

Marjut Nätyнки

VENOUS MALFORMATION
CAUSATIVE MUTATIONS
AFFECT TIE2 RECEPTOR
TRAFFICKING, DOWNSTREAM
SIGNALING AND VASCULAR
ENDOTHELIAL CELL
FUNCTIONS

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



ACTA UNIVERSITATIS OULUENSIS
D Medica 1350

MARJUT NÄTYNKI

**VENOUS MALFORMATION CAUSATIVE
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F101 of the Faculty of Biochemistry and Molecular Medicine (Aapistie 7), on 8 April 2016, at 10 a.m.

UNIVERSITY OF OULU, OULU 2016

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Acta Univ. Oul. D 1350, 2016

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ISBN 978-952-62-1138-1 (Paperback)
ISBN 978-952-62-1139-8 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2016

Nätynki, Marjut, Venous malformation causative mutations affect TIE2 receptor trafficking, downstream signaling and vascular endothelial cell functions.

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

Acta Univ. Oul. D 1350, 2016

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Abstract

Venous malformations (VMs) are localized defects in vascular morphogenesis which can seriously impede or even threaten the patient's life. VMs are characterized by enlarged, torturous vein-like channels lined by unevenly distributed smooth muscle cells. A large number of mutations in the endothelial TIE2 receptor tyrosine kinase have been found from more than half of the lesions screened, thus providing a common genetic cause. TIE2 has a crucial role in vascular development, remodeling and quiescence. However, the molecular and cellular abnormalities caused by TIE2-mutations in endothelial cells and how they relate to VM formation have been unknown.

The aim of this study was to examine how VM-specific mutations affect the molecular characteristics of TIE2-receptor downstream signaling and cellular functions. Because no effective treatment has been available for VMs, a better understanding of the molecular basis of their pathology should enable the development of more potent and non-invasive treatments as well as provide a better understanding of vascular morphogenesis in general.

The results demonstrate that the TIE2-VM forms have both common and specific effects on TIE2 and the endothelial cells (ECs) expressing them. Mutation-induced TIE2 autoactivation leading to loss of normal EC monolayer organization due to extracellular matrix (ECM) fibronectin deficiency was found to be a common change. This was shown to occur through chronic activation of the mitogen-activated protein kinase (MAPK) pathway, which also caused activation of the proteolytic plasminogen system. Also, most mutations altered TIE2 trafficking and angiopoietin ligand regulated TIE2 functions, albeit through different mechanisms. Using RNA-screening we showed that the most common sporadic TIE2-VM mutation dysregulates genes affecting vascular development, cell migration and ECM remodeling. PDGFB, a major attractant of vascular mural cells, was found to be strongly attenuated due to chronic activation of Akt, which also increases EC survival, by the TIE2 mutant receptors.

To conclude, the results in this thesis reveal genetic, molecular and cellular alterations which may potentiate VM formation. This data provides new information on the pathological mechanisms behind abnormal vascular morphogenesis and should assist the development of new molecular treatment strategies for VM patients.

Keywords: angiopoietins, endothelial cells, fibronectin, intracellular signaling, ligand-independent kinase activity, pathological mutations, PDGFB, receptor trafficking, TIE2 receptor tyrosine kinase, venous malformations

Nätyнки, Marjut, Laskimoepämuodostumia aiheuttavat TIE2-reseptorin mutaatiot johtavat molekyyli- ja solutason muutoksiin verisuonten endoteelisoluissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Biokemian ja molekyyli- ja lääketieteen tiedekunta; Biocenter Oulu; Solujen ja soluväliaineen vuorovaikutuksen huippuyksikkö

Acta Univ. Oul. D 1350, 2016

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Laskimoepämuodostumat ovat paikallisia verisuoniston kehityksen häiriöitä. Riippuen niiden koosta ja anatomisesta sijainnista ne voivat aiheuttaa merkittävää haittaa. Epämuodostumat koostuvat laajentuneista, laskimonkaltaisista verisuonista, joissa sileiden lihassolujen kerros on puutteellisesti järjestäytynyt. Yli puolessa tutkituista laskimoepämuodostumista havaitaan mutaatioita verisuonten sisäpinnan endoteelisoluissa ilmenevässä TIE2 reseptorityrosiinikinaasissa, joka säätelee verisuonten kehitystä, muokkausta ja fysiologista toimintaa. TIE2-mutaatioiden aiheuttamia molekyyli- ja solutason muutoksia tai niiden yhteyttä epämuodostumien syntyyn ei ole aikaisemmin tunnettu.

Tämän tutkimuksen tarkoituksena oli selvittää, miten laskimoepämuodostumista löydetty mutaatiot vaikuttavat TIE2-reseptorin toimintaan molekyyli- ja solutasolla sekä TIE2-reseptorista alkavaan solunsisäiseen viestintään. Koska pysyvää hoitomuotoa laskimoepämuodostumille ei tunneta, voisi tieto niiden taustalla olevista patologisista mekanismeista edesauttaa parempien, ei-kajoavien hoitomuotojen kehittämisessä ja antaa myös yleisesti uutta tietoa verisuoniston kehityksestä.

Väitöskirjan tulokset osoittavat, että mutaatiot vaikuttavat TIE2-reseptoriin ja sitä ilmentäviin endoteelisoluihin mutaatioille yhteisillä sekä mutaatiokohtaisilla tavoilla. Mutaatioille tyypillinen TIE2-reseptorin ligandista riippumaton aktivaatio aiheutti aktivaation nousun myös TIE2:sta alavirtaan olevissa viestinvälittäjissä. Tämä puolestaan johti fibronektiini-proteiinin häviämiseen soluväliaineesta, sileitä lihassoluja säätelevän PDGFB-kasvutekijän ilmenemisen laskuun ja solujen ohjelmoidun solukuoleman vähenemiseen. Useimmat tutkitut mutaatiot muuttivat myös TIE2-reseptorin sijaintia soluissa häiriten TIE2:n angiopoietiini-ligandien säätelemää toimintoja usean eri mekanismin kautta. Transkriptomin laajuiset RNA-tutkimukset osoittivat monien verisuonten kehitykseen, solujen liikkumiseen ja soluväliaineen muokkaukseen liittyvien geenien ilmentymisen muuttuneen.

Lopputuloksena tutkimus paljasti geeni-, molekyyli-, ja solutason muutoksia, jotka saattavat vaikuttaa laskimoepämuodostumien syntyyn. Tulokset antavat lisätietoa sairautta aiheuttavista mekanismeista verisuoniston kehityksen häiriöiden taustalla ja ovat hyödyksi kehitettäessä uusia lääkkeitä laskimoepämuodostumien molekkulaarisia hoitoja varten.

Asiasanat: angiopoietiini, endoteelisolut, fibronektiini, laskimoepämuodostumat, ligandista riippumaton kinaasiaktivaatio, patologiset mutaatiot, PDGFB-kasvutekijä, reseptorien liikennöinti soluissa, solunsisäinen signaali, TIE2 reseptorityrosiinikinaasi

Acknowledgements

This thesis work was conducted in Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology which along the way merged with the Department of Biochemistry to become the Faculty of Biochemistry and Molecular Medicine, University of Oulu. The work was financially supported by the Academy of Finland, the Finnish Cultural Foundation and Department of Medical Biochemistry and Molecular Biology.

I want to express my deepest gratitude to my principal supervisor Docent Lauri Eklund who has given me the possibility to dive into the fascinating world of TIE2, especially it's mutations in venous malformations, and also guided me through both master's and doctoral thesis. He is never out of great ideas offering most interesting projects to his group members. Under Lauri's guidance I have learned a lot of science and academic world and got the chance to find my likes and strengths in working life. Especially I thank him for his various and valuable comments on the first version of this thesis, which were indispensable for me to revise the text to its final form. I sincerely thank also my co-supervisor Professor Taina Pihlajaniemi for the possibility to be part of her research group, which offers an excellent environment to learn to do science.

I thank Professors Johanna Myllyharju, Seppo Vainio, Kalervo Hiltunen and Peppi Karppinen together with all other professors and group leaders of the Faculty and Biocenter Oulu for creating such a functional and highly infrastructured working environment where research is both fun and feasible.

I'm very grateful to Professor Miikka Vikkula and his group from de Duve Institute, Université Catholique de Louvain, Brussels, especially to Professor Nisha Limaye and Dr. Melanie Uebelhoer, with whom I have had the pleasure to closely collaborate. Their work on identifying mutations on TIE2 gave the foundation to this thesis project and always so interesting and useful discussions with them new perspectives and ideas for it, the collaboration leading to publishing of several fine papers. All other of my co-authors are also acknowledged for their valuable contributions.

Docent Aki Manninen and other members of my follow-up group are warmly thanked for their comments and advice on my work during these years.

Professor Paula E. North from Medical College of Wisconsin, Milwaukee and Professor George E. Davis from University of Missouri School of Medicine, Columbia are acknowledged for their valuable work as pre-examiners of this thesis.

I thank all the members of the LE-group, both past and present, for the friendly and encouraging working atmosphere, fruitful discussions and teamwork. Especially I want to thank Dr. Riikka Pietilä and M.D. Jaakko Kangas; Riikka, with whom I shared the office for so many years, for all her help and advice and our delightful discussions both on science and life outside of it, and Jaakko for sharing the TIE2-mutation project with me. Brainstorms with him and Lauri have been the moving force of this project. Sometimes it's difficult to distinguish where my work ends and his starts and vice versa.

Jaana Träskelin and Riitta Jokela, our most professional, marvelous and efficient laboratory technicians, are most deeply acknowledged for their valuable and tireless everyday work in the lab and their constant help and advice. Without them this thesis would still be years from being ready!

Anne Vainionpää, Auli Kinnunen, Teija Luoto and Irmeli Nykyri are acknowledged for all their practical help. Great secretaries make a researcher's life a lot easier! Coordinators Ritva Saastamoinen, Sinikka Eskelinen, Pirkko Huhtala and Anthony Heape are acknowledged for their valuable work, help and answers. Hanna-Mari Pesonen is acknowledged for her help during the final stages of thesis preparation. Antti Viklund is warmly thanked for his expert help with everything electronical. He has always been of invaluable and quick help when computers, microscopes and programs have disagreed with me. Dr. Veli-Pekka Ronkainen is also acknowledged for his help with microscopy.

I thank my colleagues, especially people on the 4th floor of the Kieppi building, for pleasant, kind and cooperative working environment.

Haluan kiittää vanhempiani ja veljeäni, jotka ovat antaneet minulle hyvät lähtökohdat elämässä ja ovat tukenani sitä tarvitessani. Viikko teidän kanssanne on paras tapa hetkeksi unohtaa työn huolet ja stressi!

My warmest thanks go to my children, Pihla and Aarni, who drag me out of the office even on most hectic days and bring so much bustle, joy and love to each of my days. From all my heart I thank my spouse Antti for all his love and support during our years together. During this doctoral thesis project he has encouraged me during difficulties, celebrated on times of success, pushed me forward when necessary and most of all always believed in me and this thesis, even when I have not. My dearest family, you make my life worth living for!

20.1.2016

Marjut Nätyrki

Abbreviations

ABIN2	A20-binding inhibitor of NF- κ B-2
ADAM	a disintegrin and metalloproteinase
ADAMTS	ADAM with thrombospondin motifs
AJ	adherens junction
Akt	protein kinase B
Alk	activin receptor-like kinase
ALK	anaplastic lymphoma receptor tyrosine kinase
Ang	angiopoietin (also Angpt)
ANOVA	a one-way analysis of variance
AVM	arteriovenous malformation
BBB	blood-brain barrier
BM	basement membrane
BMP4	bone morphogenetic growth factor 4
bp	base pair
CM	capillary malformation
CNS	central nervous system
C-terminal	carboxyl-terminal
CTGF	connective tissue growth factor
CXCR4	C-X-C chemokine receptor type 4
CYR61	growth factor cysteine-rich 61
Dll4	delta-like 4
DNA	deoxyribonucleic acid
Dok-R	downstream-of-kinase related protein
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EEL	external elastic lamina
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme linked immunosorbent assay
endo-H	endoglycosidase H
eNOS	endothelium nitric oxide synthase
EPC	endothelial progenitor cell
Eph	ephrin receptor
ER	endoplasmic reticulum

ErbB	erb-b2 receptor tyrosine kinase
Erk	extracellular regulated kinase
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
Fc	fragment crystallizable region of an antibody
FGF	fibroblast growth factor
FGFR	FGF receptor
Flt3	fms-related tyrosine kinase 3
FN	fibronectin
FNIII	fibronectin type III
FOXO1	forkhead box-O
GFP	green fluorescent protein
Grb	growth factor receptor-bound protein
GVM	glomovenous malformation
HB-EGF	heparin-binding EGF
HPTP β	human protein tyrosine phosphatase beta
HSP	heat-shock protein
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule 1
IEL	internal elastic lamina
Ig	immunoglobulin
IGF-IR	insulin-like growth factor 1 receptor
IRES	internal ribosome entry site
Jag1	Jagged1
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
KID	kinase insert domain
KIT	tyrosine-protein kinase KIT
LEC	lymphatic endothelial cell
LIC	local intravascular coagulation
MAPK	mitogen-activated protein kinase
MC	mural cell
MMP	matrix metalloproteinase
mRNA	messenger RNA
mTOR	mechanistic/mammalian target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
N-cadherin	neural cadherin

Nck	non-catalytic region of tyrosine kinase
NF- κ B	nuclear factor kappa B
NO	nitric oxide
NT	non-transduced
N-terminal	amino-terminal
OMIM	online Mendelian Inheritance in Man
PAI-1	plasminogen activator inhibitor 1
PAK	p21-activating kinase
PC	pericyte
PCR	polymerase chain reaction
PDGFB	platelet-derived growth factor b
PDGFR	platelet-derived growth factor receptor
PECAM1	platelet endothelial cell adhesion molecule 1
PGF	placental growth factor
PI3K	phosphatidyl-inositol-3 kinase
PIK3CA	PI3K catalytic subunit p110 α
PM	plasma membrane
PMA	phorbol 12-myristate 13-acetate
PTB	phosphotyrosine binding domain
P-TIE2	phosphorylated TIE2
P-Tyr	tyrosine phosphorylation
pY	phosphotyrosine
qPCR	quantitative PCR
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S1P	sphingosine-1-phosphatase
S1P ₁	S1P receptor
SAPK	stress-activated protein kinase
SDF-1 α	stromal-derived factor 1-a
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
ShcA	Src homology 2 domain containing protein
Shh	sonic hedgehog
SH-PTP2	Src homology 2 protein tyrosine phosphatase (also Shp2)

shRNA	short hairpin RNA
Smac	second mitochondrial-derived activator of caspases
SMC	Smooth muscle cell
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TEK	tunica interna endothelial cell kinase
TGF β	transforming growth factor beta
TF	tissue factor
TIE/Tie	tyrosine kinase with Ig and EGF homology domains
TIMP	tissue inhibitor of metalloproteinase
TJ	tight junction
TNF α	tumor necrosis factor alfa
tPA	tissue type plasminogen activator
TrkA	nerve growth factor receptor
TSP-1	thrombospondin-1 protein
uPA	urokinase type plasminogen activator
VCAM-1	vascular adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VE-PTP	vascular endothelial protein-tyrosine phosphatase
VM	venous malformation
VMCM	venous malformations, multiple cutaneous and mucosal
vSMC	vascular smooth muscle cell
VVO	vesico-vacuolar organelle
WT	wild type
Y	tyrosine

List of original publications

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

- I Limaye N*, Wouters V*, Uebelhoer M[#], Tuominen M[#], Wirkkala R, Mulliken JB, Eklund L, Boon LM, and Vikkula M (2009) Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. *Nat Gen* 41(1): 118–124.
- II Uebelhoer M, Nätyнки M, Kangas J, Mendola A, Nguyen H-L, Soblet J, Godfraind C, Boon LM, Eklund L, Limaye N, and Vikkula M (2013) Venous malformation-causative *TIE2* mutations mediate an AKT-dependent decrease in PDGFB. *Hum Mol Gen* 22(17): 3438–3448.
- III Nätyнки M*, Kangas J*, Miinalainen I, Sormunen R, Pietilä R, Soblet J, Boon LM, Vikkula M, Limaye N, and Eklund L (2015) Common and specific effects of *TIE2* mutations causing venous malformations. *Hum Mol Gen* 24(22): 6374–6389.

* and [#], equal contributions.

Nätyнки née Tuominen.

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1 Introduction

The vasculature is the first organ system to develop, which highlights its critical role of transferring oxygen and nutrients to and metabolic waste from all tissues of an organism. The growth and remodeling of vasculature (via vasculogenesis and angiogenesis) are essential during both embryonic development and adult life. In addition, vascular insufficiencies and uncontrolled growth are strongly involved in a variety of pathological conditions. As a result researchers, clinicians and the biomedical industry are highly interested in understanding physiological vessel growth and its role in health to develop treatments and pharmaceuticals to better control the vasculature in different diseases.

Venous malformations (VMs) are congenital localized defects of the vascular morphogenesis. They are composed of enlarged torturous, slow-flow venous-like channels lined by an uneven layer of vascular mural cells. VMs can vary in size and severity from almost unnoticeable birth marks to life-threatening conditions. Conventional treatments for VMs are surgery and sclerotherapy, both of which are invasive and non-lasting with lesions commonly recurring. Better knowledge of the cellular and molecular functions behind VM formation is needed to develop new treatments for VM patients.

Genetic studies have revealed that the vascular receptor tyrosine kinase TIE2, which is necessary for normal development, maintenance and remodeling of vasculature, is mutated in over 50% of VM lesions. Several different mutations have been identified and most show a common gain-of-function effect on TIE2 activation. However, the more specific effects of how uncontrolled TIE2 activity due to mutations in TIE2 may cause VM formation have not been characterized.

The aim of this thesis was to investigate how VM-identified TIE2 mutations affect the normal functions of the receptor, its downstream signaling and the endothelial cells the receptors locate in. The results show that mutation-induced TIE2 activation affects the downstream signaling of the receptor, resulting in increased cell survival, decreased secretion of mural cell attractant PDGFB and altered deposition of the extracellular matrix also modifying the morphology of endothelial cells. In addition, a non-activating deletion form was identified, possibly creating a wild type silencing null-allele and thus enabling the lesion causing potential of an inherited mutation on the other allele.

Because TIE2 is frequently mutated in VM lesions, understanding the cellular effects of its mutations could offer valuable new knowledge about the detailed pathology of VM formation. This, in turn, may aid the development of more

potent, non-invasive molecular treatments for VM patients. By observing the role of TIE2 in normal vasculature a better understanding of normal vascular functions is acquired at the same time.

2 Literature review

2.1 Development of the blood vasculature

Vasculature consists of blood vessels and lymphatic vessels. The blood vasculature is a hierarchical sequence of differently sized and functionally differentiated blood vessels called arteries, veins and capillaries. It is the first functional organ system to form during embryogenesis, and it supplies oxygen and nutrients to growing tissues beyond the limit of diffusion. Functional vasculature is also needed for immune response, regulation of the body temperature, maintenance of blood pressure and removal of metabolic waste products from tissues. (Patel-Hett & D'Amore 2011). Formation of the blood vasculature occurs through two different processes: vasculogenesis, the *de novo* formation of blood vessels from angioblasts, and angiogenesis, the growth of new blood vessels from pre-existing ones, which are then followed by remodeling and differentiation of capillaries, veins and arteries.

2.1.1 Vasculogenesis

Vasculogenesis is the *de novo* formation of the first primitive network of *in situ* differentiating endothelial cells (ECs) of blood islands (Risau *et al.* 1988). These blood islands differentiate from the embryonic as well as the extra-embryonic mesoderm at around the same time as the start of gastrulation in embryogenesis, which corresponds to embryonic day (E) 6.5–7 in mice. The blood island progenitor cells are called hemangioblasts for their ability to differentiate to both endothelial and hematopoietic cell lines. These cells differentiate depending on their location; central cells become blood cell precursors and peripheral cells become angioblasts. (Patel-Hett & D'Amore 2011, Risau *et al.* 1988). The latter differentiate into ECs and, as numerous blood islands grow and fuse, assemble into a continuous tubular network named the primary capillary plexus, which consists of quite uniformly sized vessels (Jones *et al.* 2001). Development proceeds with the formation of vascular lumen and deposition of a basal membrane (BM), a specialized extracellular matrix (ECM) structure, around the primary vessels. This capillary plexus is then connected to the developing heart before it starts beating to form the cardiovascular circulatory system. (Patel-Hett & D'Amore 2011).

Vasculogenesis is controlled by numerous growth factors and their receptors, including fibroblast growth factors (FGFs), hedgehog/Patched-pathway, neuropilins, transforming growth factor beta (TGF β) and vascular endothelial growth factors (VEGFs) (reviewed in (Patel-Hett & D'Amore 2011)). The last of these are characterized the best and perhaps represent the most important early signaling system for blood vessel growth.

The VEGF ligand family consists of nine secreted glycoproteins which interact with three main receptors (VEGFR1–3) (Patel-Hett & D'Amore 2011). VEGFR2 expression represents the first marker for developing ECs during vasculogenesis (Yamaguchi *et al.* 1993). Knocking out VEGF-A or its receptors VEGFR1 or VEGFR2 in mice results in embryonic lethality between E8.5–9.5. VEGFR1 deficiency leads to excessive EC proliferation, whereas VEGFR2 or VEGF-A deficient mice die due to reduced vascular development. Because VEGF-A binds to both these receptors but induces EC proliferation, migration and survival only through VEGFR2 even though its association to VEGFR1 is ten times stronger, it is suggested that the main role of VEGFR1 is to negatively regulate blood vessel formation by competing VEGF-A/VEGFR2 interaction. (Ho & Fong 2015). In VEGFR2 deficient mice blood islands do not form, so vasculogenesis and organized blood vessels are absent and also the hematopoietic progenitors are severely reduced in number (Shalaby *et al.* 1995). Instead, VEGFR3 is mainly characterized in lymphangiogenesis, promoting lymphatic EC (LEC) survival, proliferation and migration together with its ligands VEGF-C and VEGF-D (Secker & Harvey 2015).

2.1.2 Angiogenesis

During embryogenesis the homogenous primary capillary plexus formed by vasculogenesis is expanded and remodeled by angiogenesis, the process where new vessels arise from pre-existing ones, to form the final hierarchy of differently sized functional blood vessels. In adulthood vascular ECs are mainly quiescent (so called phalanx cells) in healthy individuals. However, angiogenesis does occur under certain normal conditions, such as the healing of wounds or the female reproductive cycle (Wong *et al.* 1997) and pathological conditions, such as tumor growth or various ischemic and inflammatory diseases (Carmeliet & Jain 2000).

Angiogenesis can be divided into two major mechanisms: sprouting angiogenesis, where new vessels grow out from existing ones, and non-sprouting

angiogenesis, where capillaries are split into two by transluminal tissue pillars (Patel-Hett & D'Amore 2011, Ribatti & Crivellato 2012, Fig. 1). However, also other mechanisms have been suggested, albeit they function only during tumor development (De Spiegelaere *et al.* 2012).

Sprouting angiogenesis

Sprouting angiogenesis is often induced by hypoxia in growing tissues. ECs change from their quiescent state into an active, proliferating and migratory state. Vessel sprouts are tethered towards hypoxia induced angiogenic signals, e.g. VEGF-A gradient. (Jeltsch *et al.* 2013).

Sprouting angiogenesis proceeds through several stages: 1) the BM is degraded on sites where EC processes project towards the surrounding ECM; 2) interendothelial junctions are weakened and ECs start to migrate into the ECM; 3) a solid cord of ECs forms while the lumen takes shape, the BM is deposited and the pericytes are recruited behind the migrating front; and 4) contacting sprouts fuse (anastomose) to form functional capillary loops (Ribatti & Crivellato 2012).

A variety of proteinases mediate the proteolytic degradation of the BM when angiogenesis begins, the major groups of which include the matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), ADAMs with thrombospondin motifs (ADAMTSs) and plasminogen activators (Theocharis *et al.* 2015). The first three of these groups are regulated through inhibition by tissue inhibitors of metalloproteinases (TIMPs), and the balance between proteinases and TIMPs determines whether the BM is degraded or protected (Arpino *et al.* 2015).

Vessel sprouting requires specification of ECs into two morphologically and functionally different types: tip cells and stalk cells (Gerhardt *et al.* 2003). A tip cell is polarized and extends long filopodia into the surrounding ECM. Typically one tip cell exists per sprout (Siekmann *et al.* 2013) and it responds to the VEGF-A gradient by migrating towards it outward from the parent vessel, a response mediated by VEGFR2 (Ruhrberg *et al.* 2002). Tip cells are slow proliferating and show strong expression of platelet-derived growth factor b (PDGFB) and VEGFR2 (Gerhardt *et al.* 2003). VEGF-A/VEGFR2 interaction induces expression of delta-like 4 (Dll4), a ligand for Notch, and activation of actin polymerization. Dll4 binds to Notch-receptors of adjacent cells downregulating their VEGFR2 receptors and preventing them from specializing into tip cells.

(Hellström *et al.* 2007). The strength by which ECs inhibit their neighboring cells resolves which EC specifies into a tip cell and starts to lead sprouting. This method enables the ECs to change roles when Dll4 secretion levels fluctuate. (Siekman *et al.* 2013). Actin, instead, is needed for filopodia formation leading to migration, which increases tip cell exposure to VEGF-A and further strengthens lateral inhibition of adjacent cells. When filopodia from two tip cells from opposite directions meet, the cells fuse to form a new capillary loop, a process called anastomosis. Dll4/Notch signaling partakes also here as the tip cells inhibit each other, thus enabling neighboring cells to flip into tip cells to form new sprouts. (Bentley *et al.* 2009).

Also stalk cells bind VEGF-A through VEGFR2, but their response is concentration-dependent proliferation (Gerhardt *et al.* 2003). In addition to being responsible for the elongation of the newly forming sprout, stalk cells are also responsible for the lumen formation, BM deposition and pericyte recruitment (Ribatti & Crivellato 2012). Two different mechanisms for lumen formation have been suggested: cord hollowing and cell hollowing. In cord hollowing blood vessel lumen forms between adjacent ECs through specific junction establishment, formation and increase of extracellular space between the ECs and changes in cell shape (Strilić *et al.* 2009). In cell hollowing ECs accumulate pinocytotic vacuoles which form the lumen through intra- and intercellular fusion without cytoplasmic mixing (Kamei *et al.* 2006, Ribatti & Crivellato 2012).

Tip and stalk cells respond differently to VEGF-A through the same receptor VEGFR2, and the difference can be partly explained by different splice variants of VEGF-A, which include both soluble and ECM-binding forms. ECM-binding form VEGF189 gives rise to VEGF-A gradient and guides tip cell migration. Soluble form VEGF121, instead, creates the concentration to which stalk cells respond. (Ruhrberg *et al.* 2002).

Non-sprouting angiogenesis

Non-sprouting angiogenesis, also called intussusceptive or splitting angiogenesis, gives rise to new capillaries by internal division of pre-existing ones. This division occurs through insertion of transcapillary tissue pillars. First the ECs of opposing walls of a capillary contact each other to form an interendothelial bridge. As EC junctions are remodeled, the pillar is hollowed and enlarged so that pericytes and ECM fibers can move in to form a pillar core. These pillars can then grow in diameter to form a capillary mesh or fuse to form larger vessels. Because

this process happens through EC rearrangements and does not require EC proliferation, it is a quick way to remodel blood vessels. (Burri & Tarek 1990, De Spiegelaere *et al.* 2012, Djonov *et al.* 2000a, Djonov *et al.* 2000b). One signaling route identified in non-sprouting angiogenesis is the angiopoietin (Ang)/TIE pathway, where TIE2 mediates EC–ECM contacts needed for formation of transcapillary pillars, and TIE1 inhibits vessel splitting by inhibiting EC stretching leading to cell membrane fusion (Patan 1998).

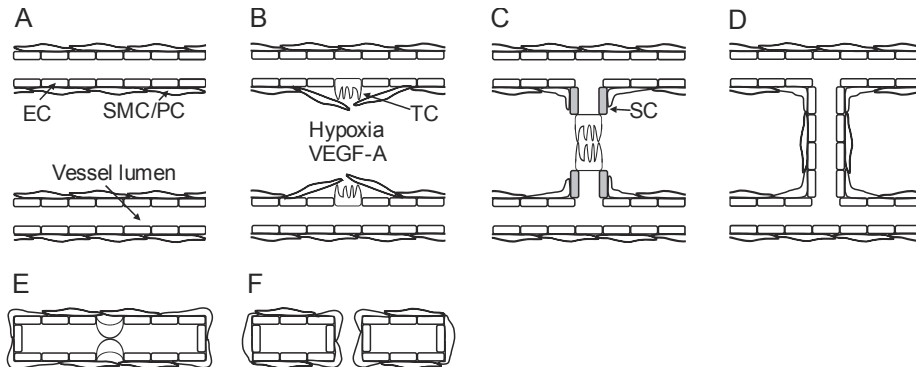


Fig. 1. Schematic presentation of sprouting (A–D) and intussusceptive (E–F) angiogenesis. A) Quiescent blood vessels. B) Angiogenic signal (e.g. hypoxia driven VEGF-A) induces sprouting angiogenesis. BM underneath the ECs is degraded and the mural cells (SMC, PC) detach, tip cells (TC) differentiate and start migrating towards the VEGF-A gradient. C) Stalk cells (light grey, SC) proliferate and differentiate behind the tip cell. Vessel lumen formation, BM deposition and mural cell recruitment occur around the stalk cells. Contacting tip cells anastomose. D) Vessel maturation is finalized and quiescence regained. E) Intussusceptive growth starts with the ECs sinking inwards to touch each other. F) Remodeling of cellular junctions forms a transluminal tissue pillar into which the mural cells migrate. Tissue pillars grow and fuse together to split the vessel in two.

2.1.3 Vessel maturation and stabilization

Newly formed blood vessels mature through stabilization by formation of adherens junctions (AJs) and tight junctions (TJs) between ECs (Dejana 1996), recruitment of vascular smooth muscle cells (vSMCs) and pericytes (PC) and construction of a BM (Jones *et al.* 2001).

Between the contacting ECs AJs are formed first and they support the following formation of TJs. Both of these junctions are composed of

transmembrane proteins that form homophilic interactions in *trans*, between the cells, forming a zipper-like structure along the cell border. Major components of the AJs are the vascular endothelial (VE-) cadherin and catenins, of the TJs the claudins and occludin. Both junction types bind to the cytoskeleton and to signaling molecules to regulate EC permeability and integrity. AJs have a role in directing the morphogenesis of vasculature and TJs function as barriers regulating the passage of proteins, lipids, solutes etc. between the ECs to and from the vascular lumen. (Dejana 1996, Dejana *et al.* 2009).

The presence of vSMCs/PCs alone is not sufficient for vessel stabilization. Instead, an array of reciprocal signaling pathways between endothelial and mural cells (MCs) is required. These include e.g. PDGFB/ platelet-derived growth factor receptor beta (PDGFR β) and Ang/TIE2 pathways (Armulik *et al.* 2011) and are described below. The interactions between ECs and MCs stabilize blood vessels also by inducing increased production of BM proteins and by enhancing expression of integrins functioning as cellular receptors for ECM components. When the integrins are inhibited, the BM is weakened leading to increase in vessel lumen diameter. The same happens when the ECM constituent fibronectin (FN) or pericyte-derived TIMP-3, both of which regulate collagen type IV, a major component of vascular BM, is inhibited. (Stratman *et al.* 2009). In neovessels TIE2 activation participates in vessel caliber regulation. Ang1-mediated TIE2 activation induces secretion of apelin from ECs. In the presence of VEGF-A apelin enhances EC proliferation and promotes EC-EC aggregation resulting in vessel enlargement. At the same time the production of nitric oxide (NO) from the ECs is increased, possibly causing the surrounding mural cells to relax. (Kidoya *et al.* 2008).

Reciprocal communication between endothelial and mural cells

Intercommunication between ECs and MCs is mandatory to regulate vascular formation, maturation, stabilization and remodeling. Main signaling pathways participating in this reciprocal crosstalk are summarized below and presented in Fig. 2.

PDGFB is secreted from the ECs on sites of active angiogenesis and binds to PDGFR β -receptors of the surrounding mural cells (Hellström *et al.* 1999). PDGFB signaling is crucial for pericyte recruitment to blood vessels (Lindahl *et al.* 1997) and depletion of PDGFB or PDGFR β in mice is perinatally lethal due to vascular dysfunction culminating into pericyte deficiency (Betsholtz *et al.* 2004,

Levéen *et al.* 1994, Soriano 1994). Secondary defects include EC hyperplasia and alterations of EC morphology and junctions, which result in dilated hyperpermeable blood vessels (Hellström *et al.* 2001). During embryogenesis mural cell progenitors initially appear around certain vessels independently of PDGFB, but during subsequent sprouting and vessel enlargement EC derived PDGFB is required to induce pericyte proliferation and co-migration (Hellström *et al.* 1999). The tip cell leading the vessel sprout secretes more PDGFB than the following stalk cells (Gerhardt *et al.* 2003) ensuring mural cell recruitment and vessel stabilization just behind the sprouting tip. The secreted PDGFB is bound to the EC surface or the surrounding ECM to direct the correct organization of pericytes and secure their vicinity to the underlying ECs (Lindblom *et al.* 2003). As PDGFB signaling participates in securing vessel stabilization, its attenuation is needed for angiogenesis to start. When angiogenesis is stimulated by VEGF-A-mediated VEGFR2 activation, VEGFR2 directly binds to PDGFR β , leading to loosening of pericyte coverage and, as a result, to blood vessel destabilization (Greenberg *et al.* 2008).

Both endothelial and mural cells express TGF β and its receptor activin receptor-like kinase (Alk) 5. In addition, a related receptor Alk1 is expressed in the ECs. (Goumans *et al.* 2002, Goumans *et al.* 2003). TGF β is produced in a latent form and its activation is dependent on collaboration between the two cell types (Sato *et al.* 1990). This signaling route mediates *de novo* induction of mural cells from an undifferentiated mesenchyme and the proliferation and differentiation of both ECs and MCs (Armulik *et al.* 2011). Alk1 and Alk5 seem to have opposing roles since Alk1 supports cell proliferation and migration, while Alk5 inhibits them to promote differentiation and vessel maturation. However, Alk5 is required for Alk1 activity and activated Alk1 inhibits Alk5, producing a loop to precisely regulate the activity and quiescence of blood vessels. (Goumans *et al.* 2003). The net effect might also depend on the strength and length of the TGF β stimulus because Alk1 responds to low concentrations of TGF β but transiently, whereas Alk5 needs high concentrations or a long stimulus. Thus, Alk1 may dominate initially promoting angiogenesis and Alk5 later on ensuring maturation. (Goumans *et al.* 2002). In addition, TGF β also forms an autoregulatory loop in ECs promoting its own expression, synthesis and release (Carvalho *et al.* 2004).

The Ang/TIE signaling pathway has been implicated in both the SMC/PC recruitment and the vessel destabilization through paracrine and autocrine regulation, respectively. SMCs and PCs produce Ang1, which activates TIE2 on

the EC surface resulting in pericyte recruitment and maturation and the quiescence of blood vessels (Sundberg *et al.* 2002). However, this interaction does not seem to be direct, but instead is proposed to influence some other EC–MC signaling pathway, like TGF β or HB-EGF (see below). The occurrence of an indirect effect is supported by the finding that pericytes gather normally to the vasculature of conditional Ang1 knockout mice (Jeansson *et al.* 2011). On the other hand, Ang2 is expressed by the ECs on sites of vessel remodeling and acts as a destabilizing factor loosening the interactions between the ECs and MCs (Maisonpierre *et al.* 1997) which leads to pericyte detachment (Feng *et al.* 2007, Hammes *et al.* 2004).

VEGF-A can directly induce pericyte proliferation and migration under hypoxia, and also stimulate ECs to produce NO, which promotes migration and recruitment of mural cells and SMC relaxation. Pericytes, in turn, produce VEGF-A to serve as a survival and/or stabilizing signal for ECs. (Ribatti *et al.* 2011). Ang2 may potentiate some of the angiogenic effects of VEGF-A. VEGF-A stimulus increases Ang2 production, and Ang2 may destabilize vessels making them more responsive to VEGF-A (Oh *et al.* 1999).

Ang1 enhances EC expression of heparin-binding epidermal growth factor (HB-EGF). HB-EGF binding on ErbB-receptors on vSMCs promotes cell proliferation and migration. In addition to the Ang/TIE pathway, HB-EGF may also collaborate with the PDGFB pathway. (Armulik *et al.* 2011).

Sphingosine-1-phosphatase (S1P) and its endothelial receptor S1P₁ also mediate interactions between ECs and MCs. Activation of S1P₁ promotes neural (N-) cadherin trafficking to plasma membrane enhancing EC–MC contacts, and also leading to the upregulation of VE-cadherin, thus strengthening the EC–EC junctions. (Allende *et al.* 2003, Luo & Radice 2005, Paik *et al.* 2004).

Other signaling pathways implicated in the EC–MC crosstalk include stromal-derived factor 1-a (SDF-1 α)/CXCR4, sonic hedgehog (Shh)/Patched, Jagged1 (Jag1)/Notch3 and Ephrin/Eph. The first two participate in pericyte recruitment and may collaborate with PDGFB and Ang1, respectively. Jag1 is expressed by the ECs and its contact with the Notch3-receptor on the pericytes induces their differentiation and maturation. Ephrins mediate the pericyte association with the endothelium. (Armulik *et al.* 2011).

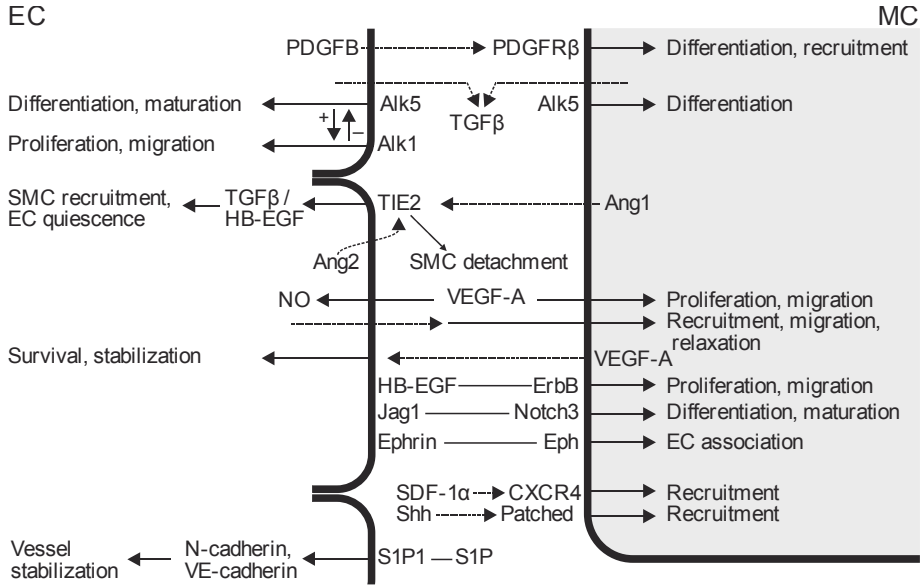


Fig. 2. Signaling pathways utilized in EC–MC communication and vessel stabilization. Dashed arrow indicates production, solid arrow effect, line direct interaction, + induction and – inhibition. EC; endothelial cell, MC; mural cell (SMC or PC). Abbreviations of signaling molecules as mentioned in the text.

2.2 Structure and function of the blood vessel wall

The wall of large blood vessels, arteries and veins, has a three layered structure surrounding the lumen. The innermost layer, *tunica intima*, consists of an EC layer surrounded by the BM and a middle layer, *tunica media*, of vSMCs and ECM. The outermost layer, *tunica adventitia*, is made of connective tissue and contains nerves and capillaries which supply the vessel. Capillaries, instead, merely consist of a layer of ECs surrounded by the BM and sparsely located pericytes. The looser structure enables the transfer of oxygen, nutrients and waste products between blood and the surrounding tissues. Fig. 3 presents an illustration of the blood vessel wall structure and its differences between large and small vessels.

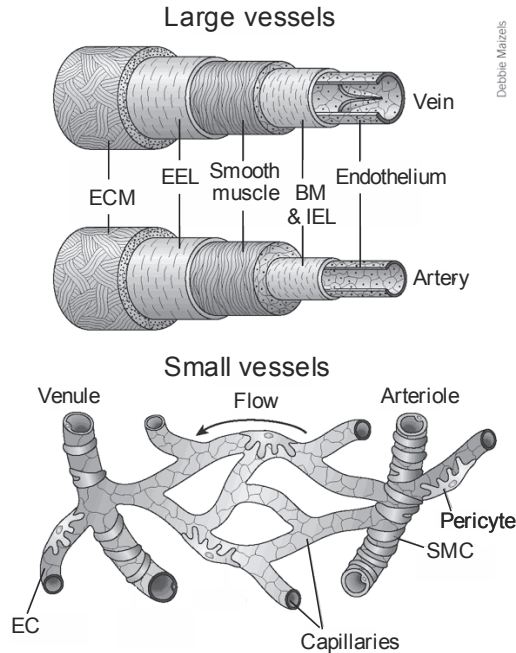


Fig. 3. Structure of the blood vessel wall. Adapted from Cleaver & Melton 2003 and reprinted with permission from Macmillan Publishers Ltd. ECM; extracellular matrix, EEL; external elastic lamina, BM; basement membrane, IEL; internal elastic lamina, EC; endothelial cell, SMC; smooth muscle cell.

2.2.1 Vascular endothelial cells

The vascular lumen is lined with the endothelium, a single layer of ECs constructing a physical barrier between blood and extravascular tissues. ECs are a very heterogenic cell population, varying on levels of morphology, function, gene expression and antigen composition to accommodate the needs of the surrounding tissue or organ. The endothelium of arteries and veins is continuous, but that of capillaries may also be fenestrated or discontinuous. The former is characteristic of organs involved in filtration or secretion, like the glomeruli of the kidney or the exocrine and endocrine glands, and differs from the latter found for instance in the liver in the way that the fenestrations are smaller and have a diaphragm. (Aird 2012).

ECs are highly dynamic and metabolically active. They participate in many physiological processes, such as the control of vasomotor tone, blood cell trafficking, maintenance of blood fluidity, basal and inducible vessel permeability, angiogenesis, hemostasis and both innate and acquired immunity (Aird 2012). Some of the vascular ECs have also been shown to have multipotent characteristics resembling those of endothelial progenitor cells (EPCs) and to be capable of transdifferentiating into vSMCs (Frid *et al.* 2002, Imamura *et al.* 2010, Moonen *et al.* 2010).

Acknowledging the transporter role of vasculature to and from tissues, there is a constitutional transfer of material across the capillary wall. Fluids and small solutes can use the paracellular route between the ECs, but macromolecules need active transfer via the transcellular route. This is mediated by caveolae, vesico-vacuolar organelles (VVOs) and transendothelial channels. (Aird 2012). Caveolae are spherical invaginations of the plasma membrane rich in caveolin1, cholesterol and sphingolipids, and capable of budding of the membrane, shuttling through the cytoplasm and fusing with the opposite cell membrane to release their contents (Simionescu *et al.* 2009). VVOs are clusters of sessile, interconnecting vesicles bridging the luminal and abluminal membranes of ECs and occasionally also adjacent cells. However, the vesicles are separated by thin diaphragms regulating the passage of macromolecules. These diaphragms can be opened by cytokines mediating vessel hyperpermeability, such as VEGF-A, histamine or serotonin, making VVOs the main route of macromolecule extravasation during situations like inflammation or tumor growth (Dvorak & Feng 2001). Transendothelial channels are proposed to be temporary endothelial pores formed by random collision and fusion of two or more vesicles (Simionescu *et al.* 1975).

EC functions are highly site- and time-dependent and modified by signals provided by circulation, ECM, MCs and other stromal cells. By producing and releasing a vast selection of vasoactive factors, the ECs themselves function on surrounding vSMCs regulating their contractile state and vessel tone and diameter as a result. (Martinez-Lemus 2012).

2.2.2 Basement membrane

The BM is a thin layer of highly cross-linked and insoluble connective tissue surrounding ECs to offer them a platform to attach to, thus directing EC polarization (Martinez-Lemus 2012). Around 50 different proteins are known to participate in BM composition. From these the main components of the vascular

BM are collagen type IV, laminin and heparan sulfate proteoglycans such as perlecan and agrin. Other components include for instance fibronectin, entactin/nidogen and non-fibrillar collagen types such as Col XV and XVIII. However, the molecular composition of the BM is unique in each tissue and can be modified according to local and momentary needs. (Glentis *et al.* 2014, Kalluri 2003). For the correct formation and maintenance of the BM, interaction between ECs and vSMCs is needed. Both cell types produce and organize BM components in addition to producing the growth factors needed for proper vascular function. (Martinez-Lemus 2012). The laminin-rich BM supports ECs and helps maintain a stable, organizational endothelium. Supportive mechanisms of the ECM include both external structural support and regulation of intracellular signaling pathways. (Davis & Senger 2005).

Launching of angiogenesis requires BM degradation, liberating ECs to proliferate and migrate. Simultaneously, growth factors sequestered by the ECM are released and connections to the surrounding vSMCs are loosened supporting EC sprouting. (Kalluri 2003). Exposure of ECs to surrounding interstitial collagens (type I and III) leads to the activation of signaling pathways resulting in the reorganization of the EC cytoskeleton and sprouting morphogenesis (Davis & Senger 2005). During BM degradation proteolytic cleavage of type IV collagen and laminin can reveal hidden binding sites and motifs that can exert both pro- and anti-angiogenic effects. For example arrestin and endostatin are inhibitors of angiogenesis that are formed from collagens by proteolytic cleavage. (Kalluri 2003). However, also during angiogenesis supportive signals from the ECM are needed for EC migration, invasion, proliferation and survival. ECM also modifies the EC cytoskeleton through ECM integrins and serves ECs with organizational cues during tube formation. Finally, the formation of a new BM is a critical regulator of lumen diameter and neovessel maturation. (Davis & Senger 2005).

The BM also participates in vessel healing after injury. The damaged vessel wall enables interactions of BM molecules with blood components and vSMCs, which induces the expression of the signals needed to anchor the immune cells to the blood vessel endothelium and allow their migration through the vascular wall. (Martinez-Lemus 2012).

2.2.3 Vascular mural cells

The pericytes of terminal arterioles, capillaries and postcapillary venules and vSMCs of bigger blood vessels are the main components of the *tunica media*.

vSMCs are separated from the endothelium by the common BM discussed above and an internal elastic lamina (IEL). The latter is a thin layer mostly consisting of degradation-resistant elastin fibers. It is not found in all blood vessels but it gives vessels the elasticity needed to accustom to pulsatile blood pressure. IEL is fenestrated, admitting direct connections between ECs and vSMCs and influencing vascular permeability. (Martinez-Lemus 2012).

The vSMCs are needed to control the vessel diameter and thus blood pressure through cell contraction and relaxation. They arrange perpendicular to the ECs twining around the vessel and organizing into one to several layers. The amount of layers increases when the vessel diameter increases. Also, in arteries there are more layers of vSMCs than in veins. So called dense bodies connect the vSMC cytoskeleton to the IEL on the luminal, and to the external elastic lamina (EEL) and the extracellular components of *tunica adventitia* on the abluminal side. These connections mediate the transmission of forces when the vessel constricts or dilates. On top, each vSMC is surrounded by its own BM and elastic fibrils allowing mechanotransduction, which is the alteration of mechanical forces into cellular responses. (Martinez-Lemus 2012).

In microvessels pericytes are embedded into the vascular BM extending longitudinally along the capillary with thin cytoplasmic processes embracing it. Pericytes are tightly connected with the ECs via holes in the BM. AJ resembling adhesion plaques locate at the edges of the pericytes, playing an anchoring role. Also gap junctions exist to allow the transfer of water and small molecules between the cell types. (Bergers & Song 2005). The density of pericytes and the proportion of EC abluminal surface covered by them vary greatly between tissues and are correlated with vessel barrier properties (dense coverage forms tight barrier), EC turnover (dense coverage for slow turnover) and blood pressure (denser coverage in lower extremities) (Armulik *et al.* 2011). Pericyte coverage is the thickest in the central nervous system (CNS) vasculature, where pericytes play a critical role in regulating the permeability of the blood–brain barrier (BBB). The presence of pericytes in CNS attenuates EC transcytosis, functionally tightens EC–EC junctions and inhibits the expression of genes known to increase vascular permeability. Pericytes also limit CNS immune surveillance and support correct polarization of astrocyte end-feet to surround CNS blood vessels. (Armulik *et al.* 2010, Daneman *et al.* 2010).

Pericytes are important regulators of vascular development, stabilization, maturation, function and remodeling, also mediating physiological and pathological repair processes (Armulik *et al.* 2005). Like vSMCs, also pericytes

are capable of vasoconstriction and vasodilation. They have also been shown to have phagocytic and macrophage-like functions and the capability to guide vessel sprouting by migrating ahead of ECs and producing VEGF-A. Some pericytes have organ specific functions, like the hepatic stellate cells of the liver which are involved in vitamin A metabolism and recruitment of inflammatory cells. (Bergers & Song 2005).

2.2.4 Perivascular ECM

The outermost layer of larger arteries and veins is the most complex compartment of the vessel wall. It consists mostly of an ECM made predominantly of longitudinally oriented collagen type I and III fibers and elastic fibers. This matrix provides structural support for the vessel and serves as a scaffold for different types of cells, like fibroblasts and immune cells, together with nerve endings and small capillaries feeding the *adventitia* and underlining *media*. This layer also functions to produce, retrieve, store and release key regulators of vessel wall function needed e.g. for the modulation of vSMC proliferation and activity, thus controlling vascular tone. The *adventitia* functions also as a storage niche for different progenitor cells, such as endothelial, mesenchymal and vSMC progenitors. It serves as the first injury-sensing component of the vessel wall. The main cell type here seems to be the fibroblasts that proliferate quickly and can adopt a strong proinflammatory phenotype secreting chemokines, cytokines, adhesion molecules and ECM proteins. They may also transform into myofibroblasts increasing their contractile capabilities and ECM production and becoming capable of migrating into the *media* and *intima* to assist healing. (Martinez-Lemus 2012, Stenmark *et al.* 2013).

2.3 Angiotensin/TIE signaling pathway

TIE2 is a receptor tyrosine kinase (RTK) expressed in ECs and their precursors (Dumont *et al.* 1992, Schnurch & Risau 1993) and in hematopoietic stem cells regulating their development, proliferation and quiescence (Arai *et al.* 2004, Iwama *et al.* 1993, Puri & Bernstein 2003, Takakura *et al.* 1998). Together TIE2 and TIE1 (also known as TEK and TIE, respectively) compose their own RTK subfamily (Dumont *et al.* 1993, Maisonpierre *et al.* 1993, Sato *et al.* 1993).

TIE2 signaling has a crucial role in the remodeling and stabilization of blood vessels during embryonic development. In adulthood it has been suggested to

regulate endothelial homeostasis, and it has also been implicated in a range of pathogenic conditions from infectious diseases to cancer. In mouse embryos, Tie2 expression is first detected at E7.5 (Dumont *et al.* 1995). It is expressed in the heart endocardium, the leptomeninges surrounding the brain and spinal cord and in both mature ECs and their progenitors, the mesenchymal angioblasts (Dumont *et al.* 1992). The necessity of TIE2 can be seen in homozygous Tie2 null mice (Tie2^{-/-} mice), which die by E10.5 due to underdeveloped vasculature and loss of EC integrity leading to hemorrhage. The embryos also suffer from growth retardation, especially in regions of the head and the heart. Their heart endocardium has a shortage of ECs and the few ECs further do not associate accordingly with the surrounding myocardium which is also underdeveloped and does not form trabecular folds. The vasculature of Tie2 deficient mice consists of uniformly sized blood vessels incapable of sprouting and branching to form the normal hierarchy of differently sized vessels, also showing a reduced amount of ECs. (Dumont *et al.* 1994, Sato *et al.* 1995).

In adult mice Tie2 is suggested to be expressed and activated through the whole hierarchy of quiescent blood vessels and upregulated on sites of vascular remodeling (Schlaeger *et al.* 1997, Wong *et al.* 1997). However, more recent data with conditional knockout of Tie2 or its ligands in mice at E12.5 or later suggests no role for the Ang/TIE2 pathway in the blood vasculature of healthy adult animals (Jeansson *et al.* 2011, Thomson *et al.* 2014).

Tie1 expression is first seen in the angioblasts of an E8.5 mouse embryo (Korhonen *et al.* 1994), later involving the heart endocardium and blood vessel endothelium of the developing embryo (Korhonen *et al.* 1992). Some leukemia cell lines and hematopoietic progenitors also express TIE1 (Batard *et al.* 1996, Partanen *et al.* 1992), whereas blood cells do not (Korhonen *et al.* 1994). In adult tissues TIE1 expression persists on a weaker level and is enhanced on sites of neovascularization (Korhonen *et al.* 1992). In addition, upregulation of TIE1 is seen in places of disturbed blood flow in vessel branching points and downstream of bifurcations in arteries (Porat *et al.* 2004). On top of normal function TIE1 expression is strongly upregulated in pathological situations like angiogenic tumor tissue (Hatva *et al.* 1995, Kaipainen *et al.* 1994) and atherosclerosis (Porat *et al.* 2004, Woo & Baldwin 2011).

In contrast to Tie2^{-/-} mice, Tie1^{-/-} embryos have normal heart structure and function and they develop a normal vessel pattern. However, the endothelium of these mice embryos fails to accomplish structural integrity leading to edema, local hemorrhage and growth retardation. (Puri *et al.* 1995, Sato *et al.* 1995). Edema

probably results from abnormalities during lymphatic development and precedes hemorrhage due to vascular defects (D'Amico *et al.* 2010). Tie1^{-/-} mice die either by E14.5 (Puri *et al.* 1995, Sato *et al.* 1995), possibly due to failure to vascularize certain organs, like kidney, brain and skin, during the midgestational organogenesis (Partanen *et al.* 1996), or as neonates because of breathing problems as the alveoli of their lungs fail to expand after birth. Thus, the main function of TIE1 appears to be to support the integrity and survival of ECs, especially on sites of angiogenic growth of new capillaries (Puri *et al.* 1995, Sato *et al.* 1995). TIE1 has also been found to regulate tumor angiogenesis, postnatal sprouting angiogenesis and EC survival (D'Amico *et al.* 2014) and to have a proinflammatory function (Chan *et al.* 2008, Chan & Sukhatme 2009).

2.3.1 Structure of TIE receptors

The main domain structures of TIE1 and TIE2 are similar (Macdonald *et al.* 2006, Partanen *et al.* 1992), although the overall amino acid identity between mouse Tie1 and Tie2 is only 46%, being highest (84%) in the kinase domains (Korhonen *et al.* 1994). The extracellular part of the receptors consists of three epidermal growth factor (EGF)-like repeats surrounded by two immunoglobulin (Ig)-like domains on the N-terminal and one on the C-terminal side followed by three fibronectin type III (FNIII) repeats (Barton *et al.* 2006, Dumont *et al.* 1993, Macdonald *et al.* 2006). Together these domains fold into a conformation resembling an arrowhead with the Ig2 domain forming the tip of the arrow. Angiopoietins, the ligands of TIE2, bind to the Ig2 domain on a lock-and-key mode without domain rearrangement and little conformational change in either the receptor or the ligand (Barton *et al.* 2006). In TIE2 the extracellular part has been shown to include nine possible N-glycosylation sites (Schnurch & Risau 1993).

The extracellular part is followed by a single transmembrane region and the intracellular part which consists of a split kinase domain separated by a short kinase insert domain (KID) and a C-terminal tail (Dumont *et al.* 1992). The C-terminal tail folds against the kinase domain when the receptor is inactive inhibiting receptor signaling (Shewchuk *et al.* 2000). A schematic of TIE receptor structure and a model of TIE2 clustering upon angiopoietin stimulation are presented in Fig. 4.

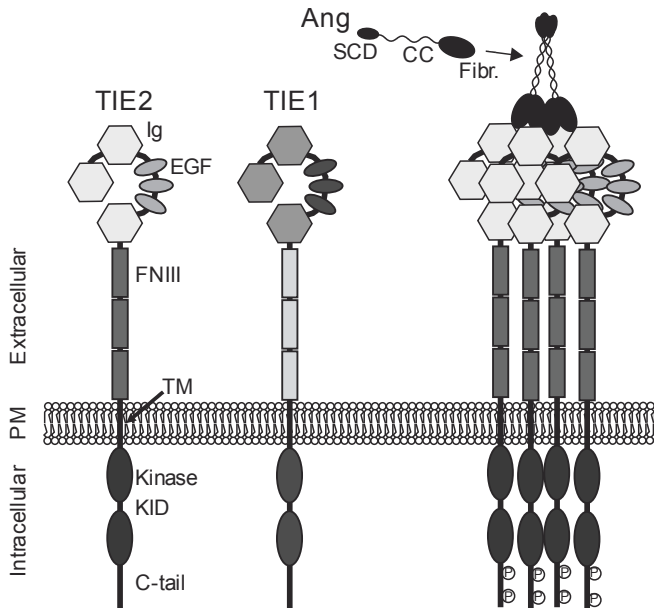


Fig. 4. Domain structure of TIE2, TIE1, and angiopoietins (Ang) and clusterization of TIE2 in response to Ang stimulus. Monomeric Angs multimerize before binding to TIE2 bringing together several receptors which then transphosphorylate each other on intracellular tyrosines (circled P). PM; plasma membrane, Ig; immunoglobulin-like domain, EGF; epidermal growth factor-like repeat, FNIII; fibronectin type III repeat, TM; transmembrane region, KID; kinase insert domain, SCD; superclustering domain, CC; coiled-coil domain, Fibr.; fibrinogen-like domain.

2.3.2 Angiopoietins, the ligands of TIE2

Angiopoietins 1 to 4 (Ang1–4, Ang3 being the mouse orthologue of human Ang4) constitute the family of ligands binding to TIE2 receptor (Davis *et al.* 1996, Maisonpierre *et al.* 1997, Valenzuela *et al.* 1999). Ang1 and Ang2 have been studied the most. The former acts as an agonist and the latter as a context-dependent agonist/antagonist. Less is known about Ang3 and Ang4 and their functions on TIE2. However, it has been suggested that Ang3 is a context-dependent antagonist like Ang2 and Ang4 an agonist like Ang1 (Valenzuela *et al.* 1999). More recently Ang4 has been linked to an increase of blood vessel permeability and the subsequent dilation of lymphatic vessels (Kesler *et al.* 2015). Thus far, no ligand directly binding to TIE1 has been found. However, it has been

shown that Ang1 and Ang4 can induce TIE1 phosphorylation, possibly through interaction with TIE2 (Saharinen *et al.* 2005).

All angiopoietins are secreted glycoproteins with a similar basic structure: a short signaling peptide to guide secretion, an N-terminal superclustering domain, which is angiopoietin specific and functions to form higher order multimers necessary for TIE2 activation, a coiled-coil domain for oligomerization, a short linker peptide and a fibrinogen-like domain that binds TIE2 (Davis *et al.* 1996, Davis *et al.* 2003, Maisonpierre *et al.* 1997, Procopio *et al.* 1999, Valenzuela *et al.* 1999). They also bind to TIE2 similarly, with only three residues contacting TIE2 differing between Ang1 and Ang2 (Barton *et al.* 2006). In addition, at least a tetrameric angiopoietin is needed for TIE2 activation. Ang2 often exists as mainly dimeric in laboratory experiments, whereas Ang1 is more frequently seen to organize into higher order multimers. (Davis *et al.* 2003, Kim *et al.* 2005, Procopio *et al.* 1999). However, it has been shown that a synthetic dimeric Ang1 variant has similar effects to the native version (Oh *et al.* 2015) and that the oligomerization pattern does not determine the agonist/antagonist effect of the angiopoietins on TIE2 (Davis *et al.* 2003, Procopio *et al.* 1999). Instead, this effect seems to be mediated by their fibrinogen-like domain (Davis *et al.* 2003, Procopio *et al.* 1999).

Ang1 is first expressed by the developing myocardium and then by perivascular cells later during development and adulthood (Davis *et al.* 1996). When Ang1 is deleted from a mouse embryo, the resulting phenotype is slightly milder but close to Tie2^{-/-} mice, supporting the idea of Ang1 being the main agonist of TIE2. Ang1^{-/-} embryos die by E12.5 and represent an immature heart endocardium poorly associating with a trabeculae-missing myocardium and a commonly less complex vasculature with more uniformly sized and less branched vessels. (Suri *et al.* 1996). However, conditional knockout of Ang1 at E13.5 or later has no immediate phenotype in mice. Only after tissue injury these animals show accelerated wound healing processes suggesting a protective role for Ang1 in situations of enhanced angiogenesis and fibrogenesis. (Jeansson *et al.* 2011).

Overexpression of Ang1 in the skin of mice leads to dermal hypervascularization with significant increase in microvessel number, diameter and branching, with capillaries representing post-capillary venules (Suri *et al.* 1998). These vessels are also more resistant to inflammation and plasma leakage (Thurston *et al.* 1999).

Overexpression of Ang2 in mice embryos causes similar but more severe defects than depletion of Tie2 or Ang1 (Maisonpierre *et al.* 1997). Depletion of

Ang2 leads to profound defects in development of the lymphatic system, but has no significant effect on vascular development during embryogenesis (Gale *et al.* 2002). Also endothelium-specific overexpression of Ang2 results in hyperplasia of lymphatic vasculature (Zheng *et al.* 2014), supporting a role for Ang2 more in lymphatic than blood vascular development during embryogenesis. However, Ang2 is necessary for postnatal vessel remodeling as demonstrated by the retinal vascularization in the eye of neonatal mice (Gale *et al.* 2002).

Conditional knockout of both Ang1 and Ang2, or the Tie2 receptor, at E12.5 in mice, however, has no effect on blood vasculature. Instead, the embryos show defective dermal lymphatic patterning and edema distinct from what is seen in Ang2^{-/-} mice. After knockout at E16.5 mice pups are born healthy but soon develop eye complications due to absence of lymphatics in the eye. (Thomson *et al.* 2014).

Together these findings indicate that Ang2 antagonizes functions of Ang1 during embryonic blood vascular development, but that the ligands function together during lymphatic vascular development. It further indicates that the Ang/TIE pathway is dispensable in the quiescent vasculature of healthy adults, but required when vascular remodeling is needed.

2.3.3 Functions of the angiotensin/TIE2 pathway

Based on a widely published model, the TIE2 signaling pathway has a dual role in regulating blood vessel maintenance and remodeling depending on the balance between Ang1 (stabilizing) and Ang2 (destabilizing). Ang1 acts as the main agonistic ligand for TIE2. *In vivo* it promotes EC survival and induces branching and vessel maturation during embryogenesis and also maintains vascular integrity in mature animals (Sato *et al.* 1995, Suri *et al.* 1996, Thurston *et al.* 2000). In addition, Ang1 has anti-permeability and anti-inflammatory effects (Gamble *et al.* 2000, Pizurki *et al.* 2003) by regulating cell junction formation and tightness e.g. through phosphorylation of platelet endothelial cell adhesion molecule (PECAM) 1 and VE-cadherin and by increasing association of β -catenin with VE-cadherin, thus preventing leukocyte transmigration (Gamble *et al.* 2000). Ang1 can also upregulate the expression of the TJ component occludin (Hori *et al.* 2004) and inhibit the production of the proinflammatory cytokine interleukin 8 (Pizurki *et al.* 2003). A method has been recently suggested where Ang1 mediates vessel integrity by inhibiting phosphorylation of occludin, promoting its interaction with other TJ components to stabilize TJs and thus blood vessels (Siddiqui *et al.* 2015).

Ang1 treatment also reduces endothelial permeability impeding transendothelial migration of cancer cells (Wu *et al.* 2015). These abilities are strengthened by studies in mice where Ang1 overexpression induces extra stable blood vessels (Suri *et al.* 1998) which are resistant to inflammation and VEGF-A-induced plasma leakage in adults (Thurston *et al.* 1999, Thurston *et al.* 2000), supporting the notion that the main role of Ang1 during adulthood is as an EC quiescence sustaining factor. However, a recent study has indicated that Ang1 is not required for pericyte recruitment and for the maintenance of vessel quiescence throughout adulthood, but is needed to regulate and constrict vascular remodeling after injury (Jeansson *et al.* 2011). Here Ang1 is posited as a “brake” balancing and modulating angiogenic activity which allows the formation of the proper number and size of blood vessels.

In vitro Ang1 has been shown to induce the survival (Daly *et al.* 2004, Fujikawa *et al.* 1999, Hayes *et al.* 1999, Kim *et al.* 2000c, Kwak *et al.* 1999, Kwak *et al.* 2000), sprouting (Kim *et al.* 2000b, Koblizek *et al.* 1998), migration (Witzenbichler *et al.* 1998), tube formation (Hayes *et al.* 1999) and network formation/stabilization (Papapetropoulos *et al.* 1999) of ECs. The effect of Ang1 on cell proliferation is controversial however, since it was first shown not to induce EC proliferation (Davis *et al.* 1996) whereas later on the opposite was indicated (Kanda *et al.* 2005).

Different cellular functions of Ang1 mirror the two sides of the molecular level effects of an Ang1 stimulus. In sparse cells mimicking angiogenic condition, Ang1 induces a migratory cell phenotype with TIE2 translocating to cell–matrix contacts and in the trailing edge of mobile cells (Fukuhara *et al.* 2008, Saharinen *et al.* 2008). As Ang1 is integrated into the ECM (Xu & Yu 2001), substrate bound Ang1 induces TIE2 to locate on the basal membrane of the ECs, promoting their spreading and migration. In confluent cells mimicking quiescent vasculature on the other hand, Ang1 stimulus induces TIE2 to quickly translocate to cell–cell junctions of contacting cells producing TIE2 interactions in *trans*, i.e. between the cells (Fukuhara *et al.* 2008, Saharinen *et al.* 2008). Also TIE1 and vascular endothelial protein tyrosine phosphatase (VE-PTP, the mouse homolog of human protein tyrosine phosphatase beta; HPTP β) are co-recruited to the same complexes that may inhibit paracellular permeability (Saharinen *et al.* 2008).

Originally Ang2 was thought to act as a natural antagonist to Ang1 in blood vessels, as it binds TIE2 with similar affinity but fails to phosphorylate it, thus competing with the activating effect of Ang1 (Maisonpierre *et al.* 1997). After embryonic development Ang2 is essential for vascular remodeling, being highly

upregulated on sites of angiogenesis or vessel regression. Sprouting is seen after Ang2 stimulus when VEGF-A is present and regression when VEGF-A is absent. (Holash *et al.* 1999, Maisonpierre *et al.* 1997). Ang2 is produced by the ECs themselves and stored in Weibel-Palade bodies, from which it can be quickly released to induce a rapid destabilization of the EC monolayer when remodeling is needed (Fiedler *et al.* 2004), thus functioning through an autocrine mechanism (Scharpfenecker *et al.* 2005). This suggests that the main role of Ang2 in ECs might be to disrupt the stabilizing effect of Ang1 to make the vessels more susceptible to other angiogenic stimuli, such as VEGF-A.

In fibroblasts ectopically expressing TIE2, Ang2 can phosphorylate TIE2 (Maisonpierre *et al.* 1997), suggesting that there is an endothelium specific inhibitory factor regulating Ang2 actions upon TIE2. Also in EPCs, Ang2 has angiogenic effects through activation of TIE2 (Kim *et al.* 2006). In addition, it has been discovered that prolonged exposure or a high concentration of Ang2 can activate TIE2 in ECs inducing TIE2-dependent survival, migration and tube formation of ECs (Kim *et al.* 2000a, Mochizuki *et al.* 2002, Teichert-Kuliszewska *et al.* 2001). In stressed ECs Ang2 also acts as an autocrine TIE2 agonist protecting them from apoptosis and maintaining vessel integrity (Daly *et al.* 2006). It is acknowledged nowadays that Ang2 functions as a context-dependent agonist/antagonist of TIE2 (Augustin *et al.* 2009, Yuan *et al.* 2009), its differences compared to Ang1 possibly depending for instance on production from endothelial versus mural cells and pure solubility versus ECM-binding (Xu & Yu 2001) as well as the cellular context and other interacting/regulating molecules present at specific moment. Actions of Ang2 might in part depend on the presence of TIE1, since TIE1 forms a heterocomplex with TIE2 and Ang2 has been indicated as incapable of dissolving this complex and blocking the TIE2 bonding site from Ang1 but not removing the inhibitory effect of TIE1 (Seegar *et al.* 2010). Also, Ang2 plays an agonistic role in LECs where less TIE1 is expressed and overexpression of TIE1 abolishes this effect (Song *et al.* 2012). In addition, Ang2 has been suggested to regulate liver regeneration through modification of TGF β and VEGFR2 levels (Hu *et al.* 2014). It has been proposed that by a similar model, the vascular niche might regulate organ regeneration also more widely (Manavski *et al.* 2014).

Another mechanism explaining context-dependent actions of Ang2 might be its TIE2-independent interaction with integrins, which are cell surface receptors that mediate cell–cell and cell–ECM interactions and participate in intracellular signaling as well. TIE2 is selectively downregulated in angiogenesis-activated tip

cells which instead show high levels of Ang2 and integrins $\alpha\beta3$, $\alpha\beta5$ and $\alpha5\beta1$. Ang2 binds to these integrins inducing EC migration and sprouting. (Felcht *et al.* 2012, Lee *et al.* 2014). Binding of Ang2 to integrins has been suggested to be mediated via different residues of the ligand than those which are necessary for Ang2 to bind to TIE2 (Lee *et al.* 2014). In addition, blocking of Ang2 inhibits tip cell formation and normal branching of the blood vessels (Felcht *et al.* 2012). Ang2 binding to $\beta1$ -integrin has also been linked to TIE2-independent EC destabilization by $\beta1$ -dependent interfering of FN fibrillogenesis in TIE2-low ECs (Hakanpaa *et al.* 2014). In diabetic mice under high glucose Ang2 binds to integrin $\alpha3\beta1$ on retinal pericytes inducing their apoptosis (Park *et al.* 2014). Also Ang1 can bind to integrins promoting e.g. myocyte survival and skin cell adhesion and survival (Dallabrida *et al.* 2005, Ismail *et al.* 2010).

To summarize, a model is suggested in which Ang1 in the quiescent blood vessel endothelium translocates TIE2 and VE-PTP to cell–cell junctions to sustain vessel integrity and possibly to promote vessel maintenance. When vessel remodeling is needed, Ang2 binds TIE2 and creates an angiogenic phenotype where Ang1 now functions to locate TIE2 to cell–matrix contacts to promote EC adhesion, spreading and migration necessary for blood vessel remodeling. (Saharinen *et al.* 2008).

In addition to blood vasculature, Ang2 is also required for the formation of the normal hierarchical lymphatic vasculature. The lymphatic vessels of Ang2^{-/-} mice fail to mature and do not express the collecting vessel phenotype. Furthermore, the postnatal remodeling of the initial lymphatic vessels is insufficient and SMCs are prematurely recruited to these vessels (Dellinger *et al.* 2008). Inhibition of Ang2 blocks formation of the button-like cellular junctions in LECs of initial lymphatics, resulting in a defective uptake of lymph, and of proper zipper-like junctions in the collecting vessels, causing their leakiness (Zheng *et al.* 2014). Conditional TIE1 depletion also affects lymphatic remodeling, hampering the formation of the collective vessels and lymphatic valves (Qu *et al.* 2015, Shen *et al.* 2014a). Ang2 also seems to have an effect in the lymphatic valves (Zheng *et al.* 2014). TIE1 deficiency causes premature SMC recruitment to the initial lymphatic vessels hampering remodeling into a hierarchical system (Qu *et al.* 2015). Ang1, on the other hand, induces lymphangiogenesis through a VEGFR3 mediated path activating LEC proliferation, vessel enlargement and generation of long filopodia, fusion of which eventually leads to new sprouts and vessel development (Morisada *et al.* 2005, Tammela *et al.* 2005). In addition, Ang1 can protect lymphatic vessel integrity during inflammation through

modulation of TJ protein expression (Kajiya *et al.* 2012). Ang1 can also rescue the lymphatic but not the blood vasculature defects seen in Ang2^{-/-} mice (Dellinger *et al.* 2008, Gale *et al.* 2002). However, the exact mechanism of how the Ang/TIE pathway regulates lymphatic development and remodeling is unclear. Even though TIE2 is expressed in LECs (Morisada *et al.* 2005, Shimoda 2009) and both Ang1 and Ang2 have agonistic effects in the lymphatic vasculature, it has also been shown that conditional knockout of TIE2 in neonatal mice has no effect on lymphatic remodeling and maturation (Shen *et al.* 2014a).

2.3.4 Downstream signaling induced by TIE2

Following ligand stimulation TIE2 receptors are clustered and phosphorylated on tyrosine (Y) residues of their intracellular domains (Davis *et al.* 2003, Kim *et al.* 2005). Adaptor proteins can then associate with phosphotyrosines (pY) through their Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Hunter 2014) coupling TIE2 activation to downstream signaling pathways. Different adaptor proteins favor different phosphorylated tyrosines on TIE2 as presented in Table 1. Three C-terminal tyrosines, Y1102, Y1108 and Y1113 (numbering follows the human TIE2 sequence in Uniprot-database) are considered as the main signaling sites of TIE2 mediating different signaling pathways (Tachibana *et al.* 2005). Other potentially important tyrosine residues include Y816 in the juxtamembrane domain and Y992 in the activation loop (Jones *et al.* 1999, Jones *et al.* 2003, Kontos *et al.* 1998). The main signaling routes following Ang1 stimulus pass through phosphatidylinositol-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Fig. 5). A short summary of TIE2 signaling is presented here; a thorough network map was recently published by (Khan *et al.* 2014).

TIE2 interaction with the p85 subunit of PI3K leads to activation of protein kinase B (Akt) regulating EC survival (DeBusk *et al.* 2004, Fujikawa *et al.* 1999, Kim *et al.* 2000c, Kontos *et al.* 1998), migration (Fujikawa *et al.* 1999), tube formation (Saito *et al.* 2003) and sprouting (DeBusk *et al.* 2004, Kim *et al.* 2000b). Through the MAPK signaling family, Ang1 promotes EC proliferation (Kanda *et al.* 2005), migration (Kim *et al.* 2002b) and both pro- and anti-apoptotic effects. TIE2 activation leads to phosphorylation of extracellular regulated kinase (Erk) 1/2 and p38 MAPKs, the former being mediated through PI3K-pathway and representing the anti-apoptotic and the latter the pro-apoptotic pathway. Even though both the anti- and pro-apoptotic pathways are activated

simultaneously, the net result is anti-apoptotic, as the strong effects of PI3K and Erk1/2 pathways suppress the pro-apoptotic effect of p38. (Harfouche *et al.* 2003). Akt also mediates activation of β -catenin which enhances Dll4 expression, supporting extracellular deposition of collagen IV through the Dll4/Notch pathway that may stabilize blood vessels (Zhang *et al.* 2011).

Factors mediating Ang1-induced signals from cell membrane into the nucleus have been studied less. However, the transcription factor forkhead box-O 1 (FOXO1) functioning downstream of Akt is one such mediator. FOXO1 is phosphorylated after Ang1 induced Akt activation. This inhibition excises it from the nucleus and/or prevents it from binding to DNA leading to the downregulation of many genes associated with vascular destabilization and remodeling and to inhibition of FOXO1-mediated EC apoptosis, increasing survivin expression. (Daly *et al.* 2004).

STATs, the signal transducers and activators of transcription factors, interact and are regulated by TIE2 activation. They are cytoplasmic transcription factors which upon activation dimerize and relocate into the nucleus where they start transcription. TIE2 activates STAT3 and STAT5 (Korpelainen *et al.* 1999) both of which have been implicated to stimulate angiogenesis (Wincewicz *et al.* 2007, Yang *et al.* 2009).

TIE2 signaling induced by Ang2 is less studied and more weakly understood than signaling induced by Ang1. However, Ang2 has been shown to elicit a TIE2 dependent increase in phosphorylation of PI3K/Akt, Erk1/2 and p38, where the net effect is the promotion of EC survival (Harfouche & Hussain 2006, Kim *et al.* 2000a). Through PI3K/c-Fes Ang2 promotes EC migration and through c-Fyn tube formation (Mochizuki *et al.* 2002).

Table 1. Phosphorylated tyrosines of TIE2 functioning as binding sites for downstream signaling molecules. pY; phosphotyrosine.

Molecule	Major binding site	Reference(s)	Other binding sites	Reference(s)
Dok-R	pY1108	Jones <i>et al.</i> 2003		
Grb2	pY1102	Huang <i>et al.</i> 1995, Jones <i>et al.</i> 1999	pY1113	Huang <i>et al.</i> 1995
Grb7	pY1102	Huang <i>et al.</i> 1995, Jones <i>et al.</i> 1999		
Grb14	pY816, pY1102, pY1108	Jones <i>et al.</i> 1999, Sturk & Dumont 2010		
p85/PI3K	pY1102	Kontos <i>et al.</i> 1998	pY897, pY1039, pY1068, pY1108, pY1113	Huang <i>et al.</i> 1995, Jones <i>et al.</i> 1999
ShcA	pY1102	Audero <i>et al.</i> 2004		
SH-PTP2	pY1113	Huang <i>et al.</i> 1995	pY816, pY897, pY899, pY1012, pY1024, pY1102, pY1113	Huang <i>et al.</i> 1995, Jones <i>et al.</i> 1999

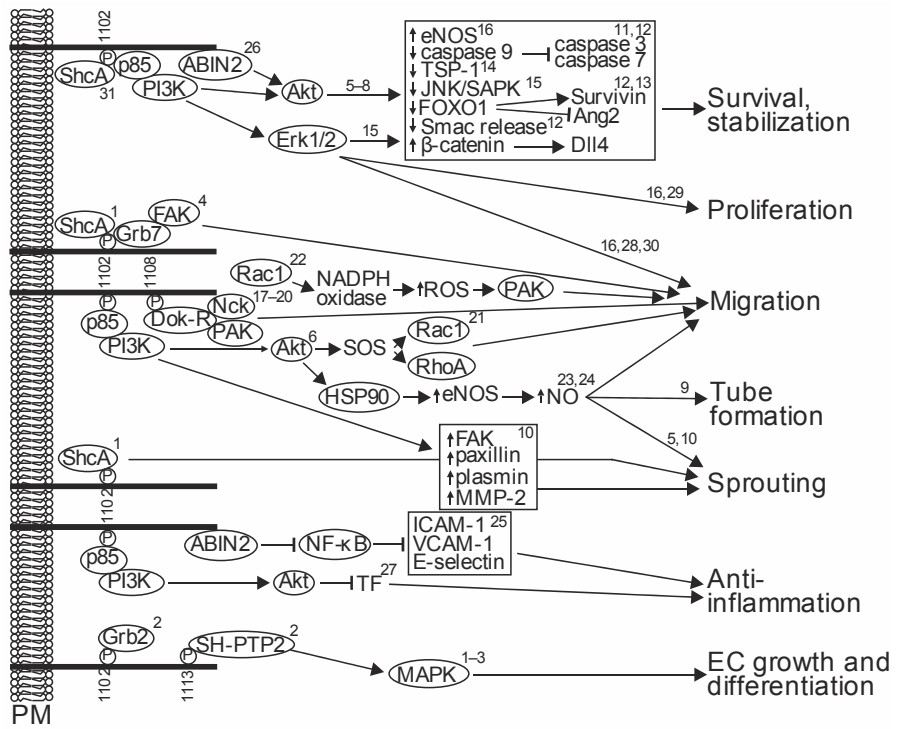


Fig. 5. Ang1-induced TIE2 downstream signaling. Strong black lines indicate TIE2 intracellular domain and circled P phosphorylated tyrosine residues. ABIN2; A20-binding inhibitor of NF- κ B-2, Akt; protein kinase B, Ang; angiotensin, DII4; delta-like 4, Dok-R; downstream-of-kinase related protein, eNOS; endothelium nitric oxide synthase, Erk; extracellular regulated kinase, FAK; focal adhesion kinase, FOXO1; forkhead box-O 1, Grb; growth factor receptor-bound protein, HSP; heat-shock protein, ICAM-1; intercellular adhesion molecule 1, JNK; c-Jun N-terminal kinase, MAPK; mitogen-activated protein kinase, MMP; matrix metalloproteinase, NADPH; nicotinamide adenine dinucleotide phosphate, Nck; non-catalytic region of tyrosine kinase, NF- κ B; nuclear factor kappa B, NO; nitric oxide, PAK; p21-activating kinase, PI3K; phosphatidylinositol-3 kinase, PM; plasma membrane, Rac1; Ras-related C3 botulinum toxin substrate 1, RhoA; Ras homolog gene family, member A, ROS; reactive oxygen species, SAPK; stress-activated protein kinase, ShcA; Src homology 2 domain containing protein, SH-PTP2; Src homology 2 protein tyrosine phosphatase, Smac; second mitochondrial-derived activator of caspases, SOS; son of sevenless, TF; tissue factor, TSP-1; thrombospondin-1, VCAM-1; vascular adhesion molecule 1. References: 1) Audero *et al.* 2004, 2) Huang *et al.* 1995, 3) Jones *et al.* 1999, 4) Han & Guan 1999, 5) DeBusk *et al.* 2004, 6) Fujikawa *et al.* 1999, 7) Kim *et al.* 2000c, 8) Kontos

et al. 1998, 9) Saito *et al.* 2003, 10) Kim *et al.* 2000b, 11) Cardone *et al.* 1998, 12) Harfouche *et al.* 2002, 13) Papapetropoulos *et al.* 2000, 14) Niu *et al.* 2004, 15) Harfouche *et al.* 2003, 16) Fukuhara *et al.* 2008, 17) Jones & Dumont 1998, 18) Jones *et al.* 2003, 19) Master *et al.* 2001, 20) Kiosses *et al.* 1999, 21) Cascone *et al.* 2003, 22) Harfouche *et al.* 2005, 23) Babaei *et al.* 2003, 24) Chen *et al.* 2004, 25) Hughes *et al.* 2003, 26) Tadros *et al.* 2003, 27) Kim *et al.* 2002a, 28) Kim *et al.* 2002b, 29) Kanda *et al.* 2005, 30) Yoon *et al.* 2003, 31) Morris *et al.* 2006.

2.3.5 Regulation of TIE2 signaling

Regulation of TIE2 signaling is a complex process which manifests itself in many different mechanisms. The structure of TIE2 itself is autoregulatory, because in the inactive state of the receptor the tyrosine 897 is phosphorylated preventing receptor dimerization and/or phosphatase recruitment (Shewchuk *et al.* 2000). Also, the C-terminal tail of an inactive receptor folds into an autoinhibitory conformation against the kinase domain blocking the substrate binding site and the C-terminal signaling tyrosines. Upon ligand binding, a conformational change occurs in the intracellular domains exposing the hidden sites and enabling signaling. (Niu *et al.* 2002, Shewchuk *et al.* 2000).

Angiopoietins have been suggested to regulate TIE2 signaling by inducing receptor internalization and degradation upon ligand binding with Ang1 provoking a faster response than Ang2. The ligands are implicated to be released from the cell membrane and to retain their activity. (Bogdanovic *et al.* 2006). Angiopoietins have also been implicated to present a negative regulatory feedback system, downregulating TIE2 expression (Hashimoto *et al.* 2004). In addition, it has been prompted that as Ang1 activates TIE2 it simultaneously downregulates TIE2 signaling by enhancing association of VE-PTP to TIE2. (Winderlich *et al.* 2009).

VE-PTP is the only known exclusively vascular EC specific phosphatase. Knockdown of VE-PTP leads to embryonic lethality by E10 due to severe defects in heart development and angiogenesis (Bäumer *et al.* 2006, Dominguez *et al.* 2007). VE-PTP forms complexes with TIE2 and VE-cadherin (Fachinger *et al.* 1999, Nawroth *et al.* 2002). Upon complex formation VE-PTP dephosphorylates TIE2 attenuating its activity (Fachinger *et al.* 1999). Disturbance of this function triggers enhanced EC proliferation, survival and migration and leads to enlargement of the vascular structures (Winderlich *et al.* 2009, Yacyshyn *et al.* 2009). In confluent cells, however, VE-PTP is recruited to cell-cell contacts Ang1-dependently, in overexpression cells also ligand-independently, together

with TIE2 leading to inhibition of monolayer permeability (Saharinen *et al.* 2008). In addition, it has been shown that enhancing TIE2 activity through inhibition of VE-PTP stabilizes blood vessels in the eye preventing neovascularization and vascular leakage which frequently contribute to visual impairment in several ocular diseases (Shen *et al.* 2014b). VE-PTP dephosphorylates also VE-cadherin, which also has an anti-permeability effect (Nawroth *et al.* 2002). It has been proposed that VE-PTP might function in EC-EC junction maturation securing stability and integrity of the junction complex as its deficiency results in reduced number and disorganization of AJs in ECs and subsequent vessel leakage (Carra *et al.* 2012).

Hypoxia-induced upregulation of TIE1 (McCarthy *et al.* 1998) leads to a reduction of Ang1-induced TIE2 activity and angiogenesis (Yun *et al.* 2013). TIE1 regulates TIE2 by forming heterocomplexes with it (Hansen *et al.* 2010, Kim *et al.* 2006, Savant *et al.* 2015, Seegar *et al.* 2010, Yuan *et al.* 2007). The data on complex formation is controversial since both ligand-independent (Seegar *et al.* 2010) and ligand dependent (Saharinen *et al.* 2005) mechanisms have been suggested. Also, whether (Saharinen *et al.* 2005) or not (Marron *et al.* 2000) TIE2 phosphorylation leads to transphosphorylation of TIE1 in a TIE1:TIE2 complex is unclear.

In a heterocomplex with TIE2, TIE1 has been suggested to inhibit TIE2 by attenuating Ang1 binding to TIE2 (Marron *et al.* 2007), thus fine-tuning blood vessel morphogenesis by downregulating TIE2-induced signaling (Yuan *et al.* 2007). Shedding of the TIE1 ectodomain, which is for instance induced by shear-stress, phorbol 12-myristate 13-acetate (PMA) -induced protein kinase C activation, VEGF-A or tumor necrosis factor alfa (TNF α) (Chen-Konak *et al.* 2003, Yabkowitz *et al.* 1997, Yabkowitz *et al.* 1999), restores Ang1/TIE2 interaction (Hansen *et al.* 2010, Marron *et al.* 2007). The expression level of TIE1 and thus the level of its interaction with TIE2 has been proposed to define the agonistic/antagonistic effect of Ang2 (Kim *et al.* 2006, Song *et al.* 2012).

Also the TIE2 extracellular domain can be proteolytically shed, e.g. by PMA or VEGF-A, to rapidly downregulate downstream signaling (Findley *et al.* 2007, Reusch *et al.* 2001). This has a double silencing effect by both directly inhibiting ligand binding to receptors on the cell membrane and by the shed soluble ectodomains binding the ligand from the surroundings, sequestering it from any remaining full-length receptors (Findley *et al.* 2007). Even though shedding of extracellular domains of both TIE1 and TIE2 are induced by the same molecules (Findley *et al.* 2007, Reusch *et al.* 2001, Yabkowitz *et al.* 1997, Yabkowitz *et al.*

1999), the time dependence of the shedding and turnover are different: VEGF-A treatment decreases TIE1:TIE2 ratio acutely but increases it chronically, so first supporting Ang1/TIE2 signaling and then dispelling it, preventing short-term fluctuations of VEGF-A from causing vessel destabilization. TNF α instead causes a slow decrease in the TIE1:TIE2 ratio with increasing duration causing chronic enhancement of TIE2 signaling. (Singh *et al.* 2012).

In a recent publication a model was suggested where a delicate balance prevails between TIE1 and TIE2 determining the net positive or negative effect of TIE1 on TIE2 in the vasculature. The writers hypothesize that in quiescent ECs TIE1 is downregulated and TIE2 supports EC survival and vessel integrity. When sprouting is needed, TIE1 is balanced with TIE2 in the stalk cells and they function together to support EC survival, whereas in the tip cells TIE1 is upregulated counter-regulating TIE2 cell surface presentation and enabling a migratory EC phenotype. (Savant *et al.* 2015).

Finally, also integrins participate in TIE2 regulation. Integrin $\alpha 5\beta 1$ interacts with TIE2 allowing a lower concentration of Ang1 to induce TIE2 activation when $\alpha 5\beta 1$ is bound to fibronectin (Cascone *et al.* 2005).

2.4 Receptor tyrosine kinase trafficking in human disease

Mutations in RTKs are linked to a large number of human congenital syndromes and diseases. Mutations can be inherited or sporadic and cause e.g. dwarfism, craniosynostosis, or cancer. Both gain-of-function mutations causing chronic receptor activation and loss-of-function mutations resulting in non-functional or dominant negative receptors have been identified. (Robertson *et al.* 2000). The pathology behind RTK related diseases seems to be commonly related to mechanisms affecting receptor trafficking, such as the subcellular site of activation and/or ligand-dependent clustering and translocation. These include alterations in receptor phosphorylation (i.e. activation), dimerization and glycosylation caused by the mutation.

RTK families share a similar structure with an extracellular ligand-binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain. Ligand binding commonly results in conformational changes that induce and stabilize receptor dimerization. Receptor activation causing mutations can locate in the extracellular, transmembrane or kinase domain, resulting in increased ligand-binding affinity, ligand-independent dimerization, decreased ligand-mediated downregulation, stabilization of ligand-induced dimers or

directly ligand-independent activation of the kinase domain. Also modifications of specific phosphotyrosines participating in receptor signaling can have a similar effect without increasing the actual receptor activity. (Gibbs & Legeai-Mallet 2007, Robertson *et al.* 2000).

Genetic defects affecting pathways synthesizing glycans, mainly the N-linked and O-linked, cause a range of diseases. Nearly all proteins traveling through the endoplasmic reticulum (ER) and the Golgi apparatus are N-glycosylated and this modification can determine or influence protein folding, stability, trafficking, localization and oligomerization. A lack of all N-glycans is lethal in organisms varying from yeast to mammals. (Freeze 2006).

A frequent RTK trafficking alteration due to immature glycosylation seems to be intracellular retention in the ER and/or Golgi that may prevent the receptor's function as a plasma membrane receptor for extracellular ligands. Commonly, ligand-independent constitutive phosphorylation of the receptor prevents its full glycosylation, leading to folding defects and possibly also protein aggregation. Misfolding impairs further protein trafficking to the cell surface and the receptors remain intracellular. The phase where the glycosylation pathway is stopped defines whether the receptor is retained in the ER or in the Golgi. RTKs for which immature glycosylation dependent ER/Golgi retention has been shown include FGFR2 in craniosynostosis, FGFR3 in dwarfism, Flt3, EGFR, ErbB2, ErbB3, IGF-IR and PDGFR α in cancer, anaplastic lymphoma kinase (ALK) in neuroblastoma and KIT in gastrointestinal stromal tumors. (Bahlawane *et al.* 2015, Choudhary *et al.* 2009, Contessa *et al.* 2008, Gibbs & Legeai-Mallet 2007, Hatch *et al.* 2006, Lievens & Liboi 2003, Lievens *et al.* 2004, Mazot *et al.* 2011, Tabone-Eglinger *et al.* 2008). The human nerve growth factor receptor (TrkA), instead, displays a reciprocal mechanism where glycosylation of the receptor is necessary to prevent ligand-independent receptor phosphorylation and activation. Here hypoglycosylation of the receptor leads to constitutive activation and intracellular retention. (Watson *et al.* 1999).

Normally, an ER checkpoint notices misfolded proteins and directs them to be bound by scaffold proteins like calnexin and calreticulin. If this binding does not help proteins to fold correctly, they are sent to proteasome for degradation. (Braakman & Bulleid 2011). For disease causing RTK mutants an increased degradation is sometimes shown (Hatch *et al.* 2006) but in other cases the mutation stabilizes the receptor resisting degradation and resulting in higher level of intracellular accumulation, possibly also affecting the severity of the disease (Lievens *et al.* 2004). In some cases it has been shown that silencing the

autoactivating effect of disease causing mutations restores full glycosylation and cell surface location of the receptor (Mazot *et al.* 2011, Schmidt-Arras *et al.* 2005).

As glycosylation deficient receptors are commonly ligand-independently activated in the intracellular compartments they are retained in, it has also been suggested that they may be able to bind and activate downstream signaling molecules, possibly differing from the conventional signaling pathways of the receptor in question, from these wrong locations. Actually, this kind of aberrant signaling has been shown for Flt3, PDGFR α , TrkA, KIT, FGFR2 and FGFR3. (Bahlawane *et al.* 2015, Choudhary *et al.* 2009, Hatch *et al.* 2006, Lievens & Liboi 2003, Lievens *et al.* 2004, Schmidt-Arras *et al.* 2005, Watson *et al.* 1999, Xiang *et al.* 2007).

2.5 Venous malformations and other vascular anomalies

Vascular anomalies are congenital lesions of abnormal vascular development. They are divided into vascular tumors and vascular malformations. The former are not always apparent at birth, grow rapidly by cellular hyperplasia and do not necessarily infiltrate surrounding normal tissues but may sometimes destruct them. The latter are further divided into slow-flow and fast-flow lesions and characterized by the vessel type involved. Malformations are usually present at birth, grow slowly, are infiltrative and destructive, never spontaneously regress and continue to expand with time. Classification of vascular anomalies is shortly summarized in Table 2 and venous malformations (VMs) are further discussed below.

With an incidence of around 1/10 000 (Brouillard & Vikkula 2007), venous anomalies are among the most common deformities seen in vascular clinics. 94% of these are sporadic VMs, 1% dominantly inherited cutaneomucosal VMs (VMCM, OMIM 600195) and 5% glomuvenous malformations (GVM, OMIM 138000) (Boon *et al.* 2011). VMs are congenital, local, slow-flow defects of vasculature. They are composed of enlarged, tortuous, venous-like channels lined by a disorganized, uneven layer of SMCs (Brouillard & Vikkula 2007, Vikkula *et al.* 1996). VMs vary from small, localized and asymptomatic to extensive, infiltrative and life-threatening (Cahill & Nijis 2011) causing pain, disfigurement, functional impairment and organ dysfunction. They grow proportionately with the individual and may enlarge during puberty and pregnancy. (Boon *et al.* 2011). Local intravascular coagulation (LIC), characterized by high D-dimer level, is

seen in almost 50% of VM patients. This coagulopathy can cause acute pain and formation of thrombi, which often calcify to form phleboliths. Severe forms may enhance hemorrhage during surgery if not treated correctly. (Boon *et al.* 2011, Dompmartin *et al.* 2008, Dompmartin *et al.* 2009).

Both inherited and sporadic mutations of the endothelial receptor TIE2 have been identified in VMs, comprising over 50% of the sporadic cases (Calvert *et al.* 1999, Shu *et al.* 2012, Soblet *et al.* 2013, Wouters *et al.* 2010, Ye *et al.* 2011, Zhou *et al.* 2015). Some of these mutations are always encountered alone, others always in *cis*, meaning on the same allele, with certain other mutation. Some mutations have been identified only as inherited, others only as sporadic and some as both inherited and sporadic (Soblet *et al.* 2013). The most common inherited mutation is R849W, and the most common sporadic is L914F (publication I). All identified missense and nonsense TIE2-VM mutations have been reported to be ligand-independently hyperphosphorylated in cultured cells (Calvert *et al.* 1999, Soblet *et al.* 2013, Vikkula *et al.* 1996, Wouters *et al.* 2010).

Treatment for VMs consists of surgery, sclerotherapy and laser therapy (Brouillard & Vikkula 2007). Elevation and compression garments are utilized to relieve symptoms and impede further growth, and low-molecular-weight heparin to improve pain from thrombosis (Richter & Friedman 2012). However, the treatment available is not potent, nor lasting and malformations tend to recur.

Table 2. Classification of vascular anomalies. The most common vascular tumor and the simple vascular malformations are presented with some additions. A thorough list can be found from www.issva.org. The table is based on Behr & Johnson 2013a, Behr & Johnson 2013b, Boon *et al.* 2011, Richter & Friedman 2012, Uebelhoer *et al.* 2012 and Wassef *et al.* 2015.

Vascular anomaly	Characteristics	Incidence	Affected genes
Tumors			
Hemangiomas	Consist of hyperplastic ECs.	1:10–25	<i>VEGFR2</i> <i>TEM8</i>
Congenital	Present at birth, proliferative phase in uterus. Rapidly, partially or noninvoluting.		
Infantile	Present shortly after birth, rapid proliferation followed by a plateau phase and involution.		
Malformations			
Slow-flow			
Capillary malformation (CM)	Consist of dilated capillary-like channels. Present at birth, generally persists throughout life. Flat, reddish cutaneous patches with irregular borders, may thicken and darken with time.	3:1000	
CM-AVM	Multifocal capillary malformations with an arteriovenous malformation.		<i>RASA1</i>
Hereditary hemorrhagic telangiectasia	Cutaneomucosal and gastrointestinal telangiectasias, often associated with multiple arteriovenous malformations.	1:5000–8000	<i>ENG</i> <i>ALK1</i> <i>SMAD4</i>
Venous malformation (VM)	Consist of enlarged venous-like channels, soft and compressible bluish mass. Venous congestion and thrombosis. Focal, multifocal or diffuse.	1:10 000	<i>TIE2/TEK</i>
Blue rubber bleb nevus syndrome	Multiple, small VMs affecting skin (especially palms and soles, commonly hyperkeratotic) and gastrointestinal tract.		
Glomuvenous malformation	Nodular, raised, purple-blue, multifocal, hyperkeratotic, often cobblestone-like lesions mostly on extremities.		<i>glomulin</i>
Cerebral cavernous malformation	Solitary or multiple nodular aggregates of closely packed veins in brain. May also affect spinal cord, retina and skin.	5:1000	<i>KRIT1</i> <i>Malcavernin</i> <i>PDCD10</i>
Lymphatic malformation	Consist of dilated lymphatic vessels with insufficient communication to the overall lymphatic system. Mostly congenital, rarely occurring after trauma or infection. Soft, fluid-filled swelling or soft and noncompressible masses under skin.	1:2000–4000	<i>VEGFR3</i> <i>FOXC2</i> <i>SOX18</i>

Vascular anomaly	Characteristics	Incidence	Affected genes
Fast-flow			
Arteriovenous malformation (AVM)	Anomalous tangle of vessels shunting blood from the arterial to the venous system without an intervening capillary bed. Lesions are pulsatile and warm.		
Arteriovenous fistula	A single connection between an artery and a vein without an intervening capillary bed. Lesion may be pulsatile and warm.		
Combined malformations	Different combinations of the aforementioned simple malformations in a single lesion.		

3 Aims of the study

Genetic studies have identified mutations of the endothelial TIE2 receptor tyrosine kinase in venous malformations. However, how those mutations affect the molecular and cellular functions of TIE2 and how this contributes to VM formation has remained unknown. The goal of this thesis project was to investigate how the different mutations affect TIE2 receptor functions at the cellular and the molecular level. To accomplish the goal, the following specific research targets were set:

1. Mutation-induced changes in TIE2 receptor trafficking and responses to ligand
2. Effects of mutated TIE2 on EC monolayer formation
3. Effects of TIE2 mutations on downstream signaling

4 Materials and methods

The materials and methods used in this thesis are summarized in the table below. Full descriptions and references can be found from the original publications I–III and their online supplements.

Table 3. Materials and methods used in the original publications.

Level	Method	Publication(s)
RNA	Allele-specific PCR	I
	Gene silencing with short hairpin RNA (shRNA)	III
	Microarray	II
	Quantitative real-time PCR	II, III
	Reverse transcription PCR	I, II, III
	RNA extraction	I, II, III
DNA	Semiquantitative minisequencing assay (SNaPshot)	I,III
	DNA constructs and cloning techniques	I, II, III
	DNA extraction	I
Protein	Biotinylation assay	III
	Enzyme linked immunosorbent assay (ELISA)	II, III
	Glycosylation analysis	III
	Immunoprecipitation	I, III
	ECM protein analysis	III
	Pharmacological inhibition	II, III
	Protein synthesis inhibition	III
	Total protein concentration quantification	III
	SDS-PAGE and Western blot	I, II, III
	Cell and tissue	Angiopoietin binding assay
Cell culture		I, II, III
Cell lysis		I, II, III
Cell proliferation assay		II
Cell viability assays		II
Fibrin gel assay		III
Fluorescence-activated cell sorting (FACS)		I, III
Histological analysis		III
Immunofluorescence staining		I, II, III
Immunohistochemistry		I, II, III
Microscopy		I, II, III
Retrovirus production and cell transduction		I, II, III
Transfection		I, II, III
Other		Computational analysis
	Quantitative image analysis	I, II, III

Level	Method	Publication(s)
	Reagents and antibodies	I, II, III
	Spheroid transplantation assay in mice	III
	Statistical analysis	I, II, III

5 Results

5.1 TIE2-VM mutations have both common and distinct effects on TIE2 on molecular, subcellular and cellular levels (I, II & III)

To study the effects of TIE2-VM mutations, human umbilical vein endothelial cells (HUVECs) were retrovirally transduced with a total of 23 TIE2 mutations identified in VMs and compared with a wild-type form (TIE2-WT) and non-transduced (NT) HUVECs. We included five inherited mutations (found in the blood of the affected individuals; R849W, Y897S, R918C, A925S and K1100N), six sporadic (found in lesions but not blood) double mutations in *cis* (in the same allele; Y897C-R915C, Y897F-R915L, Y897H-R915C, Y897S-S917I, T1105N-T1106P and T1105N-G1115X), their constituting single mutations, the most common sporadic mutation L914F, a C-terminal deletion occurring alone in lesions (R1099X) and an extracellular deletion form (Del-TIE2) found in combination with R849W substitution. The single mutations constituting different double mutations are thought to be predisposing instead of VM causing as they have not been found alone in VM lesions. Thus, comparing a double mutation to its constituting single mutations should assist the identification of any VM causing alterations. R849W, Y897S and R918C have been reported both as inherited and sporadic (Calvert *et al.* 1999, Soblet *et al.* 2013, Vikkula *et al.* 1996, Ye *et al.* 2011). Most of the TIE2 mutations identified in VMs are missense mutations but also two nonsense mutations (R1099X and G1115X) and a deletion (Del-TIE2) were studied. The locations of TIE2 mutations identified in VMs are presented in Fig. 6.

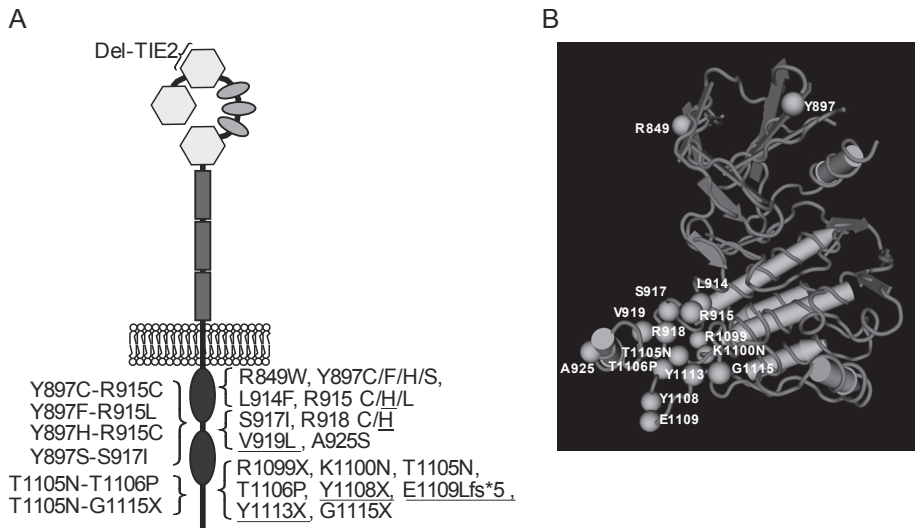


Fig. 6. Localization of TIE2 mutations identified from venous malformations. A) A schematic presentation; the underlined mutations were not studied in this thesis, and only the studied double mutations are shown. B) A 3D model of TIE2 protein intracellular structure showing how most of the VM-mutations gather on the same area of TIE2. Cn3D (<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>) was used to construct the model. fs*5; a frame-shifting mutation ending in a stop codon at position 5.

5.1.1 Phosphorylation levels of TIE2-VM mutations show high variation (I, II & III)

Common to published TIE2-VM mutations is an increase in ligand-independent tyrosine phosphorylation (P-Tyr) (Soblet *et al.* 2013, Vikkula *et al.* 1996, Wouters *et al.* 2010). In retrovirally transduced HUVECs, an increase in TIE2 P-Tyr was observed in all but three mutant forms: A925S, T1105N and Del-TIE2. For A925S and T1105N a relatively weak level of autoactivation has previously been reported in non-ECs (Soblet *et al.* 2013, Wouters *et al.* 2010). In our settings, however, they showed even lower autophosphorylation levels than TIE2-WT. The Del-TIE2, in turn, was retained intracellularly and failed to phosphorylate at all.

Most of the mutations studied caused an increase in TIE2 autophosphorylation, but the level was highly variable: ten mutations showed mild (< 5), four mutations moderate (5 to 10) and six mutations high TIE2

phosphorylation (> 10 times over TIE2-WT). The response to activating Ang1 ligand in terms of increase in P-Tyr varied also: Seven mutations either failed to increase phosphorylation after stimulus or the increase was very low (< 1.5-fold unstimulated form), indicating dysfunctional ligand-mediated regulation of TIE2. Only five mutations out of 22 responded to Ang1 on the same level as TIE2-WT (at least 2.3-fold increase).

In certain double mutations the single constituent mutations showed an additive effect on TIE2 P-Tyr (namely Y897C-R915C, Y897H-R915C and Y897S-S917I). The autoactivation state of TIE2 was lowest in single Y897 substitutions, higher in R915C and S917I and highest in the resulting double mutation actually seen in lesions. However, for the other double mutations studied (Y897F-R915L, T1105N-T1106P and T1105N-G1115X), no significant increase in autoactivation state was observed when compared to the more strongly phosphorylated constituent single mutation (R915L, T1106P or G1115X). This suggests that an increase in autoactivation alone is not sufficient to turn a predisposing single mutation into a VM causative one.

5.1.2 Extracellular deletion prevents TIE2 function as a plasma membrane located receptor by intracellular retention (I)

We identified a somatic “second-hit” alteration on the WT allele of a VMCM patient carrying an inherited R849W mutation. An in-frame deletion of 129 bp (Del-TIE2) corresponds to amino acids 122–165 of the extracellular Ig2 domain, which participates in ligand binding (Barton *et al.* 2006). Unlike other TIE2-VM mutations studied, Del-TIE2 was not hyperphosphorylated *in vitro* nor was it able to enhance R849W hyperphosphorylation *in trans*. In immunofluorescent staining, Del-TIE2 showed intracellular retention in the ER, thus abolishing TIE2 from the plasma membrane making it incapable of binding Ang1 and responding to the ligand stimulus by increased phosphorylation or receptor clustering and subcellular translocation.

5.1.3 TIE2-VM mutations distinctly affect subcellular location and activation of TIE2 (I & III)

Unlike other RTKs, TIE2-WT shows unique ligand-mediated compartmentalization in EC–EC and EC–ECM contact sites (Fukuhara *et al.* 2008, Pietilä *et al.* 2012, Saharinen *et al.* 2008). Because Del-TIE2 was seen to be

retained intracellularly, we examined the localization of the most common inherited (R849W) and sporadic (L914F) TIE2-VM mutations in HUVECs next. In unstimulated cells, TIE2-WT and R849W were distributed evenly on the plasma membranes with an occasional minor increase in perinuclear area, probably comprising of newly synthesized receptors. Immunostaining with specific antibodies detecting phosphorylated TIE2 (P-TIE2) showed no signal in TIE2-WT or R849W. Instead, L914F showed accumulation in both the ER and the Golgi, in the latter of which it was also ligand-independently phosphorylated. However, intracellular retention of L914F was not total as observed in Del-TIE2 since staining was also seen on the plasma membrane.

Previously it has been shown that after Ang1-stimulus TIE2 is quickly clustered and translocated to the cell–cell junctions of contacting cells and the cell rear of mobile cells (Fukuhara *et al.* 2008, Saharinen *et al.* 2008). We observed this in the majority of TIE2-WT and R849W expressing cells ($85.8 \pm 3.6\%$ and $84.8 \pm 7.6\%$, respectively). However, ligand-induced TIE2 translocation was inadequate in cells expressing L914F ($12.5 \pm 4.1\%$). Instead, a punctuate immunostaining pattern was found, where TIE2-L914F clusters were randomly dispersed over the plasma membrane and incompletely translocated to junctions and cell edges. In response to Ang1 both R849W and L914F were also seen to accumulate and activate more in retraction fibers than what was seen with TIE2-WT. Retraction fibers are thin, ECM-bound projections of the rear end of crawling cells forming on sites where the cell body is becoming detached from the ECM but focal adhesions persist. Also the pattern of phosphorylation in retraction fibers was different between the mutants and TIE2-WT. As TIE2-WT was typically activated in distal ends and intersections of the retraction fibers, particularly R849W was seen to be strongly activated all along the fibers. L914F activation dispersed along the fibers also, but on lower levels than R849W.

To investigate whether other TIE2-VM mutations affect TIE2 localization and compartmentalization similarly to R849W and L914F, we retrovirally overexpressed 20 different missense and nonsense mutations in HUVECs. We found out that three TIE2 trafficking abnormalities identified with R849W and L914F were common to several other TIE2-VM mutations. Accumulation and phosphorylation in the Golgi was common to 13 out of 23 mutations, being the most frequently identified alteration in TIE2 compartmentalization. Punctuate Ang1-induced clustering indicating incomplete translocation was seen with 11 mutations. Similarly, abnormally high translocation and activation in retraction fibers in response to Ang1-stimulus was also observed in 11 mutations. The ER

retention, however, was absent from all of the 20 additional mutations studied, thus being specific for Del-TIE2 (total retention) and L914F (partially retained). Comprehensive comparison of 23 mutations also revealed a new type of abnormality in TIE2 location in three out of four double mutations containing a substituted Y897. In these cells only very faint TIE2 immunostaining intensity and an attenuated response to Ang1 were observed on the plasma membrane. However, no ER retention was observed as in Del-TIE2 or L914F, suggesting a different mechanism for the diminished plasma membrane localized TIE2. Most of the mutations studied showed one to four of the five trafficking abnormalities. None showed them all and interestingly one mutation – Y897C-R915C – was also left without a localization abnormality.

5.1.4 Defective glycosylation and premature clustering, but not increased phosphorylation, may contribute to mutated TIE2 receptor retention in the Golgi (III)

Intracellular retention in the Golgi was observed in 13 of the TIE2 mutations studied. To examine the molecular mechanisms behind this abnormality we studied the possible effects of increased phosphorylation, glycosylation and intracellular dimerization/clustering previously implicated in altered RTK trafficking as stated in the literature review (Bass *et al.* 1998, Contessa *et al.* 2008, Hatch *et al.* 2006, Lievens *et al.* 2004, Schmidt-Arras *et al.* 2005, Tabone-Eglinger *et al.* 2008).

First we studied the role of receptor hyperphosphorylation by combining the highly phosphorylated most common sporadic TIE2-VM mutation L914F in *cis* to a kinase-inactivating mutation K855R (Huang *et al.* 1995). Our findings showed that hyperphosphorylation of the TIE2-VM receptors does not contribute to intracellular retention as also the kinase-negative L914F accumulated on the perinuclear area in the Golgi, even though P-TIE2 was abolished.

Premature dimerization of insulin receptors leads to less efficient maturation and intracellular retention of dimeric or aggregated receptors (Bass *et al.* 1998). In addition, bimolecular fluorescence complementation assays in transfected HEK293T cells indicate that R849W may ligand-independently form dimers more efficiently than TIE2-WT (Yamakawa *et al.* 2013). To investigate the effects of premature intracellular TIE2 clustering on TIE2 trafficking, TIE2 and multivalent Ang1-ligand were overexpressed together in HUVECs. This caused TIE2 to be retained and activated in the Golgi similarly than L914F. However, the same

effect was not seen with a dimeric TIE2-WT_Fc fusion protein, perhaps suggesting that the dimeric, but not the aggregated form of TIE2 is capable of translocation from the ER/Golgi to the plasma membrane.

N-linked glycosylation is commonly needed in receptor maturation and regulation of protein trafficking (Ohtsubo & Marth 2006). The extracellular part of TIE2 contains nine possible sites for N-glycosylation (Schnurch & Risau 1993). Glycosylation analysis of L914F revealed a more dominant endoglycosidase H (endo-H) sensitive, immature form (Hurt *et al.* 2013) compared to TIE2-WT. This indicates that L914F is processed less efficiently into a mature, endo-H-resistant glycoprotein possibly affecting its trafficking.

5.1.5 Increased protein turnover rate reduces plasma membrane localized Y897 double mutant TIE2 (III)

Three out of four double mutations containing residue Y897 substitution (Y897F-R915L, Y897H-R915C and Y897S-S917I) showed relatively low plasma membrane localization compared to corresponding single mutations. To investigate why, bicistronic TIE2-WT- and Y897F-R915L-IRES-GFP constructs were expressed in HUVECs allowing TIE2 quantification (mRNA and protein level) and transfection efficiency normalization to simultaneously expressed GFP. Without ligand stimulation, the amount of Y897F-R915L was about half of that of TIE2-WT. After an Ang1 stimulus, the TIE2-WT reduced by 36%, probably due to internalization as previously described (Bogdanovic *et al.* 2006). Surprisingly, this decrease was not seen with Y897F-R915L. To study the plasma membrane TIE2 population that should be able to react to extracellular Ang1-ligand, plasma membrane protein fractions were extracted in a biotinylation assay. Here, Y897-R915L was found to be 63% less than TIE2-WT.

To compare protein and mRNA levels of Y897F-R915L to TIE2-WT and corresponding single mutations Y897F and R915L, all these TIE2 forms were expressed as bicistronic TIE2-IRES-GFP constructs. The TIE2 protein/mRNA ratio was greatly reduced in Y897F-R915L (~70% decrease) but not in other forms studied. Next, protein synthesis was blocked with cycloheximide. A significantly faster disappearance of Y897F-R915L over TIE2-WT was seen and the constitutive single mutations showed a trend for an additive effect. This indicated an accelerated turnover rate for the double mutant receptors.

TIE2-WT signaling has been shown to be regulated by shedding of the extracellular domain (Findley *et al.* 2007, Reusch *et al.* 2001) and therefore a

possible effect of TIE2 shedding on lowered levels of the Y897 double mutant receptors was also studied. We found that levels of soluble TIE2 were elevated in conditioned medium from both Y897F-R915L and R915L expressing HUVECs compared to TIE2-WT. Thus, shedding might represent an additional mechanism behind the reduction of plasma membrane TIE2 that may also hamper ligand-controlled functions of the receptor.

5.1.6 TIE2 mutations result in resistance to apoptosis, but not increased cell proliferation (II)

To investigate the possible mechanisms causing the abnormally enlarged VM vessels, we examined the overall effects the commonly inherited R849W and most frequent L914F have on EC proliferation and survival. Mutant expressing HUVECs showed a significant increase in cell number over time, which, however, did not correlate with increased proliferation. Instead, an augmented resistance to cell death under both normal and serum starved culture conditions was observed. This result is in accordance with previous data of R849W hyperactivating the anti-apoptotic Akt (Morris *et al.* 2005). The response seen, however, was stronger in L914F than in R849W expressing HUVECs.

5.2 TIE2 hyperphosphorylation causes changes in downstream cellular signaling (II & III)

We used microarray technology to identify genes differentially expressed between confluent cultures of non-transduced (NT) HUVECs and HUVECs transduced with WT and mutant (R849W and L914F) TIE2. The inclusion of NT HUVECs allowed us to track genes dysregulated simply due to overexpression of TIE2, rather than specific mutant forms of the receptor.

A one-way analysis of variance (ANOVA) identified 743 genes differentially expressed between the four groups. In keeping with the ‘stronger’ molecular phenotype associated with L914F when compared with R849W, which is likely due to more highly induced phosphorylation, these are mainly attributable to differences between L914F and the other forms. R849W, in contrast, was similar to TIE2-WT. A Tukey *post hoc* test revealed that 41% of the genes (n = 306) are different between L914F and TIE2-WT cells, and 16% of the genes (n = 116) between NT and TIE2-WT cells, with only 5% of the genes (n = 39) distinguishing between R849W and WT cells. A direct comparison of L914F to

WT identified 80 genes with significant changes in expression of at least 2-fold (34 genes up, 46 genes down in L914F). A significant proportion of these differentially regulated genes is involved in vascular development/angiogenesis and cell migration. Interestingly, preponderance of these genes favoring EC migration, proliferation or tube formation was downregulated by L914F. Among these downregulated genes were the growth factors cysteine-rich 61 (CYR61), connective tissue growth factor (CTGF), bone morphogenetic growth factor 4 (BMP4) and placental growth factor (PGF). Also the TIE2 ligand Ang2 was downregulated. The effect, however, was not exclusively pro-quiescence, as illustrated by the downregulation of anti-angiogenic thrombospondin-1 (TSP-1). In addition, L914F led to the upregulation of many metalloendopeptidases including members of the ADAMTS family, several of which have a role in ECM remodeling (Lin & Liu 2010, Porter *et al.* 2005). The same cutoff of 2-fold difference yielded no genes that distinguish R849W from WT.

5.2.1 Ligand-independent phosphorylation of TIE2-VM forms induces activation of downstream signaling pathways (II & III)

Ligand stimulated TIE2-WT phosphorylation activates downstream signaling pathways, including PI3K/Akt and MAPK, important for cell survival and for cell migration and proliferation, respectively (Fukuhara *et al.* 2008, Kim *et al.* 2000c, Papapetropoulos *et al.* 2000, Yoon *et al.* 2003). We investigated the activation (phosphorylation) state of Akt and Erk1/2, which represent these pathways, in cells expressing TIE2-VM mutations. Both were commonly phosphorylated, however, at variable levels. Generally Akt showed stronger activation than Erk1/2 and in five mutants Erk1/2 activation was not observed. The noticed elevated activation of downstream signaling molecules, Akt and Erk1/2, mostly reflected the autoactivation state of TIE2. However, also the mildly phosphorylated A925S and T1105N caused an increase in Erk1/2 and both Akt and Erk1/2 activation, respectively. To our surprise, also the nonsense (premature stop codon) mutation R1099X, which lacks the major tyrosines implicated in normal TIE2/Akt signaling (Jones *et al.* 1999, Kontos *et al.* 1998), induced a high Akt but low Erk1/2 activation.

For the most common mutations R849W and L914F the phosphorylation level of STAT1 was also measured because R849W has been previously shown to activate it (Hu *et al.* 2008, Huang *et al.* 2013, Korpelainen *et al.* 1999). Our findings demonstrated that L914F induces more pronounced STAT1

hyperphosphorylation than R849W, echoing the autoactivation level induced by the mutants. However, the consequences of STAT1 activation and its possible role in VM formation are unknown.

5.2.2 L914F and R849W cause a decrease in endothelial PDGFB secretion through phosphorylation of Akt and FOXO1 (II)

Analysis of our microarray data predicted transcription factor FOXO1 to be significantly inhibited by L914F based on downregulation of significant proportion of its target genes comprising 12.5% of the 80 genes differentially expressed between L914F and TIE2-WT. Experiments confirmed that both mutations for which the transcriptome was analyzed, R849W and L914F, inhibit FOXO1 by phosphorylating it. R849W had a weaker effect than L914F, correlating with a lower level of TIE2 phosphorylation.

Among the FOXO1 target genes affected by TIE2-VM mutations was the vSMC attractant PDGFB. The transcriptional downregulation of PDGFB was shown to be actually accompanied by a significant reduction of protein secretion by the mutant cells. A slight inhibition of FOXO1 by TIE2-WT compared to NT was also followed by a mildly lowered production of PDGFB. The Ang1 stimulus shown to cause inhibition of FOXO1 and downregulation of PDGFB (Daly *et al.* 2004) caused a significant decrease in PDGFB secretion from TIE2-WT and NT cells. However, the magnitude of this effect did not match that of the mutant forms, as Ang1-stimulated NT and WT cells nevertheless secreted significantly more PDGFB than untreated R849W or L914F cells.

Because normal Ang1-induced inhibition of FOXO1 goes through activation of Akt (Daly *et al.* 2004) and our results show that TIE2-VM mutations increase Akt phosphorylation, we wanted to confirm whether Akt has a role in chronic depletion of PDGFB caused by the TIE2-VM mutants. With an Akt inhibitor (MK2206) we showed a significant decrease in Akt phosphorylation by the TIE2-VM mutants which was accompanied by a decrease in FOXO1 phosphorylation (i.e. inhibition) and a corresponding increase in PDGFB secretion.

To validate whether similar effects on PDGFB secretion could be seen in fixed samples from VM lesions, eight surgically resected VM tissues positive for the L914F mutation were analyzed for PDGFB expression relative to VE-cadherin to normalize for the total EC content. The results were compared with surgically resected GVMs, vascular lesions both clinically and genetically distinct from the VMs. In qPCR the VMs were found to express significantly lower levels

of PDGFB mRNA than GVMs. To verify, immunohistochemistry was done for paraffin-embedded L914F-VM tissue. Malformed veins with VM characteristics showed lower levels of PDGFB staining intensity than adjacent normal veins and arteries. Quantification of anti-PDGFB staining normalized to lumen length confirmed that VMs are indeed surrounded by significantly less PDGFB than normal veins, GVMs and arteries.

5.2.3 Chronic activation of MAPK signaling results in a decrease in ECM fibronectin and loss of EC cobblestone morphology (III)

During the activation and localization studies it was noticed that all examined TIE2-VM missense and nonsense mutations altered the normal endothelial cobblestone monolayer morphology with varying degrees. This alteration correlated positively with the TIE2 autoactivation level since lower phosphorylated mutants showed mildly affected monolayers, and more strongly activated mutations resulted in a loss of the EC monolayer organization with highly elongated and overlapping fibroblastic-looking cells.

To investigate the role of TIE2 ligand-independent phosphorylation in this phenotype, the Y897F-R915L mutant was combined in *cis* with the kinase-inactivating mutation K855R (Huang *et al.* 1995). In HUVECs expressing this construct, the monolayer morphology recovered to a normal cuboidal cobblestone appearance. However, even though this indicated that cell monolayer perturbation is dependent on TIE2 phosphorylation, a one week prolonged Ang1 stimulus did not alter the TIE2-WT monolayer, indicating that the loss of EC monolayer organization was not due to the chronic activation of normal Ang1-stimulated TIE2 signaling, but rather represented a mutation specific effect.

Live cell microscopy revealed the altered cell behavior behind the loss of EC monolayer morphology. The TIE2-WT HUVECs showed a parallel mode of cell migration with reduced cell motility in confluent cell islets that formed a typical cobblestone layer. In striking contrast, random cell migration was evident in mutated TIE2 HUVECs, with elongated, overlapping cells that failed to form a cobblestone pattern, suggesting loss of communication between the contacting HUVECs expressing the mutated TIE2.

Because both monolayer alteration and an increased Akt and/or Erk1/2 activation were seen to be common to the examined TIE2-VM mutations, we then investigated whether there may be a link between the two. Treating cells with a pharmacological inhibitor of Erk1/2 (PD98059) was sufficient to restore the

perturbed monolayer back to normal. PI3K/Akt inhibitor (LY294002), however, had no clear effect, neither alone or in combination with Erk1/2 inhibition.

Since depletion of FN from the ECs can induce a similar monolayer phenotype than we saw in TIE2-VM expressing cells (Cseh *et al.* 2010), we analyzed the amount of both cellular and ECM FN in different stages of cell confluence. This revealed that ECM FN, but not cellular FN, was diminished when Y897F-R915L cells reached confluence and practically lost at later stages of confluence. In TIE2-VM immunostaining experiments ECM FN was seen to be weak and fragmented with most of the signal coming from inside the cells, probably representing newly synthesized proteins. A reduced ECM FN was also seen in L914F and R849W, indicating a common effect. Because ECM protein reduction did not correlate with cellular protein nor mRNA levels, this suggested increased FN protein degradation and/or decreased ECM deposition.

To determine whether FN depletion is enough to alter the HUVEC monolayer, we knocked-down FN in TIE2-WT cells. This resulted in the loss of cobblestone morphology closely resembling what was seen with TIE2-VM mutants. Finally, to confirm that normalization of Y897F-R915L monolayer morphology by Erk1/2 inhibition is accompanied by increased FN, the ECM fraction of Erk1/2 inhibited Y897F-R915L HUVECs was analyzed. Inhibition of Erk1/2 efficiently increased ECM FN levels in these cells but did not alter FN mRNA levels significantly, thus confirming the link between TIE2-VM, Erk1/2, ECM FN deficiency and loss of EC monolayer morphology.

To look for potential proteolytic pathways activated by mutant-induced Erk1/2 signaling, we used a fibrin gel model for angiogenesis (Korff & Augustin 1999). During culture, the mutant EC spheroids quickly expanded radially to form large thin-walled structures with a few sprouts in striking contrast to the sprouting activity and low volume increase of the TIE2-WT spheroids. This expansion of mutant spheroids was completely abolished by the serine protease inhibitor aprotinin, which led us to measure levels of urokinase type (uPA) and tissue type (tPA) plasminogen activators, which are potentially relevant targets of aprotinin action. Transcription of both uPa and tPa was highly elevated in Y897F-R915L cells; inversely plasminogen activator inhibitor 1 (PAI-1) was downregulated. This dysregulation of plasminogen regulation could be restored back to near normal levels with an Erk1/2 inhibitor. The most common sporadic TIE2-VM mutation L914F and the commonly inherited R849W also showed dysregulation of the plasminogen system and resulted in spheroid expansion in fibrin gel, indicating a common change due to mutations in TIE2.

5.3 Light microscopical and ultrastructural analysis of VM tissue samples from patient biopsies and a mouse transplantational assay (III)

To validate how well our *in vitro* cell culture results hold true *in vivo*, we investigated human VM tissue samples surgically resected from patients and from a VM mouse model. For the patient samples normal tissue adjacent to the lesion and for the mouse studies TIE2-WT expressing cells were used as controls.

The ultrastructural analysis of biopsies from patients carrying Y897F-R915L or L914F revealed an uneven EC lining characterized by cellular elongations and an increase in EC cytoskeletal filaments, which in addition were not correctly aligned within the cells. In addition, profound changes in the perivascular ECM were observed including for instance a very wide layer of BM-resembling ECM, with randomly oriented and isolated collagen fibrils and erratically distributed SMCs. The data indicates that the TIE2-mutations cause profound alterations in the EC shape and the perivascular ECM in patients, similar to those found in retrovirally transduced HUVECs.

The capacity of mutant TIE2 expressing HUVECs to form lesions *in vivo* was tested in a VM mouse model based on EC transplantation in immunocompromised SCID mice modified from Laib *et al.* 2009. Based on a macroscopic examination, the vascularized plugs closely resembled human VM lesions and in the histological sections pathognomonic enlargement of the vessel size was evident. On the ultrastructural level, abnormalities in EC morphology, SMC layering and perivascular ECM were seen, with effects being stronger with more strongly phosphorylated TIE2-VM forms. In addition, comparison of Y897F-R915L to its single constituent mutations indicated a synergistic effect in both vessel enlargement and the severity of ultrastructural alterations.

To investigate the cellular mechanisms that may relate to the growth of abnormally enlarged vessels, we measured cell length, and the extent of apoptosis and proliferation in TIE2-WT and Y897F-R915L vascular structures. In Y897F-R915L-containing vessels, the ECs were twice as long and apoptotic nuclei were observed less frequently than in the TIE2-WT vessels without an increase in cell proliferation. EC elongation may contribute to an increased vessel diameter of the VM lesions in the absence of EC proliferation, as proposed for enlarged vessels in arteriovenous malformations (Murphy *et al.* 2014); it may also be secondary to a loss of ECM support.

6 Discussion

VMs are pathological errors of vascular morphogenesis. Over half of the lesions screened are TIE2 mutation positive (Soblet *et al.* 2013, Wouters *et al.* 2010). Published point mutations generally share the ability to increase TIE2 tyrosine phosphorylation (i.e. activity) to variable degrees. However, at the cellular and molecular level the alterations these mutations have on TIE2 functions are poorly known. In this thesis 23 mutated TIE2 receptors were studied to find out how they affect TIE2 activation, trafficking and downstream signaling. Their effects on the ECs were also investigated.

6.1 Dysregulated TIE2 localization likely attenuates receptor response to extracellular ligands

Angiopoietin ligand stimulus induces TIE2 receptor clustering, activation and compartmentalization into specific subcellular locations leading to location-dependent downstream signaling cascades (Fukuhara *et al.* 2008, Pietilä *et al.* 2012, Saharinen *et al.* 2008). Analysis of 23 TIE2-VM mutations revealed that receptor trafficking was abnormal in 22 cases. Detailed analysis of the mechanism indicated that mutations can reduce the plasma membrane located TIE2 via various mechanisms, namely via intracellular retention, enhanced turnover and shedding of the extracellular domain of the TIE2 receptor.

Del-TIE2 was found to lack 43 amino acids from its extracellular ligand binding Ig2 domain and was identified as a somatic mutation in the WT allele of a patient carrying the inherited R849W allele. Del-TIE2 was shown to remain in the ER. It is possible that deletion makes TIE2 receptors unable to fold correctly, which prevents secretion from the ER (Braakman & Bulleid 2011). However, even if Del-TIE2 receptors could be secreted from ER and normally translocated to cell membrane, they would likely be non-functional as they could not bind Ang1 in the co-immunoprecipitation assay. Thus, Del-TIE2 probably functions as a null allele locally eliminating wild type TIE2 and promoting the VM causing potency of inherited R849W.

13 out of 23 mutated receptors studied, including the most common somatic L914F, showed an accumulation and ligand-independent activation in the Golgi. Our findings indicate that this accumulation may be due to incomplete glycosylation and/or increased clustering of the receptors and, interestingly, occurs independently of the mutant-induced increase in TIE2 phosphorylation. In

addition to preventing the TIE2 receptors from binding ligand on the cell membrane, intracellular accumulation might also enable induction of mutation-specific signaling pathways spatially from the cellular subdomains. Similar dysfunction has been shown for mutations of several other RTKs in different diseases. For example mutated, highly active FGFR3 and PDGFR α both fail to mature and accumulate in ER. From there these receptors recruit and activate the STAT signaling pathway, which is not utilized by the corresponding WT receptors. (Bahlawane *et al.* 2015, Lievens *et al.* 2004). Similar mislocalized signaling may occur with intracellularly retained, hyperactive TIE2-VM receptors and may contribute to VM formation. In addition, Golgi retention may also participate in the incomplete translocation in response to Ang1 via reducing the plasma membrane located TIE2. Eight of the 11 TIE2-VM mutations with this characteristic also showed Golgi retention, suggesting that access to the ligand may contribute.

In the case of Y897F-R915L we found an accelerated protein turnover rate and increased shedding of the TIE2 extracellular domain. Enhanced turnover diminishes the amount of receptors on plasma membrane, attenuating ligand-controlled function. Shedding of the extracellular domain may have a dual inhibitory effect on silencing ligand-control. It eliminates ligand-binding receptors from the cell membrane and also increases the amount of soluble (shed) TIE2, which may exert an additional inhibitory effect by competing for ligand with the membrane-bound TIE2 receptors. Also the finding that Ang2 levels are decreased in TIE2-VM cells supports the hypothesis that ligand-regulated functions of TIE2 might be abnormal in the VM tissue.

The significance of altered TIE2 receptor trafficking caused by VM-mutations was augmented by our findings with Y897 double mutations and their single constituents. These indicated that an increase in TIE2 phosphorylation alone is likely insufficient to transform a predisposing single mutation into a disease-causing allelic double mutation. Generally, VM-causative mutations (found in lesions) showed defective receptor clustering and translocation in response to Ang1 more often (8 out of 13, 62%) than mutations not found in lesions alone (3 out of 9, 33%).

None of the localization and compartmentalization characteristics found was common to all of the examined TIE2-VM mutants. This indicates that many types of alterations hampering the normal TIE2 function potentiate VM formation and that mutations in TIE2 may utilize several different mechanisms to reduce normal

TIE2 function as a cell membrane receptor for angiopoietins and/or induce mislocalized ligand independent signaling.

6.2 TIE2 ligand independent activation affects downstream signaling which might contribute to VM formation

Contrary to previous data, two of the TIE2 missense mutations investigated in this thesis project, A925S and T1105N, failed to increase TIE2 tyrosine phosphorylation in retrovirally transduced HUVECs. In addition, also several other mutation forms showed markedly lower hyperphosphorylation levels in our experiments than what has been reported before in Cos or Sf9 cells (Calvert *et al.* 1999, Soblet *et al.* 2013, Vikkula *et al.* 1996, Wouters *et al.* 2010). One reason might be that we used ECs representing the natural environment of TIE2 receptors and also expressing endogenous TIE2 and EC-specific TIE2 signaling regulators, such as TIE1 (Savant *et al.* 2015, Yun *et al.* 2013) or VE-PTP (Winderlich *et al.* 2009, Yacyshyn *et al.* 2009), that may be lacking in Cos-cells representing fibroblastic cells from the kidney or in Sf9 insect cells not normally expressing TIE2. In our model we use retroviral mediated overexpression of TIE2-VM forms. The results were compared to TIE2-WT or non-transduced cells, however, TIE2 gene targeted VM models would be necessary to validate our model.

The most common inherited mutation R849W has been shown to cause hyperphosphorylation of Akt downstream of TIE2 (Morris *et al.* 2005). Our results show that also other TIE2-VM mutations induce chronic activation of Akt and on a higher level than R849W, the weak activator A925S being the exception. Akt is known to increase EC survival (DeBusk *et al.* 2004, Fujikawa *et al.* 1999, Kim *et al.* 2000c) possibly inducing the higher numbers of ECs and vessel enlargement seen in VMs. Interestingly, constitutive activation of Akt has been shown to cause vascular malformations in mice, lesions characterized by wide endothelial lumens and patchy association of vSMCs (Perry *et al.* 2007). In addition, we found that L914F induced greater resistance to cell death than R849W and had one of the strongest hyperactivating effects on Akt, reflecting its survival enhancing role.

In Ang1-induced TIE2-WT, Akt may represent a major signaling pathway. However, TIE2-WT has also been reported to signal through the MAPK signaling pathway (Yoon *et al.* 2003). In our analysis MAPK Erk1/2 activation was increased in 17 out of 22 mutations, demonstrating that this pathway is also

commonly enhanced by mutated TIE2. Importantly, MAPK inhibitor (PD98059) decreased phosphorylated Erk1/2 to WT levels and restored EC monolayer organization, plasminogen/plasmin activation system, and ECM FN, thus indicating that chronic (albeit low) Erk1/2 phosphorylation may have an important role in VM pathogenesis.

A recent finding that somatic activating mutations in *PIK3CA*, the gene encoding the catalytic subunit p110 α of PI3K downstream of TIE2, also cause VMs increases interest for the role of Erk1/2 as *PIK3CA* mutants were shown not to affect activation of Erk1/2, although they caused similar morphological alteration with reduced ECM FN in ECs as seen with TIE2 mutations, which also could be rescued with a specific inhibitor for p110 α (Limaye *et al.* 2015). This begs the question whether there are other MAPKs or other signaling molecules linking these pathways and affecting the FN deposition.

In transcriptome wide analysis of L914F HUVECs, we identified dysregulated genes that have previously been linked to vascular development, cell migration and ECM synthesis and/or turnover. These effects may have a pro-quiescence rather than an angiogenesis stimulating effect, thus resembling Ang1 stimulation of TIE2-WT to stabilize the vasculature. R849W induced a similar perturbation, although on a much weaker level possibly due to the lower TIE2 phosphorylation it induces, suggesting shared pathogenic mechanisms. In addition, this “pro-quiescence” effect is in line with the observation that TIE2-VM cells do not proliferate more in culture (at some points in time even less so than TIE2-WT). Immunohistochemistry of VM tissue samples resected from patients confirms similar results showing only low levels of proliferation in VM tissue (Rossler *et al.* 2013).

6.2.1 Chronic activation of Akt downstream of mutated TIE2 decreases PDGFB secretion from ECs

Our analysis of the transcriptome data from the most common sporadic TIE2-VM mutation L914F expressing HUVECs revealed that the transcription factor FOXO1 is strongly inhibited due to increased TIE2 activation. Normally, FOXO1 inhibition is mediated through Akt activation induced by TIE2 phosphorylation in response to an Ang1-stimulus (Daly *et al.* 2004). Our results also showed that increase in ligand-independent TIE2 phosphorylation due to VM-mutations commonly results in chronic activation of Akt. This suggests that FOXO1 inhibition might be a common alteration due to mutated TIE2 in VMs.

Among the genes dysregulated by FOXO1 inhibition was the major EC-secreted mural cell attractant PDGFB, of which the levels were shown to be decreased in TIE2-VM HUVECs. Blood vessels of mice deficient in PDGFB or its receptor PDGFR β show insufficient ability to recruit mural cells (Levéen *et al.* 1994, Lindahl *et al.* 1997, Soriano 1994). VMs are also characterized by an uneven layer of mural cells, possibly suggesting that a decrease in PDGFB production induced by TIE2-VM forms could play a role in VM formation. PDGFB- and PDGFR β -deficient mice further show EC hyperplasia, vessel enlargement, abnormal EC shape and dysregulation of junctional proteins (Hellström *et al.* 2001). This is in line with our findings of TIE2-VM expressing HUVECs showing increased numbers of ECs and perturbed morphology, as well as with pathognomonic enlargement of channels in VMs, providing increasing evidence for a role of PDGFB signaling deficiency in VM formation. In addition, it has been suggested that PDGFB may affect human venous SMCs more strongly than arterial SMCs, perhaps due to differential receptor distribution (Li *et al.* 2006, Li *et al.* 2011). This might induce the vein-specific location of mutated TIE2 expressing and PDGFB downregulating vascular lesions.

VM tissue resected from patients and endogenously expressing mutated TIE2 showed a decrease in PDGFB production, which is in line with the identified drop in serum levels of PDGFB in the circulation and draining veins of lesions from patients with large VMs. Similar situation has not been seen in patients with other vascular malformations. (Redondo *et al.* 2010). Thus, methods to normalize PDGFB production in the endothelium of malformed veins, such as inhibition of chronic TIE2 or Akt activity, could prove beneficial to VM patients, at least to reduce VM regrowth, regularly seen after surgical resection.

The benefits of increasing PDGFB expression in a VM could be further investigated using VM-mouse models and either pharmacological PI3K/Akt inhibitors or experimentally expressing PDGFB under viral promoter in transplanted ECs to avoid Akt/FOXO1 -dependent downregulation of endogenous *Pdgfb*-gene. In addition, as SMC recruitment to the blood vessels is mediated by several different signaling pathways, it would be interesting to investigate whether these are also affected in the PDGFB-low VM tissue. In fact, it was recently shown that TGF β , which also participates in EC-SMC communication, is downregulated in VMs (Ren *et al.* 2014), suggesting that insufficient SMC recruitment to VM blood vessels is mediated by dysregulation of several signaling pathways. TGF β and TIE2 were also indicated to have a balancing role over each other since Ang1-treatment downregulated TGF β and treatment with

TGF β suppressed TIE2 activity (Chen *et al.* 2014). Thus, TIE2 hyperphosphorylation and TGF β downregulation may function together in VM formation, supporting and enhancing each other's defective effects.

6.2.2 Loss of EC monolayer organization due to TIE2-VM mutant autoactivation occurs through ECM modulation

Upon reaching confluence, normal ECs form a dense monolayer with typical cobblestone morphology. In TIE2-VM expressing HUVECs this was lost to varying degrees, implying a common alteration. In addition, similar defects were seen in patient tissue. According to our studies this defect was due to increased Erk1/2 activity in response to mutation induced ligand-independent TIE2 activity resulting in depletion of ECM FN. FN functions as a scaffold for the proper assembly of many ECM and BM components (Kostourou & Papalazarou 2014), and also potentiates Ang1-induced TIE2 signaling through binding to endothelial $\alpha 5\beta 1$ -integrin which interacts with TIE2 (Cascone *et al.* 2005). In mouse embryos, lack of FN results in defective morphogenesis including dilated and malformed vessels and loss of endothelial-mesenchymal contact (George *et al.* 1997). This suggests that reduction of ECM FN may have a role in VM formation. It would be intriguing to study this further for instance by using shRNA methodology to deplete FN from HUVECs that would subsequently be used in a transplantation assay in mice.

Our studies also revealed that TIE2-VM forms lead to a dysregulation of the plasmin proteolytic system, more specifically to upregulation of uPA and tPA and downregulation of PAI-1 mRNAs. In addition to fibrinolysis, plasmin can directly degrade many ECM proteins including FN and BM laminin, and can induce activation of various pro-matrix metalloproteinases that degrade ECM components (Baramova *et al.* 1997, George *et al.* 1997, Horowitz *et al.* 2008, Houard *et al.* 2003, Liotta *et al.* 1981). It has also been shown that both uPa and tPa can directly act on the FN to degrade it (Marchina & Barlati 1996). The possible role of uPa and tPA in our experimental model could be studied further by upregulating their expression in HUVECs without concurrent expression of any TIE2-VM form.

mRNA expression profiling revealed that TIE2 mutations strongly increase the expression of ADAMTS peptidases that are also involved in perivascular ECM modeling. Most of the ADAMTSs upregulated by L914F mutation on TIE2 belong to the aggrecanases/proteoglycanases subgroup (ADAMTS1, 4, 5 and 9),

ADAMTS18 being an orphan enzyme. All these aggrecanases/proteoglycanases cleave versican, which is an essential ECM component during embryogenesis providing structural support and influencing adhesion, migration and proliferation of many cell types. ADAMTS activity has also been linked to atherosclerosis and arthritis, ADAMTS4 and 5 to the inhibition of neovascularization and ADAMTS1 to cleavage of TSP-1 (Kelwick *et al.* 2015), a downregulation of which increases EC survival (Niu *et al.* 2004). Because the effects of ADAMTSs are multiple and complex, it may prove difficult to solve the exact role of their upregulation due to TIE2-VM forms. However, they could affect VM formation for instance through cleavage of the surrounding ECM abolishing its support for the developing vasculature. One way to start towards understanding of the function of the various ADAMTSs in our model could be to silence or to overexpress them individually or in combinations in cultured HUVECs to see how their dysregulation affects the ECs in general and their morphology in particular.

ECM FN deficiency and dysregulated ECM processing might destabilize the vascular wall, decreasing mechanical support and thereby contributing to the development of abnormally expanded venous channels. In addition, it has been shown that the junctional proteins VE-cadherin and N-cadherin are downregulated in the VM endothelium (Chen *et al.* 2014), which could further weaken the vascular wall, possibly abolishing the normal contact inhibition of ECs to potentiate vascular enlargement.

Rapamycin (an mTOR protein kinase inhibitor) can increase PAI-1 and decrease tPA expression (Muldowney *et al.* 2007), the opposite effects caused by mutated TIE2. In a recent publication, HUVECs that we retrovirally transduced to express mutant TIE2 were used to show that rapamycin reduces TIE2 mutation induced Akt activation in cultured ECs and VM lesion size in immunodeficient mice, also improving symptoms in six severely affected VM patients. In addition, rapamycin treatment reduced D-dimer levels in three out of five patients. However, in clinical use long-term rapamycin treatment may cause significant side-effects. It has potent immunosuppressant actions, increases risk for cancer and reduces the size of existing VM lesion only slightly. In addition, commercially available TIE2 tyrosine kinase inhibitor showed no effect on VM growth in mice (Boscolo *et al.* 2015), thus making the identification of TIE2 VM-specific molecular and cellular pathways necessary for the development of better treatments.

To conclude, our results show that TIE2-VM forms chronically activate downstream signaling molecules Akt and Erk1/2, which are part of the normal

Ang1-induced TIE2 signaling. This might suggest a model where mutated TIE2 constitutively activates normal TIE2 signaling and the enhanced, non-regulated signal mediation would affect vascular morphogenesis to cause VMs. However, in our experiments prolonged Ang1-stimulation (7 days) of TIE2-WT HUVECs could not disturb the EC monolayer morphology as seen with the TIE2-VM forms, indicating that the defects we found may not be solely due to enhancement of normal Ang1-induced TIE2 signaling. Because mutated RTKs accumulating in ER and Golgi have been shown to induce abnormal signaling (Bahlawane *et al.* 2015, Lievens *et al.* 2004), and also many TIE2-VM forms show retention in these compartments, similar mutant-specific spatial signaling might also be possible in VM formation. Thus, it could be hypothesized that the mutated TIE2 receptors utilize both the normal Ang1-induced TIE2 signaling pathways as well as mutant-specific routes to potentiate VM formation.

6.3 Future perspectives

Our studies have revealed new mechanisms that may participate in VM formation and may prove beneficial for the development of new molecular treatments for VM patients. I expect that pharmaceuticals will be the future way to treat VM patients based on both the non-lasting results of current treatments and the primary results of rapamycin on difficult-to-treat VM patients (Boscolo *et al.* 2015). In addition, by studying alterations caused by mutated TIE2, we can also learn from the normal vascular morphogenesis. However, our data also provokes new interesting questions.

Our results indicate roles for decreased PDGFB production, ECM FN reduction and enhanced activation of the plasminogen/plasmin system in VM formation. As previously discussed, the role of these could be studied further with cell culture experiments and transplantational mouse assays. In addition, the intracellular retention of several TIE2-VM forms suggests the possibility of mutation-specific signaling pathways starting from the ER or Golgi as previously indicated for many other RTKs (Bahlawane *et al.* 2015, Choudhary *et al.* 2009, Hatch *et al.* 2006, Lievens & Liboi 2003, Lievens *et al.* 2004, Schmidt-Arras *et al.* 2005, Watson *et al.* 1999, Xiang *et al.* 2007). This possibility would be most intriguing to study further since it may provide a solid foundation for the development of new molecular treatments. To provide the foundation, a more thorough analysis of cellular signaling should be done, extending it also to signaling molecules not identified as part of the normal TIE2 signaling pathways.

A wide phosphoproteomics analysis could be done for example where all phosphorylated proteins of a cell sample would be isolated and blotted with 2D electrophoresis. By comparing samples from TIE2-WT and TIE2-VM expressing cells, differences may be noticed and the corresponding proteins identified by mass spectrometry. Effects of possible candidates could then be further analyzed in cell culture and mice assays.

We studied TIE2-VM forms in HUVECs, endogenously expressing TIE2 and molecules that interact with it and regulate its functions. It would be interesting to test what happens when similar levels of TIE2-WT and TIE2-VM forms are expressed together in the HUVECs. Would the TIE2-VM form have a dominant-negative effect over TIE2-WT, or could TIE2-WT suppress or reduce effects caused by TIE2-VM? Would mutations causing lower ligand-independent phosphorylation show milder or even absent effects compared to more strongly autophosphorylating mutations, reflecting possibly distribution between pre-disposing and disease-causing mutations? In addition, co-overexpression of TIE2-VM forms with TIE1 or VE-PTP, known to interact with TIE2, could reveal whether they are capable or incapable of interacting with the mutated forms of TIE2 and whether they can still regulate its functions or not. VE-PTP is known to dephosphorylate TIE2 and its deficiency leads to enlarged blood vessels (Winderlich *et al.* 2009, Yacyshyn *et al.* 2009). Might it be that VE-PTP cannot affect the mutated TIE2? TIE1 is indicated to be able to downregulate Ang1-induced TIE2 signaling (Yun *et al.* 2013); does it have similar effects on TIE2-VM forms? Further characterization of molecules normally interacting with TIE2 could reveal new mechanisms that are altered or non-functional in TIE2-VM positive endothelial cells, which would further better our understanding of the mechanisms behind VM formation.

Analysis of whole transcriptomics revealed a lot of genes whose expression was altered between TIE2-WT and L914F. It would be interesting to test the effect of dysregulation of for example the different ADAMTSs that participate in ECM processing or different growth factors (CYR61, CTGF, BMP4, PGF) regulating a broad range of cellular activities and morphogenetic functions. Also the unclear effects of STAT1, upregulated in R849W and L914F HUVECs, could be further investigated. Testing could be started by either overexpression or silencing, for instance through retroviral transduction and shRNA methodology, respectively, of the corresponding genes in cultured endothelial cells. Possible interesting findings could then be further tested *in vivo*, for example in our transplantational assay in mice or with knock-in or knockout mice.

Last, because inhibition of Erk1/2 restored the monolayer morphology, the ECM FN and the normal levels of plasminogen activators in TIE2-VM HUVECs in our experiments, it would be interesting to test whether it has any effects in our VM mouse model. Could Erk1/2 inhibition prevent formation of VM-like vascular channels in mice transplanted with TIE2-VM HUVECs? Would it affect the growth of already formed lesion-like channels or even restore them? Could Erk1/2 inhibition enhance the effects of rapamycin, which mainly lowers TIE2-VM-induced Akt levels (Boscolo *et al.* 2015)? Should Erk1/2 inhibition show beneficial effects in the VM mouse model, it could be further tested for safety to consider the possibility of pilot clinical studies.

7 Summary and conclusion

The aim of this thesis was to investigate the molecular and cellular mechanisms behind TIE2 mutation-induced formation of VMs. Taken together, our data suggests that TIE2 mutations affect several molecular mechanisms which together might potentiate VM formation: Mutation-dependent TIE2 autoactivation induces chronic activation of the major downstream signaling molecule Akt, increasing EC survival and reducing production of the major EC-secreted SMC-attractant PDGFB. Similarly, TIE2 autoactivation-dependent Erk1/2 activation negatively impacts the EC morphology, amount of ECM FN and also activates the plasminogen/plasmin protease system. In addition, VM-specific mutations commonly resulted in the loss of plasma membrane localized TIE2, causing attenuated responsiveness to angiopoietin ligand -mediated regulation of the TIE2 functions.

Currently available treatments for VMs mainly consist of surgery and sclerotherapy, but neither of these is lasting and lesions tend to recur. In a pilot clinical study, rapamycin, which reduced mutant TIE2-induced Akt phosphorylation in cultured ECs, also improved VM symptoms indicating that a molecular therapy of VMs can be effective (Boscolo *et al.* 2015). However, long-term rapamycin treatment may cause significant side-effects and rapamycin also increases Erk1/2 activation in ECs which potentiates cellular abnormalities in VM-ECs, thus making the search for more specific inhibitors attractive and necessary for the development of VM-targeted molecular therapies.

The results of this thesis provide new information on the pathological molecular level mechanisms that may influence VM formation and as a result turn out helpful for the development of new more potent molecular treatment strategies for VM patients. Additionally, because the data provides new knowledge on the molecular basis of abnormal vascular morphogenesis, it also enables a better understanding of the normal development of the vasculature and the role of the TIE2 receptor in it.

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- I Limaye N*, Wouters V*, Uebelhoer M#, Tuominen M#, Wirkkala R, Mulliken JB, Eklund L, Boon LM, and Vikkula M (2009) Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. *Nat Gen* 41(1): 118–124.
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