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*Riikka Pietilä*

# ANGIOPOIETIN-1 AND -2 REGULATED TIE2 RECEPTOR TRANSLOCATION IN ENDOTHELIAL CELLS AND INVESTIGATION OF ANGIOPOIETIN-2 SPLICE VARIANT 443

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF BIOCHEMISTRY AND MOLECULAR MEDICINE;*
*BIOCENTER OULU;*
*CENTRE OF EXCELLENCE IN CELL-EXTRACELLULAR MATRIX RESEARCH*

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University of Oulu Graduate School; University of Oulu, Faculty of Biochemistry and Molecular Medicine; Biocenter Oulu; Centre of Excellence in Cell-Extracellular Matrix Research

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## *Abstract*

Angiopoietins 1 and 2 (Ang1 and Ang2) are the ligands of the Angiopoietin/Tie signalling system, which is a binary pathway offering mechanisms for healthy vessels to reach and maintain their quiescence but also to rapidly respond to activating stimuli leading to a remodelling of endothelium. The latter is linked to disease settings such as inflammation and cancer where endothelial cell (EC) integrity is compromised and is often related to an increase in Ang2 expression. This study focused on the mechanisms enabling Ang1 to mediate both EC stability and migration and molecular and cellular determinants for ligand-specific functions of Ang2 and its isoform Ang2$^{443}$.

The findings revealed that Ang1 induces differential signalling depending on whether it anchors and activates Tie2 in cell-cell junctions in quiescent ECs, or in cell-matrix contacts in mobile ECs, thus leading to cellular phenotypes characteristic of resting and mobile ECs, respectively. In the second part of the thesis Ang2-Tie2 specific cell-extracellular matrix (ECM) contact sites were studied. Formation of Ang2/Tie2 EC-ECM contact sites was dependent on the collagen I and IV matrices, low Ang2 oligomerization state, α2β1-integrins, and intact microtubules. In the third part of the thesis the comparison of Ang2 mRNA splice variant Ang2$^{443}$ with full length Ang2 (Ang2$^{FL}$) revealed both redundant and ligand form–specific effects, expression of Ang2$^{443}$ increased the amount of monomeric ligand forms due to proteolytic processing and promoted transendothelial migration of cancer cells *in vitro*. On the other hand, both Ang2$^{443}$ and Ang2$^{FL}$ were stored in endothelial Weibel-Palade bodies (WPBs), similarly induced Ang2-specific Tie2 cellular redistribution, and were mostly comparable in developmental angio- and lymphangiogenesis.

*Keywords:* alternative mRNA splicing, Angiopoietin-1, Angiopoietin-2, cell adhesion, cell-cell junctions, endothelial cell, extracellular matrix, proteolytic processing, Tie2, vasculature, Weibel-Palade body

**Pietilä, Riikka, Angiopoietiinikasvutekijä 1 ja 2 säädelty Tie2 reseptorin paikantuminen endoteelisoluissa sekä Angiopoietiini 2 isomuoto 443:n ominaisuuksien tutkiminen.**
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Biokemian ja molekyylilääketieteen tiedekunta; Biocenter Oulu; Solujen ja soluväliaineen vuorovaikutuksen huippuyksikkö
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## *Tiivistelmä*

Angiopoietiinit 1 ja 2 (Ang1 ja Ang2) ovat Ang/Tie signalointireitin kasvutekijöitä. Ang1 kasvutekijää tarvitaan sydämen ja verisuoniston sikiöaikaiseen kehittymiseen, se vähentää Tie2 reseptorin kautta verisuonten läpäisevyyttä, mutta edistää myös yksittäisten endoteelisolujen liikkumista. Saman Tie2 signalointireitin toisen kasvutekijän Ang2:n ilmeneminen johtaa verisuonten läpäisevyyden kasvuun tulehduksessa, uusien verisuonten muodostumiseen syöpäkasvaimissa ja syöpäsolujen leviämiseen elimistössä.

Väitöskirjatutkimuksessa selvitettiin niitä solutason mekanismeja, joilla Ang1 kykenee välittämään sekä endoteelisolujen tiiviyttä että liikkumista. Lisäksi tutkittiin niitä molekyyli- ja solutason mekanismeja, joilla Ang2 ja sen isomuoto $Ang2^{443}$ välittävät kasvutekijäspesifisiä vaikutuksiaan.

Väitöskirjassa osoitettiin että Tie2 reseptori paikantuu verisuonten endoteelisoluissa Ang1 sitoutumisen seurauksena joko solu-soluliitoksiin, tai yksittäisissä endoteelisoluissa solu-soluväliaine rajapinnalle. Tie2:n siirtyminen solu-soluliitoksiin aktivoi soluissa signalointireittejä, jotka ovat tyypillisiä normaaleille tiiviille verisuonille ja solu-soluväliaineliitoksissa liikkuville endoteelisoluille tyypillisiä piirteitä.

Väitöskirjatyön toisessa osassa tutkittiin Ang2:lle ominaisia vaikutuksia ja Ang2-Tie2 kompleksin paikantumista erityisiin solu-soluväliaineliitoksiin. Tämä oli riippuvaista Ang2:n oligomerisaatiosta, kollageenisoluväliaineesta, α2β1-integriinistä ja normaalista mikrotubulusverkostosta.

Väitöskirjatyön kolmannessa osassa osoitettiin että $Ang2^{443}$ isomuodolla on sekä yhteisiä että isomuotospesifisiä piirteitä verrattuna kokopitkään Ang2:een ($Ang2^{FL}$). Liukoinen $Ang2^{443}$, mutta ei $Ang2^{FL}$, esiintyi yleisesti monomeerisenä ligandimuotona proteiinin multimerisaatio-osan pilkkomisen seurauksena. $Ang2^{443}$ lisäsi myös syöpäsolujen liikkumista endoteelisolujen läpi. Toisaalta sekä $Ang2^{443}$ että $Ang2^{FL}$ varastoitiin endoteelisoluissa Weibel-Palade varastokappaleisin, ne välittivät samanlaista Tie2 reseptorin paikantumista endoteelisoluissa ja toimivat pääsääntöisesti samanlaisina kasvutekijöinä veri- ja imusuonten kehityksen aikana hiiressä.

*Asiasanat:* Angiopoietiini-1, Angiopoietiini-2, endoteelisolu, mRNA:n vaihtoehtoinen silmukointi, proteolyyttinen muokkaus, solu-soluliitokset, soluväliaine, Tie2, verisuonisto, Weibel-Palade kappale

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Oulu, Finland, April 2015 *Riikka Pietilä*

# Abbreviations

| | |
|---|---|
| α-SMA | α-smooth muscle actin |
| ABIN-2 | A20 binding inhibitor of NF-kappaB activation-2 |
| Akt | protein kinase B |
| ALI | acute lung injury |
| Ang | angiopoietin |
| Angptl-4 | Angiopoietin-like protein-4 |
| $Ang2^{FL}$ | full-length Ang2 |
| $Ang2^{443}$ | Ang2 splice variant lacking exon 2 |
| bFGF | basic-fibroblast growth factor |
| BM | basement membrane |
| CAF | cancer associated fibroblast |
| CCOD | coiled-coil oligomerization domain |
| CLL | chronic lymphocytic leukaemia |
| CNS | central nervous system |
| Col I | collagen I |
| Col IV | collagen IV |
| COMP | cartilage oligomeric matrix protein |
| Dll-4 | Delta-like 4 |
| DNA | deoxiribonucleic acid |
| dpc | days post coitum |
| DR | diabetic retinopathy |
| DSCR1 | Down syndrome candicate region 1 |
| EC | endothelial cell |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| EGFP | enhanced green fluorescent protein |
| ELISA | enzyme-linked immunosorbent assay |
| EndMT | endothelial-mesenchymal transition |
| eNOS | endothelial nitric oxide synthase |
| EPC | endothelial progenitor cell |
| ERK | extracellular signal-regulated kinase |
| FA | focal adhesion |
| FAK | focal adhesion tyrosine kinase |
| Grb | growth factor binding partner |
| HPTP-β | human protein tyrosine phosphatase-beta |

| | |
|---|---|
| HBV | hepatitis virus B |
| HUVEC | human umbilical vein endothelial cell |
| ICAM-1 | intercellular adhesion molecule 1 |
| Ig | immunoglobulin |
| kDa | kilo dalton |
| LPS | lipopolysaccaride |
| MAPK | mitogen activated protein kinase |
| MMP | matrix metalloprotease |
| MMTV | mouse mammary tumour virus |
| MT | microtubule |
| NFAT | nuclear factor of activated T-cells |
| NG2 | chondroitin sulfate proteoglycan 4 |
| NO | nitric oxide |
| PAR-1 | protease activated receptor 1 |
| PC | pericyte |
| PCR | polymerase chain reaction |
| PDGFB | platelet-derived growth factor B |
| PDGFR-β | platelet-derived growth factor receptor beta |
| PECAM-1 | platelet endothelial cell adhesion molecule-1 |
| PI3K | phosphoinositide 3-kinase |
| PKC | protein kinase C |
| PMA | phorbol-12-myristate-13-acetate |
| PyMT | polyoma middle T |
| Rip1 | rat insulin promoter 1 |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcription polymerase chain reaction |
| RTK | receptor tyrosine kinase |
| SCD | super-clustering domain |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| Shp2 | SH-domain containing tyrosine phosphatase 2 |
| shRNA | short hairpin RNA |
| STAT | signal transducer and activation of transcription |
| TAg | SV40 large T antigen |
| TIE | tyrosine kinase with Ig and EGF homology domains |
| TIRF | total internal reflection fluorescence |
| TNF-α | tumour necrosis factor- α |
| VE-cadherin | vascular endothelial-cadherin |

| | |
|---|---|
| VCAM-1 | vascular cell adhesion molecule-1 |
| VEGF-A | vascular endothelial growth factor-A |
| VEGFR | vascular endothelial growth factor receptor |
| VE-PTP | vascular endothelial receptor tyrosine phosphatase |
| VM | venous malformation |
| vSMC | vascular smooth muscle cell |
| vWF | von Willebrand factor |
| WPB | Weibel-Palade body |
| ZO-1 | zonula occludens-1 |

# List of original papers

This thesis is based on the following papers, which are referred throughout the text by their Roman numerals:

I Saharinen P, Eklund L, Miettinen J, Wirkkala R, Anisimov A, Winderlich M, Nottebaum A, Vestweber D, Deutsch U, Koh GY, Olsen BR, Alitalo K (2008). Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol 10(5):527–537.

II Pietilä R, Nätynki M, Tammela T, Kangas J, Pulkki KH, Limaye N, Vikkula M, Koh GY, Saharinen P, Alitalo K, Eklund L. (2012) Ligand oligomerization state controls Tie2 receptor trafficking and angiopoietin-2-specific responses. J Cell Sci 1;125 (Pt 9):2212–2223.

III Pietilä R, Laitakari A, Elamaa H and Eklund L (2015) Angiopoietin-2 splice variant $Ang2^{443}$ promotes tumour cell migration and is proteolytically processed resulting in variant-specific monomeric ligand form. Manuscript.

# Table of contents

# 1 Introduction

The processes involved during the formation and remodelling of blood vessels are essential for embryonic development and for fundamental events in many physiological and pathological settings in adults. Tie receptors and their angiopoietin ligands combined with the family of vascular endothelial growth factors (VEGF) and their receptors control the angiogenesis from the formation of the first undifferentiated vessels to the maturation of a highly hierarchical vasculature. They further play an important role in many physiological and pathological conditions after birth.

The Ang/Tie signalling system is a receptor tyrosine kinase pathway which in humans consists of two receptors (Tie1 and Tie2) and three ligands (Ang1, Ang2 and Ang4) which act either as Tie2 agonists or antagonists in a context dependent manner. Ang1 is the primary activating ligand of Tie2 and induces increased endothelial cell (EC) adhesion, vascular structural integrity, and inhibition of vascular permeability in maturating vessels (reviewed in Augustin *et al.* 2009). Ang2 expression in ECs is tightly regulated and is normally detected during vascular remodelling and EC activation such as in wound healing (Staton *et al.* 2010), inflammation Roviezzo et al. 2005), sepsis (Orfanos *et al.* 2007), and in many types of cancers (Thurston & Daly 2012). In the endothelium Ang2 is thought to convert quiescent vessels into an activated state ready to respond to additional signals that promote sprouting angiogenesis.

Goals of this thesis were to gain a better understanding of the spatial regulation of angiopoietin signalling and Tie2 activation in vascular ECs, elucidate the complexity of Ang2 signalling at the molecular level, and to characterize a somewhat poorly studied Ang2 isoform $Ang2^{443}$ to learn whether it has functions or an expression pattern distinct from full-length Ang2.

The results show that the mechanism by which Ang1 anchors Tie2 receptors from adjacent cells into cell-cell junctions to induce Tie2 activation is unique amongst ligand/receptor systems and that the signal outcome is altered by cell confluence. This leads either to cell migration, or resting phenotype of ECs in a context-dependent manner. Results from Ang1 studies raised a fundamental question about spatial regulation of Tie2 activation by Ang1 and Ang2. The second part in this thesis showed that Ang2 regulates Tie2 localization and function. This regulation is context dependent, differs between Ang1 and Ang2 and the extracellular matrix as well as the oligomerization state of Ang2 ligand are specific mechanisms for Tie2 translocation into novel cell-matrix contacts.

The third part concentrated on Ang2 isoform Ang2$^{443}$ in mediating the following Ang2 specific effects: ligand intracellular storage, Tie2 translocation and tumour cell migration, as well as its effects during embryonic development and in the context of retina postnatally.

Ang2$^{443}$ was found to behave in a largely similar way as full-length Ang2 in the context of ligand storage and Tie2 translocation and activation. Ang2$^{443}$ was also stored into EC-specific storage organelles known as Weibel-Palade bodies (WPB). It translocated and activated Tie2 in the cell-matrix contacts and promoted *in vitro* tumour cell migration. Due to proteolytic processing there was an increase in the monomeric forms of Ang2$^{443}$, which may broaden the ligand functions. Domain mediating Ang2 interaction with vWF and localization to WPBs was found to lie in the C-terminus of Ang2. It was also demonstrated that both Ang2 isoforms colocalize with the well-known WPB protein P-Selectin, unlike reported before (Fiedler *et al.* 2004). *In vivo* studies using a novel mouse model expressing solely Ang2$^{443}$ showed that these mice develop normally and show only a slight decrease in retinal vascular area but no obvious defects in lymphatic vessels, which are both compromised in mice lacking Ang2.

# 2 Review of the literature

## 2.1 Development of vasculature

During embryonic development the cardiovascular system is one of the first organ systems to develop through several complex processes involving interaction between distinct cell lineages, the extracellular matrix (ECM), and several growth factors. ECs are at the centre of the process which organizes the heart, arteries, veins and capillaries to ensure the supply of nutrients and oxygen to developing tissues and organs. The formation of the embryonic vasculature and its expansion is divided into two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis is mainly restricted to very early embryonic development and refers to *in situ* differentiation and growth of blood vessels from mesodermal derived hemangioblasts, whereas sprouting angiogenesis is the main mechanism of vessel growth during later stages of development and after birth. The formation of mature, fully functional vasculature involves the interaction of ECs with the supportive periendothelial cells and their shared basement membrane (BM). VEGF-A and angiopoietins, representing two families of potent angiogenic growth factors, are via their receptors important molecules to control blood vessel formation, growth, and remodelling (Chung & Ferrara 2011, Jones *et al.* 2001).

### *2.1.1 Vasculogenesis*

Vasculogenesis, or *de novo* blood vessel formation (Risau *et al.* 1988), forms the heart and the first primitive vascular plexus within the embryo and also the yolk sac vasculature representing an extra embryonical membrane. During early embryonic development, in mice around 7.5 days post coitum (dpc), vascularization of the yolk sac takes place (Palis *et al.* 1995). The progenitor cells called hemangioblasts which will eventually give rise to both endothelial and hematopoietic cells migrate from the posterior primitive streak into distinct locations and assemble into aggregates called blood islands and start to proliferate and fuse to form the primitive, undifferentiated microvessel plexus (Chung & Ferrara 2011, Conway *et al.* 2001).

Vasculogenesis within the embryo itself starts in a similar manner. EC precursors called angioblasts that have migrated from the primitive streak aggregate directly into the dorsal aorta or cardinal vein and form a *de novo*

vascular network. Angioblast migration and differentiation into mature EC, and the formation of blood islands in general, is mediated largely by VEGF-A with its receptor vascular endothelial growth factor receptor 2 (VEGFR-2) (Shalaby *et al.* 1995, Eichmann *et al.* 1997, Ash & Overbeek 2000, Hiratsuka *et al.* 2005). After the first primitive plexus is formed it undergoes a complex remodelling process where growth, migration, sprouting, and pruning of vessels give rise to a functional circulatory system in a process termed angiogenesis.

### *2.1.2 Angiogenesis*

Angiogenesis is defined as the remodelling and differentiation of the primitive plexus of microvessels formed during embryonic vasculogenesis or postnatally as the formation of new blood vessels from pre-existing ones. The formation of neovessels can occur either via sprouting, intussusceptive growth (Burri & Tarek 1990), or via homing of endothelial progenitor cells (EPCs) into the vessel wall (Asahara & Kawamoto 1997). Both sprouting angiogenesis and intussusceptive growth are thought to occur during embryonic development and after birth. In intussusceptive growth, also known as splitting angiogenesis, the vessel wall extends to the lumen and causes vessel splitting. It is regarded as a rapid way of local neoangiogenesis since it does not require EC proliferation or migration. Sprouting angiogenesis on the other hand involves distinct, highly controlled steps of vessel destabilization, sprouting, proliferation, and maturation, each of which are regulated via several signalling pathways allowing vessel growth into avascular areas in response to angiogenic stimuli (Ausprunk & Folkman 1977, Chung & Ferrara 2011). Genetic gain and loss of function studies have identified the angiopoietin-Tie2 signalling axis as one important regulator of the remodelling and expansion of premature vasculature during embryonic development and also as a signalling pathway needed to regulate postnatal vessel remodelling and sprouting angiogenesis. The mechanisms of sprouting angiogenesis are discussed in more detail in the next chapter. Figure 1 schematically illustrates EC specification during sprouting angiogenesis (A) and *in vivo* in the context of retina (B).

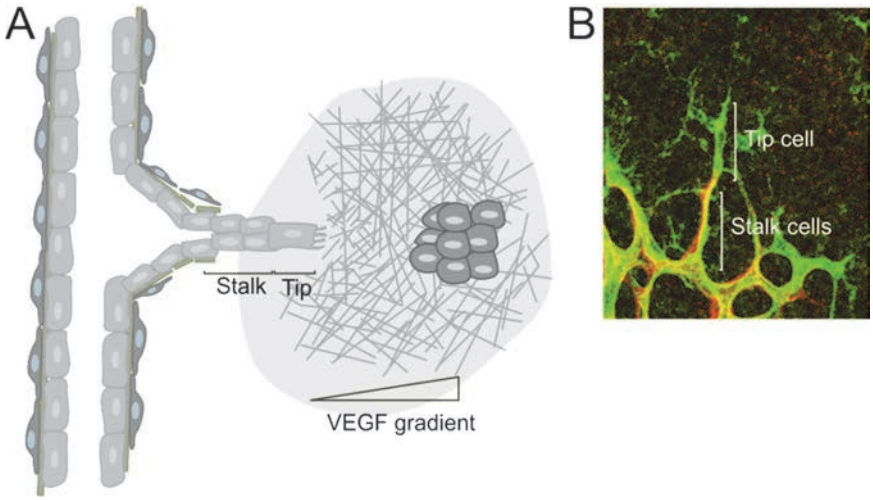


**Fig. 1. Endothelial cell specification during sprouting angiogenesis. (A) A schematic representation showing EC specification in a sprouting vessel. Tip cell with filopodial extensions guides the migration sensing ECM-bound VEGF-A gradient from the hypoxic site. Following stalk cells with forming basement membrane proliferate, form the lumen, and become stabilized as pericytes are recruited around the newly formed vessel. (B) EC specification visualized *in vivo* in the developing retina. The tip cell with filopodial extensions is stained only with isolectinB4 (green) to visualize ECs, whereas the following stalk cells show prominent expression also for BM protein Col IV (red).**

### *Vessel destabilization, sprouting, and maturation*

Cellular hypoxia, resulting from cell or tissue expansion and insufficient blood circulation, is the most important physiological factor driving either embryonic of postnatal angiogenesis. Postnatal physiological angiogenesis occurs during wound healing, endometrial growth in a menstrual cycle, or following tissue grafting and healing after injuries. Pathological angiogenesis can be induced by inflammation, tumour growth, or in a diabetic retinopathy (DR). It involves many of the same processes as physiological angiogenesis (Antonetti *et al.* 2012, Chung & Ferrara 2011, Chung *et al.* 2010).

Hypoxia induces the expression and secretion of VEGF-A (Shweiki *et al.* 1992), which can be regarded as the main molecular determinant to promote angiogenesis via binding to VEGFR-2. It has several direct stimulating effects on quiescent as well as angiogenic vessels. VEGF-A (in humans VEGF-165)

secreted from the hypoxic site diffuses into the ECM and creates a spatial concentration gradient which ECs use later as a migration guide to invade into avascular hypoxic areas (Ruhrberg *et al.* 2002). VEGF-A initiates vessel destabilization by increasing the permeability of EC-cell junctions for instance via phosphorylation of focal adhesion tyrosin kinase (FAK), leading to vascular endothelial Cadherin (VE-Cadherin) and β-catenin dissociation from junctions and an EC junctional breakdown (Chen *et al.* 2012). The expression of Ang2 in the ECs is also induced by VEGF-A, and the hypoxia response (Oh *et al.* 1999).

To facilitate vessel destabilization ECs also start to secrete factors that promote the degradation of the vascular basement membrane (BM). This occurs for instance via induction of various matrix metalloproteases (MMPs). After the endothelium at the angiogenic site has been sensitized for angiogenic sprouting via destabilization, the ECs undergo specific cellular determination as illustrated in Figure 1. Single ECs differentiate into mobile, so-called tip cells forming the leading edge of each individual vascular sprout. The function of the following ECs, referred to as stalk cells, is to proliferate and induce the formation of capillary lumen. Many factors have been identified that regulate this process of specification. The extracellular gradient of matrix bound VEGF-A isoform serves as a guidance queue for endothelial tip cells that express its receptor VEGFR-2, thus promoting the directionality of their filopodial extensions (Gerhardt *et al.* 2003, Ruhrberg *et al.* 2002), whereas Notch/ Delta-like (Dll) signalling is involved in the selection of tip cells and stalk cells. The EC that has the highest expression of Notch ligand Dll 4 in response to VEGF-A is selected as a tip cell activating Notch signalling in ECs forming the sprout to suppress their migration and sprouting, and hence destines these EC to become stalk cells (Hellström *et al.* 2007, Suchting *et al.* 2007). Specific proteins are expressed by endothelial tip cells that regulate ECM degradation, BM formation, and stalk cell behaviour (del Toro *et al.* 2010). Membrane type 1-MMP (MT1-MMP) is an example of tip cell originating secreted ECM modifying enzyme degrading ECM that allows sprout elongation and becomes down-regulated when stalk cells come into contact with pericytes (Yana *et al.* 2007, Lee *et al.* 2005, Lafleur *et al.* 2002).

When the tip cell in a growing sprout meets other sprouting vessels, cell-cell contacts are formed and forming vessels fuse together in a process called anastomosis. For example it has been suggested that macrophages play a role in this process by serving as a platform to introduce two vessel sprouts and allowing initial cell-cell contacts, although anastomosis is shown to occur also in the complete absence of macrophages albeit in a lower frequency (Fantin *et al.* 2010).

At the molecular level tip cell-macrophage interactions at sites of sprout fusion are not well understood. Possible candidate pathways include Notch signalling in the context of the retina (Outts *et al.* 2011) and the Ang2-Tie2 axis in tumours (de Palma *et al.* 2005).

The proliferation of stalk cells is necessary for the sustained growth of a newly forming vessel. Another important function of the stalk cells is the establishment of vascular lumen. This has been suggested to occur via different mechanisms based on observations in different model organisms or vascular beds. The first two models suggest the involvement of vacuoles, either intracellular vacuole coalescence involving only single ECs, or intercellular vacuole exocytosis (Kamei *et al.* 2006, Blum *et al.* 2008). A third model suggests apical membrane repulsion. In this model initial apical-basal EC polarity is established by VE-Cadherin following CD-34 sialomucin localization into cell-cell contacts. Negative charges of sialomucin would then drive the electrostatic repulsion and separation of apical membranes and the relocation of apical membrane proteins into lateral membranes (Strilic *et al.* 2009).

The final step in sprouting angiogenesis involves the recruitment of periendothelial cells around vessels to stabilize the capillary tubes. As vessels elongate the stalk cells become associated with pericytes and vascular smooth muscle cells (vSMCs) promoting a decrease in EC proliferation, migration, and vessel leakage. This has been shown to depend on signalling pathways like platelet-derived growth factor-B (PDGFB) and its receptor platelet-derived growth factor-receptor β (PDGFR-β). PDGFR-β expressing pericyte precursor cells either proliferate in the vicinity of sprouting ECs, or mature pericytes from a distance migrate in response to EC derived PDGFB surrounding newly formed vessel (Hellström *et al*. 1999). Pericytes express and secrete also Ang1 (Davis *et al.* 1996), which in turn activates Tie2 in ECs. The role of Ang1-Tie2 signalling has been considered crucial for the maintenance of endothelial integrity in terms of pericyte recruitment. However, a mouse line with global Ang1 deletion in a temporal manner revealed that while Ang1 plays a crucial role in the early development of vasculature, its deficiency does not affect pericyte recruitment in later stages of development (Jeansson *et al.* 2011).

## 2.2 Architecture of blood vessels

The structure of blood vessels differs depending on whether they are capillary vessels or large arteries or veins, but the basic constituents are the ECs, vascular

BM, and the surrounding mural cells. Thin capillaries consist solely of ECs and mural cells surrounded by the BM which separates the blood flow from the surrounding tissue. Larger vessels also have layers of smooth muscle cells in addition to the ECs and the BM to allow vessel contraction. Figure 2 illustrates the general structure of a blood vessel wall. It shows cellular sources of angiopoietin ligands as well as Tie receptors.

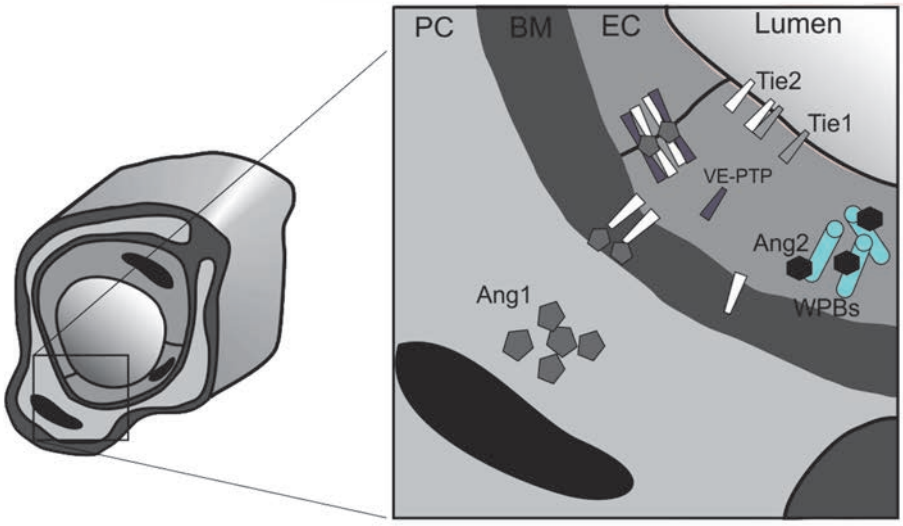


**Fig. 2. Structure of blood vessel wall showing cellular sources of Ang1 and Ang2 and Tie receptors. PC= pericyte, BM= basement membrane, EC= endothelial cell, WPBs= Weibel-Palade bodies, VE-PTP= Vascular endothelial protein tyrosine phosphatase.**

### *2.2.1 Endothelial cells*

The endothelium is the inner cellular lining of blood vessels and it plays a critical role in many physiological events such as the control of blood pressure and vascular permeability, angiogenesis, as well as innate and adaptive immunity. ECs also play an important role in diseases related to these events (Aird 2012). Depending on their localization in the body, ECs exhibit broad heterogeneity in terms of cellular morphology, function, turn-over, gene expression, and antigen composition. The normal endothelium of arteries and veins forms a continuous, uninterrupted layer of cells, whereas the endothelium in capillaries may be

continuous, fenestrated or discontinuous depending on the tissue environment. Organs that need filtration or secretion capacity, like the kidneys, the pancreas, and endocrine and exocrine organs normally display fenestrated endothelium characterized by the presence of circular pores that penetrate the endothelium and may be closed by a thin diaphragm. Discontinuous endothelium does not differ much from fenestrated endothelium except that the pores are larger in diameter and lack a diaphragm (Aird 2012).

In addition to pores, ECs also have active mechanisms to transport material across the endothelium. This transcytosis can be mediated by caveolae and vesiculo-vacuolar organelles (VVOs). Caveolae are membrane-bound, flask-shaped vesicles around 70 nm in diameter that open to the luminal or abluminal side of the cell formed by caveolin-1. Caveolae rich ECs are typically found in the capillary endothelium, but they also occur less frequently in arteries, arterioles, veins and venules, and in very low amounts in the blood-brain barrier (Simionescu *et al.* 2002). VVOs are characteristic for venular endothelium and comprise focal collections of membrane-bound vesicles and vacuoles (Dvorak & Feng 2001).

Since one of the most important roles of the ECs is to separate blood from underlying tissues they need to be tightly connected to each other and to their ECM. This is accomplished via tight cell-cell junctions and cell-matrix adhesions. ECs have two types of junctions similar to the adherens junctions (AJ) and tight junctions (TJ) of epithelial cells with different molecules but common features. The main transmembrane protein in EC AJs is VE-Cadherin (Breviario *et al.* 1995) and in TJs claudin-5 (Morita *et al.* 1999). In both junction types adhesion is accomplished via homophilic interactions of transmembrane proteins forming a zipper-like structure along the cell-cell border (Dejana 2004).

For vascular ECs adhesion to their underlying matrix is important for the regulation of apicobasal polarity, migration, proliferation, and survival. EC-ECM adhesions are mediated by integrins and other adaptor and signalling proteins that link the EC interior actin cytoskeleton to ECM proteins such as fibronectin, vitronectin, and various collagens. Currently the focal adhesions (FAs) and their variants fibrillar adhesions and focal complexes are the best characterized cell-ECM adhesions that are used by cells to sense and perceive external cues and forces. Over 50 proteins have been identified that localize into these adhesion sites and many others that affect them, even though they do not have a direct association with FA proteins. Typically cells first form adhesive contacts at the edge of their lamellipodia when coming into contact with ECM during cell

spreading or migration. The first contacts are usually dot-like focal complexes that are either transient or can further mature into FAs. The turnover of FAs is evident in migrating cells where FAs are disassembled at the leading edge to promote the formation of new protrusions and adhesions, and at the rear of the cell to induce rear retraction (Davis & Senger 2005, Geiger *et al.* 2001, Webb *et al.* 2002).

Since blood ECs are the gatekeepers of the vascular barrier there is a demand for rapid EC responses to elicit for instance an inflammatory response or wound healing. To ensure a rapid response ECs have secretory storage organelles called Weibel-Palade bodies (WPB) (Weibel and Palade 1964) for proteins that contribute to inflammation, angiogenesis, and tissue repair. WPBs are tubular structures and their main constituent is von Willebrand factor (vWF) (Wagner *et al.* 1982). vWF is a large multimeric protein essential for hemostasis and blood clotting. It also regulates inflammation by modulating leukocyte extravasation (Petri *et al.* 2010). vWF is constitutively released to circulation but also stored within WPBs, from where it can be rapidly released upon stimulation. Various other proteins have been shown to be recruited to WPBs in trans-Golgi network likely via interaction with vWF. These proteins include P-Selectin, a transmembrane protein serving as a leukocyte receptor mediating their rolling on ECs, osteoprotegerin, and Ang2 (Valentijn *et al.* 2011).

### *2.2.2 Vascular basement membrane*

Vessels ensure their quiescent state by attaching ECs tightly to each other but also to their underlying matrix. This specialized form of ECM, vascular BM, is a dynamic, three-dimensional layer of proteins, glycoproteins, and proteoglycans that provides critical support for the vascular endothelium but also regulates aspects of the activated ECs such as migration, proliferation, and survival (Kalluri 2003).

ECs in normal vessels are typically associated with continuous BM. It is assumed that endothelial and periendothelial cells themselves primarily synthesize and deposit the vascular BM components when they come into close contact with each other during angiogenesis. The BM components include collagen type IV, laminin isoforms, heparin sulphate proteoglycan perlecan, fibronectin, and entactin/nigogen. These matrix components bind various growth factors and receptors from endothelial and periendothelial cells to mediate communication between the two cell types. It is noteworthy that different cell

types secrete different patterns of matrix proteins and hence not all vascular BMs are the same (Davis & Senger 2005).

Collagen types XV and XVIII are in the minority in vascular BM (Tomono *et al.* 2002) when compared to collagen type IV, which is the most abundant collagen component of vascular BMs and, together with laminin, they contain all the information within their primary structure to allow the initiation of intermolecular self-assembly and the formation of sheet-like structures (Davis & Senger 2005). Other BM constituents such as nidogen/entactin and perlecan are not capable of self-assembly, but especially nidogen/entactin has been shown to promote collagen type IV and laminin network formation. Laminin isoforms found in endothelial and perivascular BMs include laminins 211, 411 and 511 (Yousif *et al.* 2013). Initially laminin polymerization initiates scaffold formation on the basolateral side of cells. Laminin is anchored to the cell by receptors such as integrins. After deposition of this polymer, collagen type IV polymers become associated to this initial scaffold via nidogen/entactin bridging, which leads to the anchoring of other basement membrane constituents and the formation of a fully functional BM (Yurchenco 2011).

### *2.2.3 Mural cells*

Periendothelial or mural cell is a description used for vSMCs and pericytes covering the vessel wall. vSMCs form the muscle layer that surrounds large arteries and veins, whereas pericytes wrap around capillaries, venules, and arterioles throughout the body (Armulik *et al.* 2011). Large vessels have a multi-layered vSMC layer, especially arteries that need to deliver high pressure blood from the heart to organs and tissues, and hence the main function of vSMCs is to control the calibre of blood vessels.

Pericytes are embedded in the vascular BM. Since there is no exact way to distinguish them from vSMCs or other perivascular cells, they are normally classified using criteria such as location, morphology, and protein markers. Pericytes can be found in all blood, but not in normal lymphatic microvessels (Podgrabinska *et al.* 2002) in a variable abundance.

The central nervous system (CNS) represents a classic, high integrity vascular bed with high pericyte coverage. It is estimated that in the CNS the EC to pericyte ratio is 1:1 to 3:1 and covers approximately 30% of the abluminal surface. In normal tissues the EC to pericyte ratio is estimated to vary between 1:1 and 10:1. Factors influencing pericyte coverage in different vascular beds

include differences in the demand for barrier function (large in the brain), EC turn-over (low turn-over correlates with large coverage), and ortostatic blood pressure (larger coverage in lower body parts) (Armulik *et al.* 2011).

In light of varying pericyte coverage in various tissues and organs, the physiological functions of pericytes include the stabilization of vasculature, the regulation of barrier function in capillaries, as well as the control of turn-over of ECs and capillary diameter. Although the barrier function of vessels primarily reflects differences in EC characteristics, it is nowadays recognized that pericytes play a critical role in the maturation and maintenance of for instance the tightest known vascular barrier: the blood-brain barrier (Armulik *et al.* 2010). Pericytes regulate the blood-brain barrier at the level of EC-cell junctions by increasing the transendothelial electrical resistance. In the absence of pericytes the rate of endothelial transcytosis is increased (Armulik *et al.* 2010). At the molecular level, deregulated genes in pericyte deficient vessels included mouse VEGF-A, Ang2, and Ang1 (Armulik *et al.* 2011, Daneman *et al.* 2010).

Proteins that have been identified in pericytes and that have been used to distinguish them include *e.g.* PDGFR-β (Sundberg *et al.* 1993), chondroitin sulphate proteoglycan 4 (NG2) (Ozerdem *et al*. 2001), alpha smooth muscle actin (α-SMA) (Skalli *et al.* 1989), and Ang1 (Davis *et al.* 1996). PDGFR-β plays a critical role in the recruitment of pericytes to the newly formed vessels. Sprouting ECs secrete its ligand, PDGFB, which by signalling through PDGFR-β leads to the proliferation and migration of periendothelial cells during vessel sprouting and maturation. Knocking out either the ligand or the receptor leads to a similar phenotype in which perinatal death results from the lack of pericytes (Levéen *et al.* 1994, Lindahl *et al.* 1997).

### *2.2.4 The interstitial extracellular matrix*

Vascular ECs come into contact with interstitial ECM proteins during sprouting angiogenesis. Collagen I for instance has been shown to promote both chemotactic and haptotactic migration of ECs *in vitro*. Adhesion to the ECM during angiogenesis is also crucial for the proliferation and survival of ECs to support sprout elongation (Davis & Senger 2005).

Defined as a collection of extracellular molecules secreted by various cell types, the ECM is a non-cellular component present within all tissues and organs. It is composed of fibrillar collagen types I, II, III, V and XI as well as fibrin. It offers structural integrity for cells and tissues but functions also as a highly

dynamic platform that regulates tissue homeostasis, organ development, inflammation, and disease. The general constituents of the ECM are the same as for the BM and include fibrous proteins such as collagens, elastins, laminins, and fibronectins, as well as glycoproteins, proteoglycans, and polysaccharides. Because the ECM is locally secreted by various cell types, the exact composition varies from organ to organ and tissue to tissue. Hence different ECMs have different physical, biochemical, and biomechanical properties (Davis & Senger 2005).

## 2.3 Angiopoietin-Tie signalling pathway

Tyrosine kinase with Ig and EGF homology domains (Tie) receptors (Tie1 and Tie2) and their angiopoietin ligands together form the second largely vascular EC specific signalling pathway (Partanen *et al.* 1992, Schnurch & Risau 1993). Activity of this pathway is controlled via ligand availability and at the receptor level by regulating Tie2 receptor activity via specific phosphatases and post-translational modifications. Various aspects of the Tie2 receptor are discussed below, but the cellular functions and signalling outcomes of activated Tie2 are discussed in more detail in the context of Ang1 and Ang2 and ligand-specific Tie2 signalling in following chapters.

### *2.3.1 Tie2*

#### *Structure and expression*

Tie receptors are single-pass type I transmembrane receptor tyrosine kinases (RTKs) with their N-terminus facing the extracellular space. Tie2 contains in its ectodomain two immunoglobulin (Ig) domains followed by three epidermal growth factor (EGF) domains and an Ig domain before three fibronectin type II repeats (Barton *et al.* 2006). In its cytoplasmic tyrosine kinase catalytic domain Tie2 has several tyrosine residues that have been implicated in mediating signalling upon receptor activation. These autophosphorylation sites include tyrosine Y992 in the activation loop. In mice, tyrosine 1101 mediates binding of the p85 subunit and activation of PI3K and Akt (Kontos *et al.* 1998). Tyrosine 1107 mediates Dok-R binding (Jones *et al.* 2003) and tyrosine 1112 that was shown to mediate binding of Shp2 (Peters *et al.* 2004). Phosphorylated Y897 is

implicated in Tie2 auto-inhibition (Shewchuk *et al.* 2000). A schematic presentation of Tie2 structure with indicated domains and main tyrosine residues is shown in Figure 3. The ligand binding domain in Tie2 is confined to the top of the Tie2 Ig2 domain. No major conformational changes or domain rearrangements are reported in Tie2 ectodomain upon ligand binding (Barton *et al.* 2006).

Tie2 is mainly expressed in vascular and lymphatic (Tammela *et al.* 2005) ECs as well as in certain non-ECs, such as the circulating haematopoietic cells including a sub-population of monocytes (De Palma *et al.* 2005). Tie2 distribution in adult tissues shows constitutive expression and phosphorylation in many tissues such as heart, lung, liver, and kidney in rats (Wong *et al.* 1997). Its distribution in normal microvascular endothelium has been shown to differ between arteries, veins, and capillaries in mouse mesentery. On the other hand in diaphragm Tie2 expression is the strongest at the arterial side, weak or non-detectable at the venular side, while the capillaries present a mosaic pattern of expression (Anghelina *et al.* 2005).

Hypoxia and pro-inflammatory growth factors like tumour necrosis factor alpha (TNFα) are shown to induce Tie2 protein expression in vascular ECs (Willam *et al.* 2000). At the protein level one mechanism to modulate Tie2 signalling is ectodomain shedding, where the extracellular ligand binding domain of Tie2 can be proteolytically cleaved as a response to phorbol esters and VEGF. The result is a 75 kDa soluble protein (Findley *et al.* 2007, Reusch *et al.* 2001). Mechanistically Tie2 ectodomain cleavage induced by VEGF has been shown to involve the phosphoinositide 3 kinase (PI3K) /protein kinase B (Akt) pathway (Findley *et al.* 2007). Proteases identified to mediate this process include MMP-14 (Onimaru *et al.* 2010) and Epithin/PRSS14 (Kim *et al.* 2011). Interestingly, Tie2 ectodomain shedding was shown to increase truncated Tie2 activity in the absence of angiopoietin ligands. This suggests that in steady state conditions the ectodomain might serve as an autoinhibitory structure preventing autophosphorylation. Upon ligand stimulation the autoinhibitory site becomes relieved via a conformational change, but upon ectodomain cleavage the intracellular part can undergo ligand-independent dimerization and phosphorylation (Kim *et al.* 2011).

Ectodomain shedding has been shown to occur also *in vivo* since soluble extracellular Tie2 fragments can be observed in the blood (Reusch *et al.* 2001). The cleaved Tie2 ectodomain retains its ability to bind angiopoietins, trapping

them, and hence prevent full-length Tie2 activation on the cell surface (Findley *et al.* 2007).

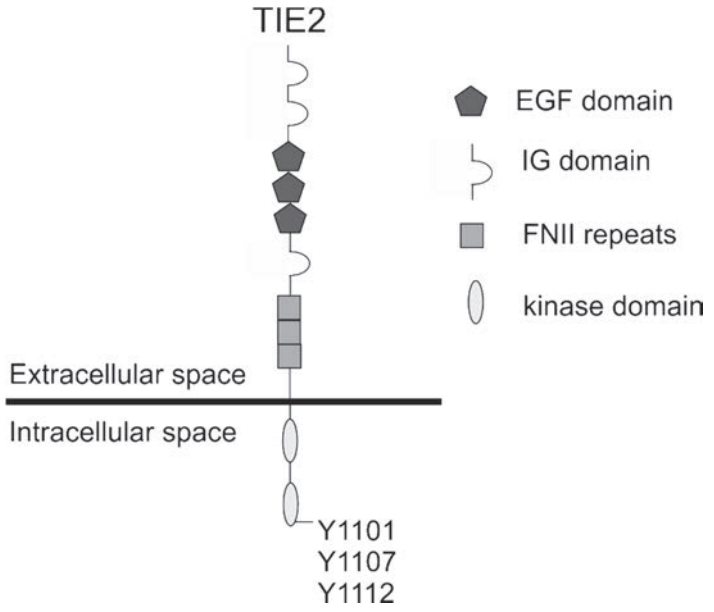


**Fig. 3. Schematic illustration of Tie2 protein structure. known tyrosine residues in mouse Tie2 that become phosphorylated upon ligand stimulation and their positions are included. Tie1 shares an identical domain structure with Tie2.**

### *Function*

Following ligand binding Tie2 dimerizes or multimerizes and becomes trans-autophosphorylated at certain tyrosine residues in its C-terminal kinase domain. In mouse Tie2, altogether 19 putative tyrosine residues can be found in the two lobes of the kinase domain (Jones *et al.* 1999). Ligand-activated Tie2 binds and activates several downstream effectors leading to various signals in the ECs, such as cell survival, migration and maintenance of barrier function leading to vascular quiescence. Tie2 binding partners and activated signalling pathways are generally illustrated in Figure 4. The main downstream signalling molecules include growth factor binding partner 2 (Grb2), Grb7, Grb14, SH-domain containing tyrosine phosphatase 2 (Shp2) (Sturk & Dumont 2010, Peters *et al.* 2004), the p85 subunit of PI3K (Kontos *et al.* 1998, Jones *et al.* 1999), and Dok-R (Jones & Dumont 1998, Jones *et al.* 2003). Following ligand binding, Tie2 has been shown to

activate via its binding partners, signalling pathways such as Akt (Kontos *et al.* 1998), extracellular signal regulated kinase (ERK) (Yoon *et al.* 2003), and FAK (Kim *et al.* 2000a). Tie2 has also been shown to activate two transcription factors from a family of signal transducers and activators (STAT). Tie2 activates STAT3 and STAT5 albeit in a weaker manner than *e.g.* VEGFR-2. Mutations in Tie2 cause the inherited disease known as venous malformation (VM), which leads to the activation of a distinct STAT transcription factor, STAT1 (Korpelainen *et al.* 1999).

A classical way to regulate RTK signalling involves receptor mediated endocytosis, where activated RTKs become rapidly internalized and ultimately sorted to lysosomes for degradation. Activated Tie2 is shown to localize to both clathrin-coated pits (Bogdanovic *et al.* 2009) as well as to caveolin-1 positive lipid rafts (Katoh *et al.* 2009, Yoon *et al.* 2003), but studies showing ligand activated Tie2 distribution along the endocytic pathway are currently lacking.

The genetic loss of function studies have revealed the importance of functional Tie2 signalling during development. Mice lacking *Tie2* gene manage to develop the primary vascular plexus but die between E10.5 and E12.5 due to insufficient expansion and maintenance of primary vessels. The plexus shows fewer ECs and branches and an incomplete number of pericytes and SMCs (Sato *et al.* 1995). Inducible gene deletion studies in adult mice suggest that Ang/Tie2 signalling is less critical in quiescent blood vasculature, but has a role in the lymphatic endothelium (Thompson *et al.* 2014, Jeansson *et al.* 2011). Mice with Tie2 overexpression in skin ECs do not develop a harmful phenotype but exhibit a significant increase in dermal vasculature, whereas excess Tie2 expression targeted towards keratinocytes leads to a psoriasis-like phenotype. This indicates that balanced and properly located Tie2 signalling is a requisite for normal embryonic and postnatal vascular development (Wolfram *et al.* 2009).

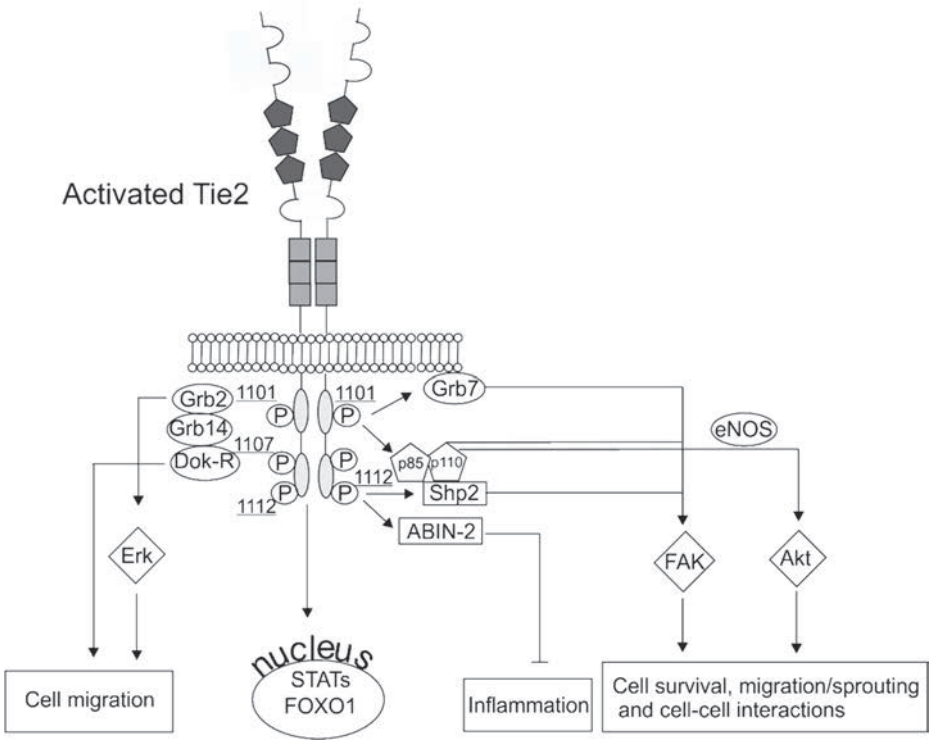


**Fig. 4. A schematic representation showing the main tyrosine residue with known binding partners of activated mouse Tie2, downsteam signalling mediators and cellular outcomes.**

### *2.3.2 Tie1*

No ligand able to directly bind and activate Tie1 has been found. Nevertheless *Tie1* deficiency causes embryonic lethality in mice (Puri *et al.* 1995, Sato *et al.* 1995). Since Tie1 has no ligand of its own, it may regulate Tie2 mediated signalling by functioning as a co-receptor. Studies have demonstrated that intracellular domains of Tie1 and Tie2 interact at the EC surface forming heterodimers (Marron *et al.* 2000). It has been proposed that Tie1 is a negative regulator of Ang1-Tie2 signalling. It has been shown that ligand-induced Tie2 phosphorylation increases when the Tie1 ectodomain is cleaved, allowing enhanced Ang1 binding to Tie2 (Hansen *et al.* 2010, Marron *et al.* 2007). Furthermore, whereas Ang1 seems to better bind Tie2 that is not in complex with

Tie1, no such requirement is shown for Ang2, where binding to Tie2 was unaffected in both Tie1 silenced as well as the Tie1 ectodomain cleaved cells (Hansen *et al.* 2010). The controversial outcome of Tie1- regulated Tie2 activity upon Ang1 and Ang2 stimulation is also reported in both vascular (Seegar *et al.* 2010) and lymphatic ECs (Song *et al.* 2012). In lymphatic ECs where Ang2 is seen as a Tie2 agonist, overexpression of Tie1 was shown to totally abolish Ang2 agonistic activity to Tie2, whereas excess Tie1 had no effect on Ang1-induced Tie2 activation (Song *et al.* 2012).

Studies using genetically modified mice where enhanced green fluorescent protein (EGFP) was placed under a *Tie1* promoter revealed that during embryonic development *Tie1* was more expressed in arterial than venous endothelium (Iljin *et al.* 2002). After birth lacZ-coupled promoter activity was observed in the arterioles, capillaries, and lymphatic cells (Iljin *et al.* 2002). Genetic studies indicate the importance of Tie1 in vascular development and vessel remodelling as well as in tumour biology. Whole body *Tie1* deficiency leads to the impairment of EC integrity causing embryonic death between E13.5 and birth (Puri *et al.* 1995, Sato *et al.* 1995). The EC defects are most profound in the capillaries. At E13.5 *Tie1* knockout embryos show no defects in their large vessels but the integrity of the microvasculature is lost and embryos die due to haemorrhages and edema (Puri *et al.* 1995). Another study demonstrates that *Tie1* deficient embryos also have defects in the lymphatic vessel development (D'Amico *et al.* 2010).

EC-specific *Tie1* deletion was shown to have a negative impact on tumour angiogenesis and growth and it also delayed normal angiogenesis of retina (D'Amico *et al.* 2014). ECs in tumour vessels were shown to have decreased survival rate and tumours in *Tie1* deficient mice were also more permissive for anti-angiogenic therapies.

At the molecular level, Tie1 knock down in cultured cells has been linked to endothelial-mesenchymal transition (EndMT) (Garcia *et al.* 2012), where ECs lose their EC markers such as CD31 and gain mesenchymal markers such as αSMA accompanied with a more motile phenotype and an increase in Col I expression. Tie1 deficiency dependent EndMT was shown to increase Erk5 phosphorylation and lead to the activation of transcription factor Slug (Garcia *et al.* 2012).

### 2.3.3 VE-PTP - a phosphatase regulating Tie2 activity

The Tie2 and VEGFR-2 that are quite selectively expressed in ECs are RTKs. Thus their activities are also regulated via mechanisms counteracting directly their kinase activity, such as via dephosphorylation by phosphatases. Vascular endothelial protein tyrosine phosphatase (VE-PTP), a mouse homolog of human protein tyrosine phosphatase beta (HPTP-β), is the only known receptor type tyrosine phosphatase that is selectively expressed in the ECs (Fachinger *et al.* 1999). It interacts directly with Tie2 (Fachinger *et al.* 1999) and VE-Cadherin (Nawroth *et al.* 2002), and Tie2 dependently with VEGFR-2 (Hayashi *et al.* 2013).

In cultured ECs VE-PTP is shown to down-regulate Ang1 and Ang2 induced Tie2 activity (Winderlich *et al.* 2009, Yacyshyn *et al.* 2009), although Ang1 is able to induce strong Tie2 activation despite of the VE-PTP activity. Ang2 on the other hand, induces very low Tie2 activation on its own, but when VE-PTP activity is blocked, Ang2 induces Tie2 activation reaching almost equal levels as Ang1 alone (Shen *et al.* 2014, Yacyshyn *et al.* 2009).

Little is known about the regulation of VE-PTP expression. Hypoxia was shown to increase VE-PTP expression both *in vitro* and *in vivo* (Shen *et al.* 2014, Yacyshyn *et al.* 2009). VE-PTP is expressed in both the arterial and the venous endothelium during embryonic development, but the predominant expression is observed mainly in the arterial side. The expression continues in adult vascular endothelium (Baumer *et al.* 2006, Dominguez *et al.* 2007). Mice null for *VE-PTP* die at E10 and exhibit many similar defects as Tie2 and Ang1 knockout mice. At E9.5 when strong remodelling of the primary plexus is ongoing in the brain and heart for example, *VE-PTP* knockout embryos show vascular defects in the areas where angiogenesis should occur (Baumer *et al.* 2006, Dominguez *et al.* 2007).

In ECs VE-PTP associates with and regulates VE-Cadherin phosphorylation (Nawroth *et al.* 2002), promoting EC-cell adhesion. Association of VE-PTP and VE-Cadherin is disrupted in inflammatory reactions to regulate vascular permeability and leukocyte transmigration. Two distinct tyrosine residues identified in VE-Cadherin that mediate these effects included T685, which increases vascular permeability, and T731, which regulates leukocyte transmigration (Vockel & Vestweber 2013, Broermann *et al.* 2011). Recently, a small molecule inhibitor (AKB-9778) against VE-PTP phosphatase activity was used to analyse the effects of VE-PTP blockage in cell culture systems, in developmental angiogenesis in zebrafish, as well as in tumour angiogenesis.

Pharmacological inhibition of VE-PTP activity led to a ligand-independent Tie2 activation and impaired developmental angiogenesis but normalized structure and function of tumour vessels (Goel *et al.* 2013). Pharmalogical inhibition of VE-PTP has been evaluated also in preclinical mouse models of retinal and choroidal neovascularization (Shen *et al.* 2014) and it has been recently tested in clinical trials in patients with diabetic macular edema (Campochiaro *et al.* 2015). In mouse models the intravitreous injection of AKB-9778 suppressed ocular neovascularization. Additionally, subcutaneous AKB-9778 also enhanced activation of the Tie2 pathway, and suppressed VEGF-induced vascular leakage and neovascularization. This indicates that the inhibition of VE-PTP and the resulting Tie2 activation may provide a new treatment strategy to stabilize retinal and choroidal blood vessels to prevent vision lost.

## 2.4 Angiopoietin ligands

Angiopoietins are secreted multimeric growth factors that were first found as ligands for Tie2, but since then have been also found to exert their effects by binding various integrins under specific conditions (Carlson *et al.* 2001). Three angiopoietins can be found in humans: angiopoietins 1, 2, and 4. They share a very similar overall structure, but differ in terms of expression pattern, efficiency for Tie2 activation, and signalling outcome. Ang1 and Ang2 are the most studied ligands in the Ang/Tie pathway mediating opposing functions on Tie2 signalling in terms of vessel integrity. Ang4 and its mouse orthologue Ang3 (Valenzuela *et al.* 1999) are less studied ligands in this pathway and little is known of their contribution to normal and pathological EC biology. Only Ang1 and Ang2 ligands and their functions are discussed below. Figure 5 illustrates the amino acid structures of Ang1 and Ang2 showing the specific protein domains characteristic for these ligands.

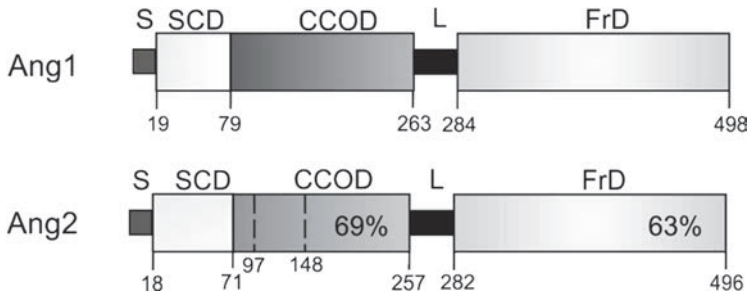


**Fig. 5. Protein structures of human Ang1 and Ang2. A short signal sequence for secretion (S) is followed by a super clustering domain (SCD) and a coiled-coil oligomerization domain (CCOD). A linker sequence (L) separates the N-terminus from a C-terminal receptor binding fibrinogen-like domain (FrD). Corresponding amino acids for each domain are shown. Dashed lines with indicated amino acids show the amino acids in Ang2 structure that are missing in Ang2$^{443}$. Percents in Ang2 CCOD and FrD domain boxes indicate amino acid identity to Ang1.**

### *2.4.1 Angiopoietin-1*

The first member of the angiopoietin family, angiopoietin 1 (Ang1), was cloned almost two decades ago in the search for a ligand for Tie2 receptor (Davis *et al.* 1996). It is a secreted, oligomeric glycoprotein inducing a strong Tie2 tyrosine phosphorylation (*i.e.* activation). Ang1 has been shown to mediate a variety of EC functions via binding and activating Tie2.

#### *Structure and expression*

All angiopoietins share a similar overall structure. They are secreted glycoproteins with different modules each having its own function. The overall structure of Ang1 is shown in Figure 5. It is a 70 kDa protein in its monomeric state with N-terminal signalling sequence ensuring protein secretion. This is followed by a small super clustering domain (SCD) that is responsible for oligomerization of various size multimers. Coiled-coil oligomerization domain (CCOD) participates in the formation of dimers, trimers, and higher order multimers. A short linker domain separates the N-terminus from the C-terminal fibrinogen-like receptor binding domain (FrD) (Davis *et al.* 2003).

When studying mechanisms of Tie2 activation in relation to Ang1 structure, it was shown that the oligomerization and multimerization of Ang1 are critical for

Tie2 binding and activation. An Ang1 form where only the linker and FrD are present is unable to either bind or phosphorylate Tie2 (Kim *et al.* 2005). Similarly, Ang1 without the SCD-domain is incapable of binding or activating Tie2. Ang1 needs to be at least a tetramer to be able to bind Tie2 (Davis *et al.* 2003).

Due to its multimeric nature, expression and purification of Ang1 for research purposes has been a tedious task. Because of the N-terminus, purified Ang1 aggregates and becomes insoluble very easily and higher oligomers have been described for Ang1 than Ang2 *in vitro*. Whether these higher oligomers exist also *in vivo* is not known however. A chimeric construct for Ang1 was developed to overcome the aggregation problem. Cartilage oligomeric matrix protein (COMP)-Ang1 has proven a competent variant to use when strong Tie2 phosphorylation levels are an object of interest. In COMP-Ang1 the oligomerization domain of Ang1 has been swapped with that of COMP. As a result, COMP-Ang1 is mainly a pentameric protein that induces stronger Tie2 activation than native Ang1 (Cho *et al.* 2004).

At the cellular level Ang1 is shown to be expressed mainly by the vascular support cells (vSMCs and pericytes) surrounding the ECs (Davis *et al.* 1996), in developing heart myocardium of both atrium and ventricle (Maisonpierre *et al.* 1997), and in certain pericytes such as the podocytes in the kidney (Satchell *et al.* 2002). Some studies also show that platelets and megakaryocytes store Ang1 and that platelet activation also induces Ang1 secretion (Li *et al.* 2001). Based on a widely published model, Ang1 activates Tie2 in a paracrine manner. Ang1 is also shown to bind to the ECM (Xu & Yu 2001) and thus pericyte derived Ang1 can be anchored to the ECM where it can bind Tie2 at the basal side of the ECs.

When it comes down to the regulation of Ang1 expression not much is known in comparison to Ang2 expression. IL-1β has been shown to down-regulate Ang1 expression in both ECs and pericytes (Fan *et al.* 2004), although ECs are not thought of as a traditional source of Ang1. Recently an organ-specific induction of Ang1 expression model was introduced in the intestine. Decreased levels of Ang1 via protease activated receptor-1 (PAR-1) are there linked to the defective vascularization of villi (Reinhardt *et al.* 2012). The actions of Ang1 are thus largely regulated via Ang2 and Tie1 or VE-PTP. Induction of Ang2 expression in ECs causes a competitive situation between the ligands leading to a decrease of Ang1 signalling in high concentrations of Ang2. Similarly, Tie1 has been shown to negatively affect Ang1 activity. Regulated ectodomain shedding of Tie1 from Tie1-Tie2 hetero complexes has been shown to increase the ability of COMP-

Ang1 to induce Tie2 activation via modulation of ligand responsiveness (Marron *et al.* 2007).

### *Function*

Ang1 is an obligatory agonist for Tie2 inducing its phosphorylation and activation and initiation of signalling cascades that have beneficial effects for the endothelium. Numerous cellular effects have been described for Ang1, namely EC migration (Cascone *et al.* 2003, Witzenbichler *et al.* 1998), tubule formation (Hayes *et al.* 1999, Kim *et al.* 2000a, Koblizek *et al.* 1998), and survival (Hayes *et al.* 1999, Kim *et al.* 2000b). Most importantly Ang1 is considered as a stabilizing factor for the maturating endothelium that helps endothelium to reach and maintain its barrier function. In cell culture systems Ang1 induces anti-permeability of ECs at a junctional level (Gamble *et al.* 2000). *In vivo* excess Ang1 results in leakage resistant vessels (Thurston *et al.* 1999) and it has been shown to counteract *e.g.* VEGF induced EC permeability (Thurston *et al.* 2000). Mechanistically this has been shown to proceed via decreasing the tyrosine phosphorylation of two well-known endothelial adhesion proteins, VE-Cadherin and the platelet endothelial cell adhesion molecule-1 (PECAM-1) and also via suppressing the dissociation of VE-Cadherin from beta-catenin, thus promoting maintenance of VE-Cadherin in EC AJs (Gamble *et al.* 2000, Wang *et al.* 2004). Because of its many protective effects regarding the endothelium, it is not surprising that Ang1 acts also as an anti-inflammatory cytokine, *e.g.* Ang1-activated Tie2 has been shown to interact with the A20 binding inhibitor of NF-kappaB activation-2 (ABIN-2) protein that mediates its anti-inflammatory effects (Hughes *et al.* 2003).

Downstream signalling pathways activated by Ang1 include PI3K/Akt that mediates its anti-apoptotic and cell survival effects (Kim *et al.* 2000b). The PI3K/Akt pathway has been shown to also induce nitric oxide (NO) production via endothelial nitric oxide synthase (eNOS) to promote Ang1-induced angiogenesis *in vitro* and *in vivo* (Babaei *et al.* 2003). Ang1-induced EC migration has also been shown to proceed via recruitment of another adaptor protein Dok-R to Tie2 (Master *et al.* 2001). Ang1 is shown as an inhibitor of a forkhead transcription factor (FKHR, also known as FOXO1) via activation of Akt (Daly *et al.* 2004), likely partially explaining its protective nature regarding the endothelium since FOXO1 target genes are known to promote EC destabilization (Daly *et al.* 2004).

The importance of Ang1 for embryonic angiogenesis has been studied using a whole mouse deletion of *Ang1* and recently by deleting *Ang1* from mice in a temporal manner during embryogenesis. Conventional *Ang1* null embryos die around E12.5 and although the vasculature has formed, they demonstrate heart defects and decreased complexity and branching of vessels with fewer ECs, dilated vessels, and less mural cells (Suri *et al.* 1996).

The conditional mouse model where *Ang1* is deleted in a temporal manner shows that Ang1 functions during embryonic development can be categorized time dependently. In conditional *Ang1* knockout mice Ang1 is shown to be a critical factor for the initiation and early steps of angiogenesis: mice where Ang1 signalling is disturbed before E10.5 die due to defects in the trabeculations in the heart similarly to conventional *Ang1* knockout mice (Jeansson *et al.* 2011, Suri *et al.* 1996). Secondly, *Ang1* deletion in a fine window between E10.5-E12.5 leads to an increase in vessel number and diameter and an overall disorganized vascular network. However, the pericyte coverage around the ECs is comparable to that of control mice. Surprisingly, *Ang1* deletion from E13.5 onwards does not cause any severe phenotype. This indicates that Ang1 is not required for the mature vasculature to maintain its anti-permeability under steady-state conditions (Jeansson *et al.* 2011). Postnatally *Ang1* deficiency leads to a compromised neoangiogenesis with faster wound closure. Wounds exhibit more fibrotic tissue, possibly due to activated TGF-β signalling (Jeansson *et al.* 2011). Collectively this suggests that Ang1 might be needed during postnatal angiogenesis to balance the growth of new vessels by stabilizing them in a stationary phase in the context of injury, but in quiescent vessels its actions are dispensable.

Interestingly, both conventional and conditional *Ang1* knockout mice demonstrate major defects in the capillary BMs of several vascular beds, suggesting the importance of Ang1 signalling for the production of ECM (Jeansson *et al.* 2011, Suri *et al.* 1996).

Effects of excess amounts of Ang1 *in vivo* have been studied using a mouse model where *Ang1* is placed under a keratin 14 promoter leading to Ang1 overexpression in skin. These mice exhibit an increase in vessel number, size, and branching (Suri *et al.* 1998), which suggests that Ang1 functions as an angiogenic cytokine also *in vivo*.

### 2.4.2 Angiopoietin-2

Expression of Ang2, unlike Ang1, is strictly controlled and is normally detected only in vessels where active remodelling occurs. In mice Ang2 is dispensable for embryonic vascular, but not for lymphatic development and is essential for postnatal vascular remodelling in the eye. Re-expression of Ang2 after birth is linked to disease states including cancer and sepsis, leading to a de-stabilization of vessels hence contributing to the pathology of these diseases.

In contrast to Ang1, Ang2 induces a weak Tie2 activation in ECs *in vitro*. It is thus classified as a Tie2 antagonist, since in the presence of Ang1, Ang2 competes for binding to Tie2 leading to a decrease in Tie2 phosphorylation and activation. In addition to Tie2, Ang2 is shown to act via other receptors including integrins in certain vascular beds. Thus actions of Ang2 are highly context-dependent and difficult to interpret *in vivo*.

#### *Structure and expression*

Ang2 is a multimeric protein with a monomeric mass of approximately 68 kDa. It has the signalling sequence, a small SCD region, and a rather large coiled-coil motif at the amino terminus. The coiled-coil motif is followed by a short linker domain that separates it from a carboxy terminal FrD that facilitates receptor binding. Unlike Ang1, Ang2 exists mainly as a disulphide-linked dimeric protein with only minor multimeric and oligomeric forms (Kim *et al.* 2009, Kim *et al.* 2005). Ang2 shares 69% and 63% homology with the corresponding Ang1 coiled-coil and receptor binding domains, respectively (Jones *et al.* 2001).

During embryogenesis and shortly after birth Ang2 expression is detected in several organs and tissues including the dorsal aorta, lungs, kidneys and pancreas (Gale *et al.* 2002, Maisonpierre *et al.* 1997). Under normal physiological conditions in adults Ang2 is detected at sites where active vascular remodelling follows the activation of the endothelium such as during the menstrual cycle (Maisonpierre *et al.* 1997) and wound healing (Staton *et al.* 2010).

Ang2 is mainly expressed by ECs. Its expression is modest in vascular ECs when compared with lymphatic ECs in culture (Podgrabinska *et al.* 2002), but angiogenic or inflammatory activation of endothelium leads to upregulation of Ang2 (Fiedler *et al.* 2004, Oh *et al.* 1999). Factors known to induce Ang2 expression in ECs include cellular hypoxia (Oh *et al.* 1999) and cytokines such as VEGF (Oh *et al.* 1999) and bFGF (Mandriota & Pepper 1998), whereas physical

stress like laminar and oscillar flow have been shown to downregulate and upregulate Ang2 expression, respectively (Li *et al.* 2014, Tressel *et al.* 2007). Shear stress was shown to induce Ang2 expression via the canonical Wnt signalling pathway (Li *et al.* 2014). At the transcriptional level Ang2 is regulated by FOXO1 so that the activated form of FOXO1 upregulates Ang2 expression, thus linking Ang1 as a negative regulator of Ang2 expression since Ang1 acts as a FOXO1 inhibitor via Akt (Daly *et al.* 2004).

In ECs Ang2 is reportedly stored in specific WPBs. It colocalizes with vWF but is excluded from WPBs containing P-Selectin (Fiedler *et al.* 2004). Ang2 from WPBs is rapidly secreted following various stimuli, including protein kinase C- (PKC) activating phorbol-12-myristate-13-acetate (PMA) and thrombin (Fiedler *et al.* 2004). Endothelial vWF was also shown to regulate the Ang2 release from ECs, since ECs devoid of vWF showed higher levels of Ang2 in cell supernatants compared with control cells (Starke *et al.* 2011). In addition to WPBs, Ang2 secretion has been shown to involve exosomes, small membrane vesicles transporting proteins, mRNAs and microRNAs, which are believed to be part of the intercellular communication machinery (Ju *et al.* 2014). Exosomal secretion of Ang2 was shown to be increased following Akt or eNOS inhibition (Ju *et al.* 2014).

### *Function*

Since its discovery Ang2 has been regarded as a negative regulator of Ang1/Tie2 signalling and classified as a Tie2 antagonist. It is considered a competitor of Ang1 for Tie2 binding and as a result it interferes with Ang1 induced Tie2 phosphorylation and causes a decrease in Tie2 activation (Maisonpierre *et al.* 1997). *In vivo* this is thought to affect EC stabilization, leading to weakened EC integrity as illustrated in Figure 6. Ang2 is indeed a weaker Tie2 agonist in vascular ECs when compared to Ang1 (Maisonpierre *et al.* 1997, Yuan *et al.* 2009). Interestingly, if Tie2 is ectopically expressed in non-ECs, Ang2 induces similar Tie2 activation as Ang1. This suggests that there are other EC-specific factors that contribute to the weak agonistic nature of Ang2 (Maisonpierre *et al.* 1997). Binding affinity of Ang2 to Tie2 has been shown to be equal to that of Ang1 (Maisonpierre *et al.* 1997) and hence receptor affinity does not explain the modest effect of Ang2 on Tie2 activation. Tie1 is either shown not to have a regulatory role in Ang2 induced Tie2 activation, since in the presence or absence of Tie1 Ang2 exhibits similar kinds of kinetics for Tie2 activation (Hansen *et al.*

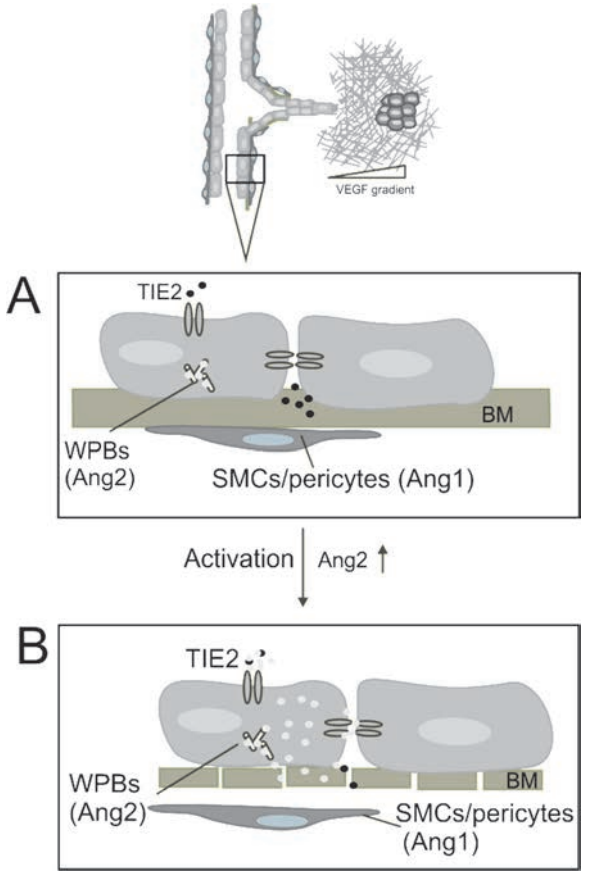


**Fig. 6. Illustration of Ang2 actions in blood endothelium. (A) In quiescent vessels Ang2 that is expressed at low levels is stored into WPBs. (B) Activation of endothelium (following hypoxia or inflammatory response for example) leads to an upregulation of Ang2 expression and exocytosis from WPBs. Ang2 replaces Ang1 as a Tie2 ligand leading to decreased Tie2 activation and increased junctional permeability. Ang2 induced detachment of pericytes and degradation of the basement membrane at EC-ECM contact sites lead to EC apoptosis in the absence, but to a sprouting angiogenesis in the presence of other angiogenic growth factors such as VEGF.**

2010), or reported as a negative regulator of Ang2 induced Tie2 phosphorylation (Seegar *et al.* 2010). Thirdly, in ECs where VE-PTP actions are prevented using a blocking antibody, Ang2 induces Tie2 activation almost equal to Ang1, suggesting that Ang2 cannot overcome the inhibitory effect of VE-PTP to Tie2 as well as Ang1 (Shen *et al.* 2014).

Findings from genetically modified mice highlight the complexity of the Ang2 antagonistic/agonistic nature. Whole mouse *Ang2* deletion has been shown to lead to a variable phenotype depending on the genetic background. Mice with a mixed C57BL/6 and 129/J background were shown to die within two weeks after birth (Gale *et al.* 2002) with more severe phenotypes when compared to mice with a C57BL/6 -background that survive until adulthood and which show low mortality (Dellinger *et al.* 2008). Ang2 null mice in both genetic backgrounds show lymphatic valve defects and abnormalities in mesenteric lymphatics resulting in chylous ascites. These lymphatic defects can be rescued by inserting *Ang1* into *Ang2* locus (Gale *et al.* 2002), which indicates that in lymphangiogenesis Ang2 functions as a Tie2 agonist. Deletion of *Ang2* also affects the blood vasculature during embryogenesis in kidneys (Pitera *et al.* 2004) and in the postnatal eye, in which hyaloid vessel regression is delayed and the growth of retinal vessels is defective. Interestingly, *Ang1* cannot complement *Ang2* functions in the retina (Gale *et al.* 2002, Hackett *et al.* 2002, Maisonpierre *et al.* 1997). This suggests ligand specific functions in the blood vasculature of the eye. Ang2 has also been shown to regulate the transformation and integrity of lymphatic EC junctions (Zheng *et al.* 2014). Furthermore, loss of both *Ang1* and *Ang2* leads to an enhanced phenotype in specific lymphatic vessels in the Schemm´s canal (a specialized vascular structure in the eye) demonstrating the cooperation between ligands in certain vascular beds (Thomson *et al.* 2014). On the other hand, excess Ang2 leads to similar vascular defects as caused by the targeted disruption of *Tie2* or *Ang1*: mice die at E9.5-10.5 with phenotypes being rounded ECs which have poor interaction with the underlying matrix (Maisonpierre *et al.* 1997). *In vivo* studies have also shown that both in normal and diabetic retina local overexpression of Ang2 induces pericyte drop out and apoptosis (Hammes *et al.* 2004). In diabetic retina this is shown to be mediated by integrin signalling (Park *et al.* 2014). Hence it can be assumed that Ang2 functions in the lymphatic endothelium are Tie2 activating, similarly to Ang1, but Ang2 exhibits both agonistic and antagonistic features depending on the blood vasculature context.

Although Ang2 induces a relatively weak Tie2 activation in vascular ECs and is thus classified in many instances as a Tie2 antagonist, it also has been shown to increase viability of ECs *in vitro*. High doses of Ang2 have been shown to protect ECs from serum deprivation-induced apoptosis (Kim *et al.* 2000c). The same anti-apoptotic effect was also observed when endogenous Ang2 mRNAs were knocked-down in ECs, or when blocking antibodies were used (Yuan *et al.* 2009).

Furthermore, Ang2 has been shown to act as autocrine protective factor in ECs in a stress-induced environment (serum withdrawal) where Ang2 expression restores low PI3K/Akt signalling, thus protecting ECs from apoptosis (Daly *et al.* 2006).

Ang2 has also been found to be important for injury-induced organ regeneration. Liver regeneration has been shown to be dependent on regulated Ang2 expression in a spatiotemporal manner where Ang2 exerts its effects on other cell types via regulating the expression of transforming growth factor-β1 (TGF-β1) (Hu *et al.* 2014). Also in zebrafish embryos Ang2 signalling is involved in both developmental angiogenesis and in angiogenesis following injury (Li *et al.* 2014). In the context of atherosclerosis, exogenous Ang2 is also found to confer atheroprotection in atherosclerosis-prone mice (Ahmed *et al.* 2009).

There is accumulating evidence that Ang2 can bind and signal via integrins in the ECs as well as in other cell types. *In vitro* studies suggest that Ang2 induces complex formation between Tie2 and αVβ3 integrin in vascular ECs promoting αVβ3 integrin internalization and degradation (Thomas *et al.* 2010). It has been suggested that Ang2 signalling via integrins is also context-dependent and occurs mainly in conditions where the high-affinity Tie2 receptor is lacking or expressed at lower levels (Carlson *et al.* 2001, Felcht *et al.* 2012, Hakanpää *et al.* 2015). Recently, Gln362 in C-terminus of Ang2 was implicated in mediating the association of Ang2 with α5β1-integrin (Lee *et al.* 2014). On the other hand, it has also been reported that integrin-binding domain of Ang2 lies in its N-terminus (Hakanpää *et al.* 2015).

### *Angiopoietin-2$^{443}$*

A splice variant of Ang2, Ang2$^{443}$, was discovered in 2000. Ang2$^{443}$ lacks the second exon corresponding to amino acids 97-148 encoding part of the CCOD (Kim *et al.* 2000d). As for full-length Ang2 (Ang2$^{FL}$), secreted Ang2$^{443}$ was reported to be mainly a dimeric protein able to bind Tie2 without inducing any Tie2 phosphorylation. Expression of Ang2$^{443}$ was evident in the primary EC lines, in some tumour cell lines, and in primary tumour tissues. Expression of Ang2$^{443}$ was relatively low (approximately 10%) in ECs when compared with Ang2$^{FL}$. Some tumour cells lines, such as the cervical cancer cell line CaSki, were shown to solely express Ang2$^{443}$ (Kim *et al.* 2000d).

After its discovery the expression of Ang2$^{443}$ along with Ang2$^{FL}$ has been reported during the regression of chicken testes (Mezquita *et al.* 2000) as well as in various pathological settings such as in chronic lymphocytic leukaemia (CLL)

patients (Maffei *et al.* 2010), in canine adenocarcinomas (Kool *et al.* 2014), in acute lung injury (ALI) (Meyer *et al.* 2011), in hepatitis virus B (HBV) treated cells and patient samples (Sanz-Cameno *et al.* 2006), and temporarily during macrophage differentiation (Kim *et al.* 2000d). However, it is not known whether $Ang2^{443}$ is the major Ang2 isoform in special cell types or growth conditions, whether its functions are different from $Ang2^{FL}$ in terms of intracellular storage, secretion, or Tie2 translocation in ECs, or whether it has disease promoting or suppressing functions compared to $Ang2^{FL}$ in tumour microenvironment or in the onset of inflammation where Ang2 functions are implicated.

### *2.4.3 Angiopoietin-2 in disease settings*

Blood vessel remodelling and angiogenesis are common features for pathologic conditions such as inflammation and cancer. Sustained tumour growth relays on neoangiogenesis, chronic inflammation has been linked to the onset and progression of cancer and inflammatory myeloid cells have been shown to contribute to tumour growth. On the other hand, sprouting angiogenesis and the recruitment of inflammatory cells are observed in both acute and chronic inflammation. All this suggests that there are common players affecting the cellular events in both pathologic settings. The activated endothelium of inflamed tissues and primary tumours both display many of the same characteristics known to promote Ang2 expression such as increased expression of TNF-α and VEGF or environmental factors such as hypoxia. As a response to these stimuli, ECs start to produce Ang2. This creates a microenvironment in which Ang2 acts to promote vessel destabilization and sprouting angiogenesis.

#### *Ang-2 and inflammation*

Evidence that Ang2 mediates vascular destabilization accompanied with increase in vessel permeability came for instance from *in vivo* studies where Ang2 administration was shown to increase edema and tissue infiltration of neutrophils (Roviezzo *et al.* 2005). Further studies with Ang2 knockout mice showed reduced EC responsiveness to inflammatory stimuli, not directly, but in a TNF-α-dependent manner decreasing the expression of the intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1(VCAM-1). This leads to impaired leukocyte adhesion on ECs following inflammation (Fiedler *et al.* 2006).

*In vitro*, TNFα, a central inflammatory cytokine, has been shown to induce both Tie2 and Ang2 expression in ECs (Kim *et al.* 2000e, Willam *et al.* 2000).

In many human inflammatory diseases (psoriasis, inflammatory bowel disease, rheumatoid arthritis) (Kuroda *et al.* 2001, Koutroubakis *et al.* 2006, Scott *et al.* 2002) as well as in sepsis (Parikh *et al.* 2006, Gallagher *et al.* 2007) and acute lung injury (ALI) (Bhandari *et al.* 2006), the levels of Ang2 in circulation are increased, which is regarded as a sign of compromised EC integrity. It has also been suggested that Ang2 levels correlate with sepsis severity (Orfanos *et al.* 2007). Serum samples from sepsis patients show elevated Ang2 levels and *in vitro* induce EC paracellular gap formation and increase EC permeability (Parikh *et al.* 2006).

Ang2 storage in WPBs also highlights the importance of regulated Ang2 secretion in response to inflammatory stimuli. WPBs are permissive for inflammatory signals and elicit a rapid exocytosis of WPB proteins preceding leukocyte trafficking. Interestingly, Ang2 and P-Selectin, a receptor for leukocytes on ECs, are stored into a different subset of WPBs (Fiedler *et al.* 2004).

### *Ang-2 actions in tumour growth and metastasis*

In many cancer types elevated Ang2 levels in the serum correlate with poor prognosis and metastasis. Table 1 summarizes human cancers that have increased Ang2 expression. Table 2 summarizes the experimental tumour models where the mechanisms of Ang2 effects in tumour growth and angiogenesis have been studied.

**Table 1. Ang2 expression in human cancers.**

| Organ | Disease state/ Cellular origin of Ang2 expression | Reference |
|---|---|---|
| Brain (glioma) | Primary glioma / invasive front | Hu *et al.* 2003 |
| Breast | MT/ tumour cells | Imanishi *et al.* 2007 |
| Blood (CLL) | ND/ CLL B-cells | Maffei *et al.* 2010 |
| Colon (CRC) | MT/ stromal cells | Goede *et al.* 2010 |
| Ovary | Ovarian neoplasm and cancer/ ND | Sallinen *et al.* 2014 |
| Pancreas | MT/ tumour cells | Schulz *et al.* 2011 |
| Prostate | MT/ tumour cells | Lind *et al.* 2005 |
| Skin | Advanced stage and MT/ ECs and melanoma cells | Helfrich *et al.* 2009 |

CLL= chronic lymphocytic leukaemia, CRC= colorectal cancer, RCC= renal cell carcinoma, MT= metastatic, ND= not described

**Table 2. Ang2 actions in experimental tumour models in mice.**

| Model | Outcome of Ang2 actions | Reference |
|---|---|---|
| Intracranial glioma cell xenografts | Increased invasiveness via α5β1 integrin and MMP-2 | Hu *et al.* 2003, Hu *et al.* 2006 |
| Breast cancer xenografts | Induces primary tumor growth and MT, *in vitro* motility is increased via α5β1 integrin | Imanishi *et al.* 2007 |
| MMTV-PyMT[1] mice | Ang2 blockage regresses TV | Mazzieri *et al.* 2011 |
| Rip1-Tag[2] mice | Ang2 blockage regresses TV | Mazzieri *et al.* 2011 |
| Ang2 overexpression in mice with tumor xenografts | Increased tumor BV and LV density, lymph node and lung MTs | Holopainen *et al.* 2012 |
| EC-specific Ang2 overexpression in mice | Increased tumor colonies in lungs | Holopainen *et al.* 2012 |
| Tumor xenografts | Ang2 blockage attenuated tumor BV and LV angiogenesis | Holopainen *et al.* 2012 |
| Tumor xenografts | Ang2 blockage inhibits tumor xenograft growth and vascularity via Tie2 | Daly *et al.* 2013 |
| DSCR1 mouse model[3] | VEGF induced Ang2 expression in lungs promotes pulmonary MT | Minami *et al.* 2013 |

MT= metastasis, TV= tumour vasculature, BV= blood vessels, LV = lymphatic vessels, [1] a genetic breast cancer model in mice where polyoma middle T (PyMT) oncogene is placed under mouse mammary tumour virus promoter to target tumour growth into mammary gland, [2] a mouse tumour model in which the SV40 large T antigen (TAg) oncogene is expressed in pancreatic beta-cells under the control of rat insulin promoter (Rip1), [3] a knockout mouse model where deficiency of Down syndrome critical region 1 (DSCR1), a natural inhibitor of calcineurin, leads to an upregulation of calcineurin signalling.

As shown in Table 1, increased Ang2 levels are associated with many different types of tumours. The specific feature of Ang2 expression in cancer is its consistent presence in tumours with metastatic lesions. Elevated serum Ang2 levels from patients with metastatic lesions seem to correlate with poor overall survival. Consistently, across experimental tumour models Ang2 blockade inhibits the metastatic potential of tumour cells whereas Ang2 overexpression increases metastatic lesions. Many mechanisms of how EC-derived Ang2 can promote tumour growth and metastatic outspread have been introduced. This highlights that many single aspects in Ang2 functions and signalling may contribute to the metastatic potential of tumour cells. One puzzling question is the source of Ang2 expression in tumours. Most studies show that the primary source in tumours are ECs and concentrate on elucidating EC-derived Ang2 functions in tumours. However, many studies also show tumour cells themselves express Ang2 both *in vitro* and *in vivo* as indicated in Table 1.

In a tumour microenvironment the EC-derived Ang2 expression likely increases due to hypoxia and increased VEGF and other cytokine levels, which initiate Ang2-mediated cellular destabilization. Ang2 is known to induce the expression of matrix modifying enzymes such as MMP-2 (Hu *et al.* 2003), which might explain the EC detachment from the underlying matrix. Interestingly, the cancer cell-EC co-culture induces Ang2 and Tie2 localization into cell-cell junctions (Holopainen *et al.* 2012) where it may compete with Ang1-Tie2 signalling leading to decreased Tie2 activation and compromise vessel integrity.

Once ECs are sensitized and the vascular sprouting into avascular tumour is initiated, Ang2 expression in the EC tip cells (del Toro *et al.* 2010) may promote both sprout elongation and branching. Ang2 actions in experimental tumour models were shown to affect not only tumour ECs but also tumour-infiltrated myeloid cells that are known to possess pro-angiogenic functions. Subpopulation of these cells expresses Tie2, which expression level was shown to decrease upon Ang2 blockage, affecting myeloid cell association with tumour blood vessels (Mazzieri *et al.* 2011). Since monocytes participate in vessel anastomosis (Geudens & Gerhardt 2011) Ang2 expression in endothelial tip cells can attract Tie2 positive monocytes, induce monocyte-EC interaction, and promote vessel branching. Secondly, in tip cells Ang2 is suggested to signal via integrins because of the low expression of Tie2 in tip cells. Ang2-integrin signalling in the tip cells could contribute to the migration of ECs and sprouting angiogenesis (Felcht *et al.* 2012).

Vessel integrity and functionality are decreased in tumour vasculature as a result of the constant exposure to angiogenic factors which can promote the tumour cell intravasation into the blood stream. However, in Ang2-dependent metastasis it is unclear whether tumour cell extravasation into the metastatic site is promoted by Ang2 released into circulation by the primary tumour, or whether induction of Ang2 expression locally in ECs at the metastatic site promotes the transmigration of cancer cells into a new environment. One of the main organs to exhibit metastatic lesions are the lungs and in many experimental tumour models tumour cells from distinct cancer types have shown Ang2 dependent metastasis into the lungs (Holopainen *et al.* 2012, Imanishi *et al.* 2007). Recently it was shown that VEGF expressed by tumour cells induces Ang2 expression in the pulmonary endothelium via VEGFR-2, calcineurin, and nuclear factor of activated T-cells (NFAT) signalling promoting particularly lung metastasis (Minami *et al.* 2013). However, other studies have shown that upregulation of Ang2 is detected following VEGFR-2 blockage, serving as a bypass route for

tumours likely via induction of other growth factor signalling pathways contributing to Ang2 expression (Rigamonti *et al.* 2014).

### *Ang-2 in diabetic retinopathy*

High glucose levels suppress Ang1 -Tie2 induced Akt signalling *in vitro* (Singh *et al.* 2010), while for instance exogenously adiministrated COMP-Ang1 has a protective effect in diabetic mice kidneys *in vivo* (Lee *et al.* 2007). On the other hand high glucose levels induce Ang2 expression (Yao *et al.* 2007), which links imbalanced Ang1-Ang2 ratio also to metabolic diseases induced by hyperglycaemia such as diabetes, where deleterious effects of sustained Ang2 expression are seen for instance in the vasculature of eye.

Diabetic retinopathy (DR) caused by complications of diabetes leads to damage of retinal vasculature and is the leading cause of visual loss in working-aged people (Antonetti *et al.* 2012). Characteristic for an early phase of DR is the loss of pericytes from the retinal vasculature. The loss of pericytes compromises the integrity of the inner blood-retinal barrier. Although the ECs are intact the pericyte loss possibly causes capillary instability and vascular leakage in the macular edema. During disease progression there are areas in the retina devoid of vessels and hence oxygen. This triggers the retina to send signals to the ECs for neoangiogenesis. This stage of disease is referred to as proliferative DR (Antonetti *et al.* 2012).

Hyperglycaemia induces pericyte apoptosis. Increased Ang2 levels have been detected in the vitreous of patients with proliferative DR (Watanabe *et al.* 2005). In DR Ang2 induced pericyte apoptosis proceeds via integrin signalling. Under high glucose Ang2 signals via integrin $\alpha 3\beta 1$ to induce pericyte apoptosis via the p53 pathway (Park *et al.* 2014).

# 3 Aims of the study

Angiopoietin 1 is known to mediate both vessel stabilization and EC migration via Tie2 activation. The first objective of this thesis was to study how Ang1 mediates these dual functions. The second part of the thesis focuses on the mechanisms of how Ang2 mediates ligand-specific functions different from Ang1. The final part of the thesis is aimed at studying Ang2 splice variant $Ang2^{443}$, particularly $Ang2^{443}$ oligomerization, storage into WPBs, Tie2 translocation into specific subcellular compartments, and possible tumour-EC interactions. A novel mouse model expressing alternative $Ang2^{443}$ was also generated. The specific aims of this study are as follows:

1. Characterization of the effects of Ang1 on Tie2 localization and formation of signalling complexes in specific subcellular domains.
2. Investigation of Ang2-specific Tie2 redistribution.
3. Characterization of $Ang2^{443}$ in terms of EC storage, oligomerization, secretion, and ECM binding, characterization of the subcellular Tie2 translocation in ECs, and an analysis of the importance of $Ang2^{443}$ in developmental angiogenesis in mice and in EC-tumour cell interaction *in vitro*.

# 4 Materials and methods

The materials and methods used in this thesis are summarized in the table below. Detailed information with references can be found in the original papers I-III.

**Table 3. Materials and methods used in the original papers.**

| Level | Method | Used in |
|---|---|---|
| DNA | DNA constructs | I, II, III |
| | Cloning techniques | I, II, III |
| RNA | RNA extraction | II, III |
| | RT-PCR | III |
| | RNA knockdown using short hairpin RNA (shRNA) | II |
| | Quantitative real-time PCR | II |
| Protein | Adhesion of Tie2-coated beads on substrate-linked angiopoietins | II |
| | Enzyme-linked immunosorbent assay (ELISA) | III |
| | Production of conditioned media | II, III |
| | Mass spectroscopy | III |
| | Immunoprecipitation | I, II, III |
| | Western blot | I, II, III |
| | Solid phase binding assay | I, II |
| Cell and tissue | Cell culture | I, II, III |
| | Cell adhesion assay | I, II |
| | Cell aggregation assay | I |
| | Cell polarity assay | I |
| | Cell spreading assay | I, II |
| | Cholesterol depletion | II |
| | Depolymerization of microtubules | II |
| | Fluorescent microsphere assay | II |
| | Immunofluorescence microscopy | I, II, III |
| | Integrin function-blocking assay | II |
| | Live cell microscopy | I, II, III |
| | Modified “wound-closure” migration assay | II |
| | Permeability assay | I |
| | Transendothelial migration (TEM) assay | III |
| | Whole-mount retina preparations | II, III |
| Other | Production of retro- and lentiviruses | I, II, III |
| | Animal models | II, III |
| | Intravitreal injections | II |
| | Reagents and antibodies | I, II, III |
| | Statistical analysis | I, II, III |

# 5 Results

## 5.1 Angiopoietin-1 induces different signalling cascades through Tie2 in cell-cell and cell-matrix contacts (I)

Angiopoietin-1 is known to promote both vascular remodelling and vessel integrity. However, at the cellular level the mechanisms mediating these dual effects were not known.

### *5.1.1 Angiopoietin-1 induces Tie2 translocation to endothelial cell-cell contacts in a trans-type decreasing endothelial permeability and inducing a signalling pathway that promotes endothelial cell stability*

EC stimulation with native Ang1 or with the pentameric Ang1 variant COMP-Ang1 induced Tie2 translocation to cell-cell contacts. This was evident in conditions mimicking the quiescent endothelium, *i.e.* in confluent EC cultures where the majority of cells form cell-cell contacts. In the presence of Ang1 Tie2 translocation occurred in cell junctions if both contacting cells expressed Tie2, showing a homotypic *in trans*-type association of the ligand-receptor complex in which the multimeric Ang1 ligand can bridge Tie2 receptors from neighbouring cells. Increased internalization of the Tie2 receptor following ligand activation was evident when a neighbouring cell was missing or when the cell-cell junction was loosened. This suggests that the stabilization of cell-cell junctions via the Ang1/Tie2 complex is occurring at the plasma membrane in junctions and when the majority of the receptor is not internalized.

Junctional Tie2 did not colocalize with known EC junctional proteins VE-Cadherin at AJs or zona occludens-1 (ZO-1) at TJs but was aligned with actin microfilaments. VE-Cadherin depletion did not affect junctional Tie2 localization, but when the actin cytoskeleton was disturbed with cytochalasin B treatment, junctional Tie2 showed discontinuous staining in the cellular junctions and a colocalization with VE-Cadherin in clusters of retracting actin microfilaments.

Association with actin cytoskeleton reduces the protein solubility against detergents. Accordingly, Tie2 was found in the detergent insoluble fraction only after COMP-Ang1 stimulation. Tie2 phosphorylation was also relatively higher in the insoluble versus the soluble fraction.

Ang1 or COMP-Ang1 stimulation led to significant Tie2 activation in cell-cell contacts and to the subsequent activation of eNOS via Akt. Activated eNOS colocalized with Tie2 only in cell-cell junctions and not in mobile cells lacking cell-cell contacts.

### 5.1.2 In the absence of cell-cell contacts Angiopoietin-1 induces Tie2 translocation to the basal cell membrane, inducing a signalling cascade that leads to a migratory phenotype

In contrast to confluent EC monolayers in sparse ECs, Ang1 and COMP-Ang1 induced Tie2 clustering to the rear of single cells. Ang1 has been previously shown to interact with ECM (Xu & Yu 2001). Furthermore, Tie2 clusters in the rear of sparse HUVECs following Ang1 stimulation were observed at the basal side of single cells. Therefore effects of Ang1 in sparse cells were studied more closely. Substrate bound Ang1 induced Tie2 localization into the cell-matrix contacts without colocalization with any known focal or fibrillar adhesion proteins such as integrins α5 or β1, paxillin, or vinculin.

Tie2 clustering at the cell rear was accompanied by polarized caveolin-1 location and microtubule organizing centre distribution in a manner typical of migratory cells. The migratory phenotype and polarization pattern were lost when Tie2 was internalized from the cell rear into the vesicular structures. Loss of caveolin-1 polarity impedes EC polarization and directional movement (Beardsley *et al.* 2005). In accordance to this, Tie2 negative ECs had an impaired caveolin-1 polarization when stimulated with COMP-Ang1.

Previous studies have shown that Ang1-induced EC migration proceeds via the Dok-R signalling mediator (Master *et al.* 2001). Consistent with this, Ang1 induced Tie2 activation at the rear of sparse cells led to the colocalization of Tie2 and Dok-R. The activation of Dok-R in these compartments provides further evidence that the mechanism of Ang1-mediated cell migration involves Dok-R.

### 5.1.3 Tie2 is necessary for the VE-PTP translocation to cell-cell junctions to decrease endothelial cell permeability

VE-PTP, an EC-specific phosphatase known to interact with Tie2 (Fachinger *et al.* 1999), contributed to Ang1 induced paracellular permeability. In confluent EC monolayers overexpressed VE-PTP was observed mainly in the vesicles. In cells overexpressing both Tie2 and VE-PTP their colocalization was observed in cell-

cell junctions. Junctional colocalization of Tie2/VEPTP increased following COMP-Ang1 stimulation. VE-PTP colocalization with Tie2 in cell-cell contacts led to a decrease in permeability of the HUVEC monolayer. The paracellular permeability further decreased following COMP-Ang1 stimulation. However, association of VE-PTP with Tie2 in the rear of sparse ECs was not observed suggesting that VE-PTP is part of the Tie2 signalling complex only in cell-cell junctions and although junctional VE-PTP leads to a decrease in Tie2 activation, the introduction of VE-PTP into junctions reduces the junctional permeability via other mechanisms also.

## 5.2 Ligand oligomerization state and specific ECM compartmentalize agonistic Ang2-Tie2 signalling in endothelial cells (II)

The first part of the thesis demonstrated that in cell-cell junctions Ang2 functioned as a Tie2 antagonist decreasing Ang1-induced Tie2 phosphorylation. It further demonstrated that under certain conditions Ang2 induces Tie2 translocation into specific structures close to the FAs resembling cell-matrix contact sites. Interestingly, these sites were formed only upon Ang2 stimulation and not in the presence of other angiopoietins suggesting ligand-specific functions.

### *5.2.1 Ang2- and collagen type specific Tie2 translocation to cell-matrix contacts*

To study the effects of Ang2 on the Tie2 receptor translocation to subcellular compartments other than EC-cell junctions, freshly plated, actively spreading sparse ECs were used. This model made it possible to also study the initial cell migration after adhesion and spreading. In this context only Ang2, and not Ang1 or Ang4, was able to translocate Tie2 into specific structures to the distal ends of FAs resembling newly formed cell-matrix adhesions. This Ang2-mediated Tie2 translocation was cell substrate specific, since it only occurred on ECs plated on Col I and IV matrixes but not on ECs plated on gelatin, poly-D-lysine, or fibronectin. Although Tie2 translocation did specially occur distally from FAs, the forming Ang2/Tie2 cell-matrix sites were neither typical FAs nor focal contacts as shown by lack of colocalization with FA proteins vinculin, paxillin, β1-integrin, signalling mediator Crk, phosphotyrosine, and actin stress fibres. This sort of

Ang2-dependent clustering was also evident in non-endothelial NIH3T3 fibroblasts on Col I, but only if Tie2 was introduced in them.

Ang2-Tie2 adhesion sites were also visible when imaging Ang2 stimulated Tie2-HUVECs on Col I matrix in living cells with total internal reflection fluorescence (TIRF) microscopy. TIRF allows the visualization of the basal cell membrane within 100 nm from the glass surface, thus confirming that the contact sites as cell-ECM adhesions were located at the basal cell surface.

### 5.2.2 Ang2-specific Tie2 translocation is not dependent on Tie2 kinase activity

To test whether the Tie2 phosphorylation state regulates Ang2 induced receptor translocation, Tie2 variants with different kinase activities were over-expressed in the HUVECs or NIH3T3 cells. Wild-type Tie2, kinase negative, intracellularly deleted, or hyper phosphorylated Tie2 were all translocated to cell-matrix contact sites upon Ang2 stimulation, indicating that Ang2-induced Tie2 translocation is not dependent on the Tie2 phosphorylation state or intracellular domain. Furthermore, the lack of Tie2 kinase activity did not alter the Ang1-induced Tie2 distribution either, showing that differences in Tie2 kinase activity do not explain differential receptor localization between Ang1 and Ang2.

### 5.2.3 Lower order oligomerization of native Ang2, α2β1-integrin and intact microtubules are critical for Tie2 translocation into cell-matrix contacts

Angiopoietins are multimeric proteins, however, Ang2 differs from Ang1 in that it exists normally mainly as lower order dimers, whereas Ang1 forms higher order multimers and oligomers in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Kim *et al.* 2005). Therefore the ligand oligomerization state was studied as a mechanism explaining why Ang2, but not Ang1 is able to translocate Tie2 into cell-matrix contacts. When normally mainly dimeric recombinant Ang2 was clustered into higher order multimers via antibody cross-linking, it inhibited the Tie2 translocation into cell-matrix contact sites. Instead, the Tie2 clustering was now highly similar to that induced by native multimeric Ang1. There was also a size-based cut-off in cargo that could be transported into Ang2-Tie2 cell-matrix adhesions. By using fluorescent beads of various size it was demonstrated that only 20 nm, but not bigger (from 100 nm

onwards) fluorescent beads could be associated with these structures following Ang2 stimulation. In line with this observation, rotary shadowing electron microscopy has shown the size of Ang1 oligomers to be roughly 200nm (Kim *et al.* 2005), suggesting that Ang1/Tie2 complexes are excluded from these EC-ECM contacts based on their larger size.

Since angiopoietin signalling, especially Ang2 related, has been suggested to proceed also via integrins it was studied whether integrins may contribute to the Ang2-mediated Tie2 clustering into cell-matrix contacts. Since Ang2-induced Tie2 trafficking was Col I and IV specific, the involvement of major collagen binding integrins α1β1 and α2β1 was examined. Although Tie2 could not be colocalized with the known FA component β1- integrin in cell-matrix contacts, blockage of β1-integrin using a blocking antibody led to a total abolishment of both FAs and Tie2 clustering at the cell-ECM contacts. Next the alpha subunits of major collagen binding integrins α1β1 or α2β1 were depleted from ECs using mRNA knockdown. Depletion of α2 integrin prevented formation of FAs and Ang2-Tie2 cell-matrix adhesions, whereas lack of the α1 subunit did not have a negative effect on the formation of FAs or Ang2-induced Tie2 clustering.

Using live cell microscopy it was demonstrated that accumulation of Tie2 into the cell-matrix contacts was vesicle-based. Since vesicular trafficking to FAs is mediated via microtubules (MT) (Kaverina *et al.* 1998), the effect of MT depletion on the formation of Ang2-Tie2 cell-matrix adhesions was studied. Indeed, Tie2 translocation into cell-matrix adhesions following Ang2 stimulation was prevented in Tie2-HUVECs lacking a normal MT network, although the formation of FAs was not affected.

### *5.2.4 Ang2 binds to ECM molecules with different affinities and substrate bound Ang2 mediates relatively weaker cell adhesion*

Next, substrate bound Ang2 effects on cell adhesion were analysed and compared to Ang1. Findings showed that when compared to matrix bound Ang1, matrix anchored Ang2 induced significantly weaker cell adhesion. This occurred independently of Tie2 activity and intracellular domain. From tested substrates Ang2 attachment to Col I was weaker when compared to fibronectin.

### 5.2.5 Ang2 and Ang1 activate partially overlapping signalling pathways in spreading cells on Col I

Both Ang1 and Ang2 activate the PI3 kinase/ Akt pathway to promote EC survival (Daly *et al.* 2006, Kim *et al.* 2000b). In addition, Ang1 exerts motility via Erk (Yoon *et al.* 2003, Kim *et al.* 2000a). Therefore the activation of these pathways following Ang1 or Ang2 stimulation was studied in conditions promoting ligand-specific Tie2 translocation to the ECM contacts. Akt was phosphorylated by both ligands, albeit Ang2 mediated somewhat weaker Akt activation than Ang1. Only Ang1 was able to induce significant Erk phosphorylation, indicating that both ligands have overlapping but also distinct cellular effects.

### 5.2.6 Only native low oligomeric Ang2 increases vessel branching in mouse retina

In order to compare the *in vivo* effects of native Ang2 to Ang2 ligands with higher oligomeric states and to native multimeric Ang1, different Ang2 variants and Ang1 were intravitreously injected into mouse eyes and their effects on retinal angiogenesis were analysed. Only native, dimeric Ang2 increased branching of the retinal vessels when compared to the control (BSA) retinas. COMP-Ang2 (pentameric) (Kim *et al.* 2009), HIS-Ang2 (multimerized using an antibody cross-linking), or native multimeric Ang1 did not induce increased retinal vessel branching when compared to the controls.

## 5.3 Angiopoietin-2 splice variant Ang2$^{443}$ promotes tumour cell migration and is proteolytically processed resulting in variant-specifc monomeric ligand form (III)

In the third part of the thesis Ang2$^{443}$, an Ang2 splice variant without part of the N-terminal CCOD was characterized and compared to the full-length Ang2 (Ang2$^{FL}$) in terms of ligand oligomerization, intracellular localization and secretion, and Tie2 translocation and activation. Ang2$^{443}$ effects on EC-cancer cell interactions *in vitro* were studied and Ang2$^{443}$ effects *in vivo* were analysed using a transgenic mouse line expressing solely Ang$^{443}$.

### 5.3.1 Ang2$^{443}$ exists in two monomeric forms due to proteolytic processing

Analysis of Ang2$^{443}$ produced as a recombinant protein in transfected COS-1 cells or in retrovirally transduced HUVECs showed that in conditioned media secreted Ang2$^{443}$ run as two different monomers under reduced conditions in SDS-PAGE when compared with secreted Ang2$^{FL}$ that appeared as a single size protein. Conditioned media analysed under non-reduced conditions from the same samples showed that Ang2$^{443}$ was mainly a dimeric protein like Ang2$^{FL}$. Interestingly, an increase in monomeric Ang2$^{443}$ forms was evident, suggesting that hetero monomers of Ang2$^{443}$ affect the relative oligomerization pattern of Ang2$^{443}$.

Under reduced conditions secreted Ang2$^{443}$ with C-terminal HIS-tag existed also as hetero monomers in conditioned media when compared to Ang2$^{FL}$ that showed a single band when an antibody against His epitope was used. The same was observed in conditioned media analysed from HUVECs transduced with retroviruses overexpressing C-terminally FLAG-tagged Ang2$^{FL}$ and Ang2$^{443}$ when using an antibody against the FLAG-tag. This indicated that possible modification in Ang2$^{443}$ takes place in the N-terminus of Ang2$^{443}$. To better analyse the modification site Ang2$^{443}$-HIS and Ang2$^{FL}$-HIS from COS-1, conditioned media were immunoprecipitated for mass spectroscopy analysis. Although the exact cleavage site could not be identified, mass spectrometric analysis of tryptic peptides indicated that lower band in Ang2$^{443}$ hetero monomers lacked the entire SCD unlike upper monomer of Ang2$^{443}$ and Ang$^{FL}$. The first identified peptide for upper monomer of Ang2$^{443}$ and Ang$^{FL}$ corresponded to amino acids from glutamine 33 (Q33) onwards. Instead, first peptide identified for lower Ang2 monomer was aspartic acid 67 (D67). Although mass spectroscopy results need further confirmation using other methods, they suggest that low molecular weight monomers of Ang2$^{443}$ may lack the SCD that in Ang2 corresponds to amino acids tyrosine 19 (Y19) to proline 70 (P70) that can be expected to impair dimerization/oligomerization of Ang2$^{443}$.

### 5.3.2 C-terminal Ang2 domain is needed for its storage in WPBs where it colocalizes with P-Selectin

Previous studies have shown that Ang2 is stored into EC-specific storage granules called WPBs. The storage of $Ang2^{443}$ into these organelles and the importance of Ang2 domains in its WPB translocation were analysed next.

Since the main constituent of WPBs is vWF, antibodies against Ang2 and vWF were first used to analyse the colocalization of $Ang2^{FL}$ and $Ang2^{443}$ with vWF in retrovirally transduced HUVECs. Using a double staining of $Ang2^{FL}$ and vWF, colocalization was observed mainly in vesicles with only few classical cigar-shaped WPBs double positive for $Ang2^{FL}$ and vWF. vWF is reported to be both constitutively secreted as well as stored to WPB. This is why vesicular colocalization of $Ang2^{FL}$ and vWF might represent a constitutively secreted pool of Ang2 and vWF. In contrast to $Ang2^{FL}$, in $Ang2^{443}$-HUVEC cells colocalization between $Ang2^{443}$ and vWF could not be detected at all. Intriguingly, when $Ang2^{443}$- and $Ang2^{FL}$-HUVECs were immunostained using only an Ang2 antibody, both isoforms could be detected in structures resembling WPBs, though in $Ang2^{443}$-HUVECs most of the signal was observed in the vesicles.

vWF in mature, cigar-shaped WPBs is extensively multimerized and becomes further condensed when packed into WPBs, which results from the distinctive cigar-shape morphology of WPBs (Valentijn *et al.* 2011). Therefore it was considered possible that the unexpected staining results (rare cigar-shaped WPBs double positive for vWF and Ang2 isoforms) could be because i) polyclonal vWF antibody binding at multiple sites in mature WPBs might block binding for Ang2 antibody in double staining, ii) there might be epitopes for polyclonal Ang2 antibody in exon 2 that is missing in $Ang2^{443}$ and iii) C-terminus of Ang2 might be partially protected in the majority of mature WPBs from antibody staining. To overcome possible problems with vWF antibody masking and avoid using the available polyclonal antibody for Ang2, either sequential antibody staining or EGPF-tagged $Ang2^{443}$ and $Ang2^{FL}$ were used next, respectively.

By using sequential staining where $Ang2^{443}$- and $Ang2^{FL}$-HUVECs were first stained with Ang2 antibody after washing and staining with highly diluted vWF antibody, an increase in the colocalization of $Ang2^{FL}$ with vWF was observed in mature WPBs. However, for $Ang2^{443}$ the signal still mainly localized in the vesicles, though some mature $Ang2^{443}$ positive WPBs could be observed. By tagging both isoforms with C-terminal EGFP-tag, colocalization of vWF and both

Ang2 isoforms in mature WPBs became evident as well as an EGFP signal in mature WPB structures in living cells.

To clarify the Ang2 domain necessary for localization into WPBs, we investigated the importance of the whole N-terminus of Ang2 in its WPB storage next. For that angiopoietin chimeras were generated where the N-terminus of Ang1 (that does not localize into WPBs, Fiedler *et al.* 2004, own observations) was fused to the C-terminus of Ang2 and *vice versa*. Results showed that the C-terminus of Ang2 is critical for the translocation into mature WPBs since colocalization of Ang1-Ang2 with vWF in WPBs was evident using a sequential staining method. The Ang2-Ang1 chimera did not colocalize with vWF in WPBs using the sequential staining method. When both chimeras were tagged with the EGFP tags, fluorescence signal of Ang1-Ang2 chimera but not the Ang2-Ang1 chimera colocalized with vWF in mature WPBs. The antibody staining or fluorescence signal of Ang2-Ang1 chimera was diffusely distributed in the HUVECs with no colocalization in any specific structures.

A previous study has shown that Ang2 is excluded from P-Selectin positive WPBs. To analyse whether $Ang2^{443}$ is similarly distributed in a specific subset of WPBs, HUVECs transduced with EGFP-tagged Ang2 isoforms were used and stained with P-Selectin antibody. Both $Ang2^{443}$ and $Ang2^{FL}$ colocalized with P-Selectin in both vesicles as well as in mature WPBs. Consistent with previous results Ang1-Ang2 chimera (with or without EGFP tag) also colocalized with P-Selectin positive WPBs, whereas the Ang2-Ang1-EGFP chimera did not.

### *5.3.3 $Ang2^{443}$ translocates Tie2 into cell-matrix contacts*

In the second part of the thesis Ang2 specific Tie2 translocation into cell-matrix contact sites was shown to be dependent on lower oligomerization state of Ang2. Next it was thus studied whether $Ang2^{443}$ could induce Tie2 translocation and activation in the same subcellular compartments.

Spreading $Tie2^{WT}$-HUVECs on collagen I matrix $Ang2^{443}$ induced Tie2 accumulation in cell-matrix contact sites as shown before for $Ang2^{FL}$. Also Tie2 activation in these compartments upon $Ang2^{443}$ stimulation was observed similarly to $Ang2^{FL}$.

### 5.3.4 Ang2$^{443}$ becomes attached to the basal cell surface upon cell spreading

Previous studies have shown that Ang1 but not Ang2 is incorporated into the ECM (Xu & Yu 2001). However, we have shown that Ang2 induces Tie2 translocation into specific cell-matrix adhesions distal from FAs on Col I. In the second part of the thesis it was also shown that recombinant Ang2 binds to ECM proteins such as Col I and fibronectin. To test whether EC-derived Ang2$^{443}$ is incorporated into the basal cell membrane, Ang2$^{443}$-EGFP distribution was analysed by spreading Ang2$^{443}$-EGFP HUVECs on gelatin and Col I matrixes and using TIRF microscopy. During cell spreading, both Ang2$^{443}$-EGFP and Ang2$^{FL}$-EGFP were observed first as vesicles in the basal cell surface following their attachment to underlying matrix as cells continued spreading. No difference was observed between gelatin and Col I matrixes. Furthermore, on Col I no ligand distribution into elongated cell-matrix adhesions representing Ang2-Tie2 adhesions were observed likely due to a relatively low Tie2 expression in HUVECs versus virally expressed Ang2-EGFP forms.

### 5.3.5 Ang2$^{443}$ is expressed at relatively low levels in endothelial and colorectal cancer cells and tumour tissue samples and its relative abundance is not changed in hypoxic or nutrient deprived conditions

Ang2 promotes tumour growth and metastasis (Daly *et al.* 2013, Holopainen *et al.* 2012, Mazzieri *et al.* 2011) and some studies suggest that tumour cells might be the source of Ang2 expression (Helfrich *et al.* 2009). Thus it was an object of interest whether Ang2$^{443}$ is expressed as a prominent Ang2 isoform in certain cancer cell lines. Selected cancer cell lines for the study included non-metastatic and metastatic breast cancer cell lines (MCF-7 and MDA-MB-231 respectively), the HCT-116 colorectal cancer cell line as well the CaSki cell line from cervical cancer metastasis that has been previously reported to express solely Ang2$^{443}$. HUVECs were used as a control since they have been shown to express isoforms with high Ang2$^{FL}$ to low Ang2$^{443}$ ratios. (Kim *et al.* 2000d).

Under basal conditions the colon cancer cell line HCT-116 was the only cancer cell line showing Ang2 mRNA expression based on RT-PCR analysis. Ang2$^{FL}$ was the dominant isoform is these cells. Analysis of protein expression from cell lysates using Ang2-ELISA indicated the same. These findings are in

contrast to previous studies where HCT-116 cells were shown to express Ang2 only after hypoxic treatment (Gu *et al.* 2006). Both Ang2 isoforms were also expressed in tumour tissue samples from different stages of CRC in a similar fashion.

Next it was analysed whether hypoxia or serum deprivation could induce Ang2 expression in Ang2 negative cancer cell lines or shift the $Ang2^{FL}$-$Ang2^{443}$ ratio in HUVECs or HCT-116 cells, since both of these physiological stress factors are prevalent in the tumour microenvironment.

PCR analysis of cells grown under hypoxia for 24 hours showed a similar Ang2 isoform expression pattern in HUVECs and HCT-116 cells as was observed in the basal conditions $Ang2^{FL}$ being the prominent isoform with no detectable Ang2 expression in other cell lines. Analysis of Ang2 protein expression using ELISA from cells kept under hypoxia for 48 hours showed increase in Ang2 expression in HUVECs but a decrease in HCT-116 cells when compared to normoxia.

In serum deprived cells no significant increase in $Ang2^{443}$ expression in HUVECs or cancer cells was observed.

### *5.3.6 $Ang2^{443}$ promotes tumour cell migration through endothelial monolayer in vitro*

Ang2 has a well-established role in tumour growth and it has particularly been shown to promote metastatic outspread of cancer cells (Holopainen *et al.* 2012, Mazzieri *et al.* 2011). To model cancer cell extravasation *in vitro* and analyse EC-derived $Ang2^{443}$ effects, a trans- endothelial migration (TEM) assay was used in which cancer cell invasion through EC monolayer was analysed. For that, HUVECs were transduced to over express either $Ang2^{443}$, $Ang2^{FL}$, or left untransduced (control cells). CaSki cells were chosen as a cancer cell line since they did not express Ang2 isoforms endogenously. In this experimental setting CaSki cells showed a significantly increased capacity for cancer cell migration through the $Ang2^{443}$-HUVEC monolayer when compared to the control cells, but only a slight increase was observed when compared to the $Ang2^{FL}$-HUVECs, indicating that $Ang2^{443}$ promotes cancer cell migration *in vitro*.

### 5.3.7 Postnatal vascularization of retina and development of mesenteric lymphatic vessels are not compromised in Angpt2$^{\Delta E2}$ transgenic mice

For the *in vivo* analysis of Ang2$^{443}$ functions, a gene targeted mouse line was generated in which exon 2 of *Angpt2* gene was replaced with a floxed neomycin-cassette. Offsprings from heterotsygote crossings followed the Mendelistic ratio (24.7% wild type, 53.6% heterotzygous, and 21.6% gene targeted). Genotyping of transgenic mice and their wild type littermates was performed using two sets of primers distinguishing both the wild type allele and the targeted allele. RT-PCR analysis with the primers expanding from exon 1 to exon 4 was performed to investigate mRNAs expressed from the targeted allele. This confirmed that the sequence corresponding exon 2 was lacking in the homozygous Angpt2$^{\Delta E2}$ mice.

In order to investigate whether functions of Ang2$^{443}$ are different from those induced by Ang2$^{FL,}$ we analysed the postnatal vascularization of retina and the development of lymphatic vessels that are Ang2 dependent next (Gale *et al.* 2002, Hackett *et al.* 2002).

Vascular development was analysed in whole mount retinas prepared from mice during the first postnatal week (P 5.5. and 6.5) and stained against isolectin B4 to visualize ECs. A trend for delayed retinal vascularization was observed in mice expressing Ang2$^{443}$. Analysis of the lymphatic vessels of mesentery showed no clinical signs of dysfunction in Ang2$^{443}$ expressing mice.

# 6 Discussion

## 6.1 Ang1 assembles distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts showing a unique requirement for a trans- association of Tie2 receptors from adjacent cells (I)

Angiopoietin 1 is a Tie2 agonist known to mediate both vessel integrity but also EC migration. However, molecular and cellular mechanisms regulating different functions had not been studied before. Findings of the first paper show that to mediate these distinct effects, Ang1 induces Tie2 receptor translocation to cell-cell junctions or to cell-ECM contacts depending on cell confluency. This led to the formation of distinct signalling complexes, which promoted EC junctional integrity and cell migration, respectively. Using various cell biology techniques it was shown that in cell-cell junctions Tie2 receptors from adjacent cells are required for Ang1-induced receptor translocation into junctions. Results of the first work were further strengthened by findings reported by another research group (Fukuhara *et al.* 2008).

One of the main findings of the study was that the multimeric Ang1 had the capacity to anchor Tie2 receptors from adjacent cells in cell-cell junctions. In fact, this was a perquisite for Ang1 to induce Tie2 phosphorylation (activation) in cell-cell junctions, since Tie2 activation was not observed if one of the contacting cells was Tie2 negative. This reflects the multivalent nature of native multimeric Ang1: Ang1 needed to be at least a trimer to induce Tie2 phosphorylation in cell-cell junctions.

Junctional Tie2 did not directly colocalize with VE-Cadherin, a known and important EC junctional protein, but instead Tie2 was seen in parallel with actin filaments. The Tie2 in the cell-cell junctions was part of a detergent insoluble fraction and was internalized only from junctions where receptors from a single cell accumulated. This was different from *e.g.* VEGFR-3 that showed rapid internalization into vesicles upon ligand stimulation. This suggested that Ang1-induced homotypic in-trans Tie2 complexes in cell-cell junctions to induce receptor activation and induction of signalling cascade without significant receptor internalization. The phosphorylated Tie2 (pTie2) /Tie2 ratio in the insoluble fraction was also higher when compared to the soluble fraction. In this respect the regulation of Ang1-Tie2 signalling in junctions seemed to differ from

a general mechanism in which RTK signalling following ligand stimulation is regulated by receptor internalization and subsequent sorting to lysosomes for degradation. Previous studies have shown that Ang1 induces Tie2 localization to both the caveolin-1 positive lipid rafts (Katoh *et al.* 2009) and clatrin-coated pits (Bogdanovic *et al.* 2009). Furthermore, Tie2 has also been shown to be internalized in response to Ang1 (Bogdanovic *et al.* 2006).

Studies showing Tie2 internalization following Ang1 stimulation have been based on biotin labelling of total Tie2 on the whole cell surface without addressing the question whether Tie2 in junctions is similarly endocytosed. Further detailed studies are needed to analyse whether Ang1-induced Tie2 clustering into cell-cell junctions may prevent Tie2 trafficking into the endocytic pathway and whether this may limit its signalling capacity into cell-cell junctions. The low internalization rate observed in cell-cell junctions is in favour of the widely published model, where constant Tie2 activation in vessels by Ang1 is thought to support EC quiescence and vessel integrity, which is better accomplished if the Tie2-Ang1-Tie2 turn over from the cell-cell junctions is low.

If the model in which Ang1 induces EC integrity mainly via translocation of Tie2 into cell-cell junctions and maintaining it there in activated state is valid, then the signalling molecules activated by Ang1-Tie2 should mainly be located at cell-cell junctions. eNOS is an enzyme responsible for production of NO and is coordinately regulated by various RTKs (Mukherjee *et al.* 2006). NO actively regulates many aspects in EC biology such as vasodilatation, permeability, and angiogenesis. Due to the short half-life of NO, its subcellular actions are mainly dictated via the subcellular localization of eNOS (Mukherjee *et al.* 2006). In cell-cell junctions but not in the rear of ECs, COMP-Ang1 induced Tie2 activation led to eNOS phosphorylation. Also other RTKs like VEGFR-2, PDGFR, and EGFR are shown to induce an upregulation of eNOS and an increase in NO production (Mukherjee *et al.* 2006). Whether Ang1 also induced increased eNOS expression was however not studied.

In this study eNOS phosphorylation was analysed by using an antibody detecting phosphorylated S1179 that is known to activate the enzyme via Akt (Sessa 2004). In later studies Ang1 has also been shown to induce eNOS phosphorylation at threonine 497 (T497), which results in the deactivation of enzyme enabling Ang1 to counteract VEGF induced eNOS activation and NO mediated permeability of ECs (Oubaha & Gratton 2009). Interestingly, this was not mediated by Akt but by atypical PKCζ. Our study did not show whether Ang1 stimulation induced NO production, the ultimate outcome of eNOS activation.

Therefore activation of eNOS at S1179 following Ang1 stimulation in cell-cell junctions might not exceed the inhibitory effect Ang1 may have at T497 via a different signalling pathway. Furthermore, under basal conditions Ang1 regulation of eNOS activity might not have a role but be an important mechanism allowing Ang1 to counteract VEGF induced permeability.

Another molecule that colocalized with Ang1/Tie2 in cell-cell junctions but not in EC-ECM contacts was VE-PTP. Translocation of VE-PTP into cell-cell junctions was dependent on Tie2 and increased following Ang1 stimulation, reflecting that it was part of the ligand-induced signalling complexes. Although VE-PTP decreased Tie2 phosphorylation, occupation of VE-PTP into cell-cell junctions led to an overall decrease in junctional permeability likely due to VE-PTP´s capability to regulate phosphorylation levels of other junctional proteins such as VEGFR-2, VE-Cadherin, and beta-catenin. Previous studies have shown that Ang1 mediated EC integrity involves the maintenance of VE-Cadherin and beta-catenin in the cell-cell junctions (Gamble *et al.* 2000, Wang *et al.* 2004) and that may be dependent of VE-PTP recruitment into junctions. Furthermore, the *in vivo* Ang1-Tie2-VE-PTP axis has been shown critical for the regulation of EC polarization in vessel sprouts. The VE-PTP activity localization into stalk cell junctions was shown to be Ang1-Tie2 dependent. It regulated VEGFR-2 junctional activity and led to a decreased VE-Cadherin phosphorylation maintaining EC polarization and promoting lumen formation (Hayashi *et al.* 2013).

In the absence of cell-cell junctions, Ang1 induced Tie2 translocation in the rear of polarized ECs in cell-matrix contact sites. Ang1 has been shown to bind ECM via its linker domain (Xu & Yu 2001), which is also present in the designed COMP-Ang1 that was also used in these studies. The substrate bound Ang1 induced Tie2 activation and promoted cell spreading and migration in a Tie2 dependent manner. Ligand-induced Tie2 translocation in the rear of single cells was accompanied with a colocalization of caveolin-1 and Dok-R. Caveolin-1 is a marker of front-rear cell polarization and important for cell migration (Beardsley *et al.* 2005, Grande-Garcia *et al.* 2007). Dok-R has also previously been shown to induce EC migration by Ang1 (Jones *et al.* 2003, Master *et al.* 2001). However, unlike in the cell-cell junctions, VE-PTP was not part of the Ang1-Tie2 signalling complex in EC-ECM contacts, suggesting that its regulatory functions may be dispensable in Tie2 positive EC-ECM contacts and that Ang1/Tie2 signalling complexes are different in the subcellular domains investigated.

## 6.2 Lower oligomerization state controls Tie2 receptor trafficking and Angiopoietin-2 specific responses (II)

When compared to Ang1, Ang2 has both opposing and redundant roles, but the molecular mechanisms responsible for its versatile functions are poorly understood. The main finding of the second study was that Ang2 induced specific cellular effects distinct from Ang1, indicating that it is not solely Ang1 blocking the antagonistic ligand. Ang2, but not the other angiopoietins, induced Tie2 translocation into novel cell-ECM contacts distal from FAs. Most importantly, a lower oligomerization state of Ang2 was critical in this translocation. If native Ang2 was clustered into a more oligomeric ligand such as Ang1, Tie2 translocation into cell-matrix contact sites was prevented. Similarly by using fluorescent beads of various sizes only the smallest beads (20 nm) could associate with Ang2-Tie2 positive cell-ECM contacts. Furthermore, there was a dependence of intact microtubules and α2 integrin in Ang2 mediated Tie2 translocation. However, no known FA proteins could be associated in the Ang2-Tie2 cell-ECM contacts. Tie2 was phosphorylated in these cell-matrix contacts leading to an increase in Akt but not Erk signalling. Neonatal retinal angiogenesis is compromised in Ang2 knockout mice showing persistent hyaloid vessels and defective retinal vascularization (Gale *et al.* 2002, Hackett *et al.* 2002) and recombinant Ang2 induces pericyte drop out in mature retina (Hammes *et al.* 2004), but the effects of recombinant Ang2 in developing retina had not been studied before. From the mice injected with various Ang2 variants, only the native Ang2 increased the number of vessel branching points. Also other studies indicated that Ang2 blocking reduces retinal branching (Felcht *et al.* 2012, Holopainen *et al.* 2012), suggesting a role for native Ang2 in active angiogenesis and vessel remodelling.

Collectively, this data shows that ligand valency resulting smaller ligand size determines Ang2 ligand-specific effects that are different from Ang1. Ang2 specific clustering of Tie2 into cell-matrix contacts distal from FAs could serve as a mechanism part of promoting EC survival during the initial steps in vessel destabilization. This is in line with the observation that Ang2 specific Tie2 clustering was dependent on cell substrate Col I and Col IV. While Col IV is the major BM collagen, ECs come into contact with ECM proteins such as Col I during sprouting angiogenesis. Ang2 has been reported to promote EC survival under stressed conditions by restoring phosphorylated Akt levels in ECs (Daly *et al.* 2006). In the cell-ECM contacts Ang2 induced Tie2 activation led to an

increase in phosphorylated Akt levels. Whether Ang2 specific Tie2 clustering and activation at cell-ECM contacts also promotes EC destabilization was not studied in detail however. Ang2 has been implicated in the induction of various matrix degrading enzymes under the angiogenic environment of tumours or during wound healing. MMPs shown to be induced by Ang2 include *e.g.* MMP-2 or MMP-9 (Bezuidenhout *et al.* 2007, Hu *et al.* 2003). However, this study did not analyse whether the Ang2 induced Tie2 activation in cell-ECM contacts was accompanied by the induction of certain MMPs. Also, it was not studied whether Tie2 clustering into cell-ECM contacts induced degradation of Col I or Col IV matrixes.

## 6.3 Angiopoietin-2 splice variant Ang2$^{443}$ promotes tumour cell migration and is proteolytically processed resulting in variant-specifc monomeric ligand form (III)

The aim of the third study was to better characterize the Ang2 isoform Ang2$^{443}$ to investigate whether the partial lack of CCOD, which is proposed to regulate the oligomerization state, induces Ang2-variant specific effects. The study focused on the ligand oligomerization state, intracellular storage into WPBs, Ang2-specific Tie2 translocation into ECM contacts, and the Ang2$^{443}$ effects on cancer cell-EC interactions. Furthermore, a novel mouse model lacking exon 2 of Ang2 thus expressing the Ang2$^{443}$ form was generated to study Ang2$^{443}$´s role in developmental angiogenesis.

Analysis of recombinant, secreted Ang2$^{443}$ in relation to Ang2$^{FL}$ under reduced conditions showed that in both ECs and non-ECs, the secreted Ang2$^{443}$ was predominantly found in two distinct monomers, whereas Ang2$^{FL}$ was found forming single-size monomers. When analysing the secreted Ang2$^{443}$ under non-reduced conditions, it mainly formed dimers similarly to Ang2$^{FL}$, but a significant increase in Ang2$^{443}$ monomers was evident. Analysis of secreted, C-terminally His-tagged Ang2$^{443}$ and Ang2$^{FL}$ showed similar formation of two Ang2$^{443}$ monomers under reducing conditions, whereas Ang2$^{FL}$ formed largely single-size monomers. This suggested that Ang2$^{443}$ upon secretion is perhaps partially proteolytically cleaved at the N-terminus, increasing monomeric forms. In mass spectroscopy analysis no N-terminal peptides for SCD could be identified in the lower molecular weight Ang2$^{443}$ monomer, which were found for monomers of uncleaved Ang2$^{443}$ and Ang2$^{FL}$.

For cleaved Ang2$^{443}$ the first identified peptide started from aspartic acid 68 (D68). Since the SCD domain in Ang2 ranges from tyrosine 19 (Y19) up to proline 70 (P70), the results suggest that the lower molecular weight Ang2$^{443}$ lacks the entire SCD. This would explain the shift in oligomerization seen in western blot, because our previous studies have shown that since designed Ang2$^{\Delta SCD}$ (lacking amino acids 1-96) forms only monomers, the SCD of Ang2 is needed for dimerization of ligand (work II). Further studies are needed to locate the precise cleavage site, the mechanisms regulating the cleavage, and to identify the function of the resulting ligand form.

Proteolytic cleavage of growth factor ligands occurs in the family of VEGF EC-specific vascular and lymphatic growth factors. VEGF-C and VEGF-D are proteolytically cleaved. This is a requirement for them to initiate signalling (Otrock *et al.* 2007). CCOD of angiopoietin-like 4 (Angptl-4) was also shown to be proteolytically cleaved resulting formation of monomeric Angptl-4 (Ge *et al.* 2004). Existence of angiopoietin monomers *in vivo* has also been described for Ang1, showing that in cardiac muscle Ang1 existed mainly as monomers exerting its effects via integrins (Dallabrida *et al.* 2008) suggesting that also *in vivo* monomeric forms of angiopoietins may have cellular effects different from oligomerized forms, and that the formation of the angiopoietin monomers might happen via the proteolytic cleavage of SCD.

When analysing the N- and C-terminuses of Ang2 more closely it was found that the C-terminus of Ang2 determines its localization into endothelial WPBs as judged by the colocalization of Ang1-Ang2 chimera but not the Ang2-Ang1 chimera with vWF. Furthermore, based on our results both Ang2$^{FL}$ and Ang2$^{443}$ colocalize with P-Selectin in mature, cigar-shaped WPBs. This is in contrast with a previous study showing that Ang2 is excluded from P-Selectin positive WPBs (Fiedler *et al.* 2004). However, also another study has shown endogenous Ang2 and P-Selectin colocalization in mature WPBs (van Agtmaal *et al.* 2012). Hence further studies are needed that determine the nature of Ang2 containing WPBs, their exocytosis upon various stimuli, and analyse whether P-Selectin is exocytosed from the same WPBs with Ang2 or not.

Problems with Ang2 detection in cigar-shaped WPBs using immunofluorescence techniques were solved either by performing antibody stainings in a sequential fashion accompanied with a highly diluted vWF antibody but most notably by using EGFP- tagged Ang2 isoforms enabling the visualization of Ang2$^{443}$ and Ang2$^{FL}$ in mature WPBs without antibodies.

In the second study lower oligomerization state of Ang2 was found to control Tie2 translocation and its ligand-specific effects. Next it was analysed whether $Ang2^{443}$ is capable of similar Tie2 translocation as reported before of full-length Ang2. The analysis of Tie2 translocation upon $Ang2^{443}$ stimulation showed that $Ang2^{443}$ also efficiently translocated Tie2 into cell-ECM contacts on Col I matrix accompanied with Tie2 activation comparable to $Ang2^{FL}$ (II). Unlike reported before (Xu & Yu 2001), analysis of spreading HUVECs transduced with EGFP-tagged $Ang2^{443}$ or $Ang2^{FL}$ showed that both isoforms translocated to the basal cell surface during cell spreading. Our previous results show that recombinant Ang2 binds both gelatin and Col I (II), but these results indicate that during cell spreading EC-derived Ang2 is also efficiently incorporated into the basal cell surface. However, a complex formation with Tie2 is required for the formation of Ang2-Tie2 positive cell-matrix contacts near FAs on Col I.

Since an increase in Ang2 is reported in many tumours and elevated Ang2 levels correlate with metastatic lesions (Holopainen *et al.* 2012, Mazzieri *et al.* 2011), whether $Ang2^{443}$ could mediate the EC-cancer cell interactions *in vitro* was analysed next. It was also studied whether hypoxia or serum deprivation can induce a relative increase in $Ang2^{443}$ expression levels in ECs or in various cancer cell lines derived from breast, colon, or cervix. While EC-derived $Ang2^{443}$ induced a significant increase in tumour cell transmigration through EC monolayer, neither hypoxia nor serum deprivation increased $Ang2^{443}$ expression in ECs or tumour cells. Unlike previously reported (Kim *et al.* 2000d), CaSki cells expressed neither of the studied Ang2 isoforms. Whether specific conditions such as EC tumour cell, or EC cancer associated fibroblast (CAF) cocultures might induce Ang2 splicing into $Ang2^{443}$ remain to be studied.

Since papers solidly describing $Ang2^{443}$ as the major Ang2 isoform are currently lacking, it raises a fundamental question of how the Ang2 splicing in EC or other cell types is regulated. Intriguingly, there are studies showing that *e.g.* following viral infection in the liver an increase in $Ang2^{443}$ expression in relation to $Ang2^{FL}$ is observed (Sanz-Cameno *et al.* 2006). Furthermore, severity in acute lung injury (ALI) following trauma has been linked to increased plasma $Ang2^{443}$ levels. Presence of $Ang2^{443}$ was shown to be dependent on the intronic polymorphism of *ANGPT2* affecting splicing (Meyer *et al.* 2011). Therefore further studies are needed to analyse whether factors such as EC-tumour cell coculture, inflammation, or postnatal angiogenic settings accompanied with inflammation such as tumour growth or wound healing could induce $Ang2^{443}$ expression or whether it is solely dependent on genetic factors. In addition,

Ang2$^{443}$ effects in angiogenic settings accompanied with inflammation deserve deeper analysis.

# 7 Summary and conclusions

The first two papers of this thesis show the importance of proper Tie2 clustering into distinct subcellular domains in the Ang/Tie signalling pathway in mediating Ang1 and Ang2 ligand-specific functions. This study revealed also for the first time the possible importance for the different oligomeric natures of Ang1 and Ang2 in Tie2 translocation.

Findings of the first study explain the mechanisms behind the capability of Ang1 to promote both the EC migration and junctional integrity. This is because the differential receptor translocation and initiation of signalling cascades in cell-ECM contacts or EC-cell junctions. Furthermore, the oligomeric nature of Ang1 enabled it to anchor Tie2 receptors from adjacent cells into cell-cell junctions. This is a requirement for a junctional Tie2 activation. It also may serve as a mechanism to restrict Tie2 activation into cell-cell junctions without significant receptor internalization. Formation of the Ang1-Tie2 complex was also important for the recruitment of VE-PTP into EC-cell junctions to regulate paracellular permeability.

The second study revealed that the lower oligomerization state of Ang2 is critical for its ligand specific functions in the Ang/Tie signalling pathway and together with the first study described a novel subcellular compartment for Ang2 signalling. Further studies will reveal whether Ang2/Tie2 clustering into these cell-matrix contacts serves as a mechanism to regulate for instance Ang2 specific EC destabilization and survival prior to sprouting angiogenesis.

The third study demonstrated that the Ang2 splice variant $Ang2^{443}$ undergoes partial proteolytic cleavage and that the resulting increase in monomeric forms may lead to broaden ligand functions. $Ang2^{443}$, albeit lacking part of the oligomerization domain, exhibited many of the same characteristics as the full-length Ang2 *in vitro* in the context of Tie2 translocation and activation. The third study also revealed that Ang2 translocation into WPBs is mediated via its C-terminal domain and demonstrated that both Ang2 isoforms colocalize in mature WPBs with P-Selectin. *In vitro* results of the third study were strengthened by the *in vivo* findings that $Ang2^{443}$ transgenic mice developed normally and showed only a slight delay in vascularization of the retina and no obvious defects in the development of lymphatic vessels. However, the novel mouse model allows the study of $Ang2^{443}$´s role in postnatal physiological and pathological angiogenesis such as in wound healing, inflammation, and tumour growth which differ from developmental angiogenesis.

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