKERCSGQE GCKSYVFCLP DPYGCSCATG WKGLQCNEAC
 301 HPGFYGPDCK LRCSCNNGEM CDRFQGCLCS PGWQGLQCER EGIPRMTPKI VDLPDHIEVN
 361 SGKFNPICKA SGWPLPTNEE MTLVKPDGTV LHPKDFNHTD HFSVAIFTIH RILPPDSGVW
 421 VCSVNTVAGM VEKPFNISVK NSGTDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT
 481 PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG
 541 KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRD ELTKNQVSLT CLVKGFYPSD
 601 IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY
 661 TQKSLSLSPG KGSGSGHHHH HH
```

Appendix (14)

BLAST N of Tie2-Wt and pTIE2-14, pTIE2-15, pTIE2-16

1- BLAST N of Tie2-Wt-Ig Fc-His-tag and pTIE2-14 -Ig Fc-His-tag

```
Query         1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
Query_228053  1    ............................................................  60

Query        61    FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
Query_228053 61    ............................................................  120

Query        121   QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180
Query_228053 121   ..............................L......I.....--..A.........  178

Query        181   AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240
Query_228053 179   ............................................................  238

Query        241   ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300
Query_228053 239   ............................................................  298

Query        301   ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360
Query_228053 299   ............................................................  358

Query        361   VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420
Query_228053 359   ............................................................  418

Query        421   VWVCSVNTVAGMVEKPFNISVKNSGTDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS  480
Query_228053 419   ............................................................  478

Query        481   RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL  540
Query_228053 479   ............................................................  538

Query        541   NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP  600
Query_228053 539   ............................................................  598

Query        601   SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN  660
Query_228053 599   ............................................................  658

Query         661  HYTQKSLSLSPGKGSGSGHHHHHH  684
Query_228053  659  ........................  682
```

2- BLAST N of artificial Tie2-Wt-Ig Fc-His-tag and pTIE2-15 -Ig Fc-His-tag

```
Query         1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
Query_94861   1    ............................................................  60
Query         61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
Query_94861   61   ............................................................  120
Query         121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180
Query_94861   121  ........................................I.....--DI..........  178
Query         181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240
Query_94861   179  ............................................................  238
Query         241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300
Query_94861   239  ............................................................  298
Query         301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360
Query_94861   299  ............................................................  358
Query         361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420
Query_94861   359  ............................................................  418
Query         421  VWVCSVNTVAGMVEKPFNISVKNSGTDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS  480
Query_94861   419  ............................................................  478
Query         481  RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL  540
Query_94861   479  ............................................................  538
Query         541  NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP  600
Query_94861   539  ............................................................  598
Query         601  SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN  660
Query_94861   599  ............................................................  658
Query         661  HYTQKSLSLSPGKGSGSGHHHHHH  684
Query_94861   659  ........................  682
```

3- BLAST N of Tie2-Wt-Ig Fc-His-tag and pTIE2-16 -Ig Fc-His-tag

```
Query          1    MDSLASLVLCGVSLLLSGTVEGAMDLILINSLPLVSDAETSLTCIASGWRPHEPITIGRD  60
Query_104651   1    ............................................................  60
Query          61   FEALMNQHQDPLEVTQDVTREWAKKVVWKREKASKINGAYFCEGRVRGEAIRIRTMKMRQ  120
Query_104651   61   ............................................................  120
Query          121  QASFLPATLTMTVDKGDNVNISFKKVLIKEEDAVIYKNGSFIHSVPRHEVPDILEVHLPH  180
Query_104651   121  ..............................L......I.....--DIA.........  178
Query          181  AQPQDAGVYSARYIGGNLFTSAFTRLIVRRCEAQKWGPECNHLCTACMNNGVCHEDTGEC  240
Query_104651   179  ............................................................  238
Query          241  ICPPGFMGRTCEKACELHTFGRTCKERCSGQEGCKSYVFCLPDPYGCSCATGWKGLQCNE  300
Query_104651   239  ............................................................  298
Query          301  ACHPGFYGPDCKLRCSCNNGEMCDRFQGCLCSPGWQGLQCEREGIPRMTPKIVDLPDHIE  360
Query_104651   299  ............................................................  358
Query          361  VNSGKFNPICKASGWPLPTNEEMTLVKPDGTVLHPKDFNHTDHFSVAIFTIHRILPPDSG  420
Query_104651   359  ............................................................  418
Query          421  VWVCSVNTVAGMVEKPFNISVKNSGTDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS  480
Query_104651   419  ............................................................  478
Query          481  RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL  540
Query_104651   479  ............................................................  538
Query          541  NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP  600
Query_104651   539  ............................................................  598
Query          601  SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN  660
Query_104651   599  ............................................................  658
Query          661  HYTQKSLSLSPGKGSGSGHHHHHH  684
Query_104651   659  ........................  682
```

Appendix (15)

PCR steps to replace native sequence leader of Tie2 ectodomain with CD5 leader

PCR-1 amplification reaction to add part of the CD5

Using (CD5-Tie2-5, BGH-R oligo)

PCR-2 extension reaction to add the rest of CD5

oligonucleotide using

(CD5 oligo and BGH-R)

Double Restriction digestion of PCR-2, Wt and R3-COMP

Using *Nhe* I/ *Not* I and *Eco*R-I

To get the PCR insert and vector

Ligation

Transformation and plasmid prep

Transfection and protein purification