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FUNCTIONAL GENOMICS OF VASCULAR ENDOTHELIAL CELLS

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Stockholm 2008

Published by Karolinska Institutet. Printed by US-AB.

ISBN 978-91-7409-178-6

When the mind is free from the clouds that prevent perception,
all is known, there is nothing to be known.

Sri Patanjali, The Yoga Sutras

ABSTRACT

Angiogenesis, the formation of new blood vessels from preexisting ones, is a process involved in normal development as well as in several pathological conditions, such as cancer, ischemic heart disease, wound healing and certain retinal complications. Antiangiogenic targeting is therefore a promising new therapeutic principle. However, few blood vessel-specific drug targets have been identified, and information is still limited about endothelial cell (EC)-specific molecular processes. Here we aimed at identifying novel key players and endothelial signaling pathways during angiogenesis, and to determine the EC-specific core transcriptome *in vivo*.

During angiogenesis, specialized endothelial tip cells lead the outgrowth of blood-vessel sprouts towards gradients of vascular endothelial growth factor (VEGF)-A. We found that Delta-like 4 (DLL4)/Notch1 signaling regulated the formation of appropriate numbers of tip cells to control vessel sprouting and branching in the developing postnatal retina. Inhibition of Notch-signaling led to excessive tip cell formation, and increased vascular density. Conversely, activation of Notch-signaling led to fewer tip cells and reduced vessel density. DLL4/Notch1-signaling between ECs therefore restricts tip cell formation in response to VEGF, leading to correct sprouting and branching patterns. We also found that blocking VEGF receptor 3 (VEGFR-3) signaling with antibodies resulted in decreased sprouting, vascular density, vessel branching, and EC proliferation. Antibodies against VEGFR-3 and VEGFR-2 in combination had additive effects. Notch inhibition led to endothelial VEGFR-3 expression and excessive sprouting, which was inhibited by blocking VEGFR-3. These findings suggest that Notch and VEGFR-3 signaling may constitute new targets for antiangiogenic therapy.

In order to identify additional candidate vascular drug targets, we combined publicly available gene expression data with own transcriptional profiles of mouse microvasculature. In this way we identified 58 gene transcripts with broad and specific expression in microvascular endothelium, of which 32 presently lack known functions in vascular biology. 7 of the 32 genes showed considerably enriched expression in the microvasculature, namely: *Eltd1*, *Gpr116*, *Ramp2, Slc9a3r2*, *Slc43a3,* and *NM_023516*. The 32 gene products were all predicted to be cell surface expressed, or implicated in cell signaling processes, and are therefore interesting as putative microvascular drug targets. We also identified yet another set of new candidate vascular targets by combining reverse- and chemical genetics. In the reverse genetics screen, 50 genes were knocked down in zebrafish and 16 of these were found to be necessary for developmental angiogenesis. In the chemical genetics screen, 28 compounds targeting 69 proteins selectively inhibited endothelial sprouting. The reverse- and chemical genetics screens identified an overlap of three members of a superfamily of serine/threonine (S/T) protein phosphatases, *Ppp1ca, Ppp1cc* and *Ppp4c,* and one compound, Endothall, targeting that family. Treatment of zebrafish with Endothall led to a dose-dependent effect on lumen formation, similar to that seen in zebrafish knock-downs of the identified S/T protein phosphatases.

The discoveries made in this study span from detailed insights into specific endothelial signaling pathways to global effects on endothelial gene expression, representing different angles of angiogenesis and vascular biology research. Overall, the results in this study contribute to the understanding of the vasculature and its transcriptome.

Keywords: vasculature, endothelial cells, angiogenesis, vascular endothelial growth factor (VEGF), Notch, transcriptome, genomics, microarray, tumor, retina, morpholino knock-down

LIST OF ORIGINAL PUBLICATIONS

I. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis.
Hellström M, Phng LK, Hofmann JJ, **Wallgard E**, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C.
Nature, 445:776-780, 2007.

II. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation.
Tammela T, Zarkada G, **Wallgard E**, Murtomäki A, Suchting S, Wirzenius M, Waltari M, Hellström M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Ylä-Herttula S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, and Alitalo K.
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III. Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature.
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Arteriosclerosis, Thrombosis, and Vascular Biology, 28:1469-76 2008.

IV Combination of reverse- and chemical genetic screens reveals novel angiogenesis inhibitors and targets.
Kalén M, **Wallgard E**, Asker N, Nasevicius A, Billgren E, Larson JD, Wadman SA, Clark KJ, He L, Karlsson-Lindahl L, Häger AK, Samuelsson T, Essner JJ, Hackett PB, and Hellström M.
Manuscript

LIST OF ABBREVIATIONS

AM	adrenomedullin
AEM	angiogenic endothelial marker
AMD	age-dependent macula degeneration
APP	aβ protein precursor
BBB	blood brain barrier
BEM	brain endothelial marker
Calcrl	calcitonin receptor-like
Cdh	cadherin
cDNA	complementary deoxynucleic acid
CG	chemical genetics
Cldn	claudin
CNS	central nervous system
CDL	CBF1/RBP-Jκ/suppressor of hairless/LAG-1
DAPT	n-[n-(3,5-difluorophenacetyl)-l-alanyl]-s-phenylglycine t-butylester)
DLL	delta-like
EC	endothelial cell
Eltd1	egf, latrophilin seven transmembrane domain containing
Eng	endoglin
Epas	endothelial PAS domain protein
Eph	eph receptor
Esam	endothelial cell-specific adhesion molecule
EST	expressed sequence tag
Flt	fms-like tyrosine kinase
Gpr	g protein-coupled receptor
GPCR	g protein-coupled receptor
GSI	gamma-secretase inhibitor
HUVEC	human umbilical vein endothelial cells
ISV	intersegmental vessel
Hif	hypoxia inducible factor
Jag	jagged
JLK-6	7-amino-4-chloro-3-methoxyisocoumarin
Kdr	kinase insert domain protein receptor
LEM	liver endothelial marker
mRNA	messenger ribonucleic acid
NO	nitric oxide
NotchICD	notch intracellular domain
Nrarp	notch-regulated ankyrin repeat protein
OIR/ROP	oxygen induced retinopathy/retinopathy of prematurity
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
Pecam	platelet/endothelial cell adhesion molecule
Ppp	protein phosphatase
Ramp	receptor activity modifying protein
Rasip	ras interacting protein

RG	reverse genetics
Robo	roundabout homolog
RT Q-PCR	real time quantitative polymerase chain reaction
SAGE	serial analysis of gene expression
Sftp	surfactant protein
SMC	smooth muscle cell
S/T	serine/threonine
TEM	tumor endothelial marker
Tie	tyrosine kinase with immunoglobulin and epidermal
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VSMC	vascular smooth muscle cell

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1 INTRODUCTION

1.1 BLOOD VESSEL STRUCTURE AND FUNCTION

Blood vessels pervade most of our organs and tissues, allowing the blood to transport nutrients and oxygen, and to remove carbon dioxide and metabolites. The blood vessels can be described as tubes of different sizes and compositions, and the main constituents are the endothelial cells (ECs) that line the blood vessel lumen, a deposit of basement membrane surrounding the ECs, and outside the basement membrane a layer/layers of mural cells, or vascular smooth muscle cells (VSMC)/pericytes), that may vary in thickness (**Figure 1**).

An important role of the ECs is to create a physical and chemical barrier between the blood and the tissue, which is essential for homeostasis. The barrier consists of junctional complexes that form connections between the ECs, briefly described below.

The tight junctions mainly consist of occludins and claudins that join the ECs at the apical (luminal) side, and anchor to the actin cytoskeleton intracellularly[1, 2]. The adherens junctions consist mainly of vascular endothelial (VE-) cadherin and connect ECs along the cell membrane towards the basolateral (abluminal) side (reviewed in[3]). VE-cadherin is intracellularly anchored to β- or γ-catenin, which propagate downstream signaling (reviewed in[1]). The gap junctions facilitate intercellular transport of small low-molecular weight molecules, and they consist mainly of connexins (reviewed in[4]).

An example of the importance of junctions is in the central nervous system (CNS), where vascular permeability is kept to a minimum. ECs in the CNS comprise the major part of the blood brain barrier (BBB). Here, the junctions (mainly the tight junctions) uphold a strong barrier, allowing only the essentials to pass, such as oxygen and glucose[5, 6]. The specialized type of mural cells, pericytes, cover microvessels in the retina, in other parts of the CNS, and in other tissues as well, although pericyte abundance may vary extensively between different organs. In the CNS, pericytes offer support and are also contributing to BBB maintenance[7].

The EC barrier function can be dynamically and locally regulated in the case of inflammation or tissue damage, as leukocytes representing the immune defense transmigrate over the blood vessel wall to reach the site where needed (reviewed in[8]).

ECs are also involved in nitric oxide (NO) signaling, which instructs the vascular smooth muscle cells to contract/relax, leading to changes in blood pressure due to the change in blood vessel diameter (reviewed in[9]).

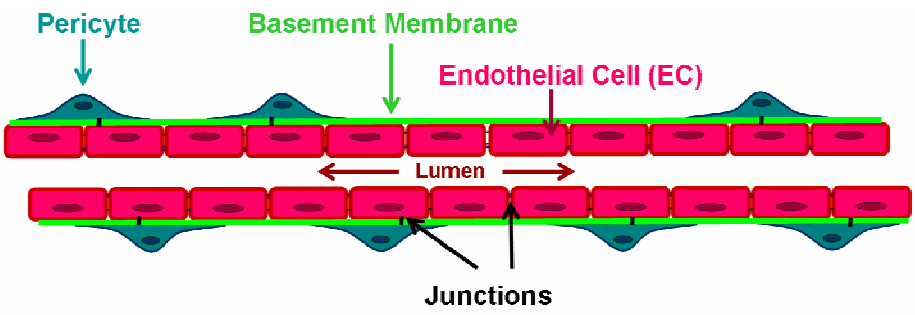

Figure 1. Schematic illustration of the basic components of a blood vessel.

1.2 ANGIOGENESIS

During embryogenesis, and also sometimes during adulthood and development of disease, there is a need for increased oxygen- and nutrient supply, which leads to formation of new blood vessels from pre-existing ones. This process is called angiogenesis, to be distinguished from *de novo* formation of blood vessels; vasculogenesis. There are three known forms of angiogenesis, splitting (intussusception), branching and sprouting (reviewed in[10]), of which sprouting angiogenesis is considered to be the most common one, and that will be discussed here.
Sprouting angiogenesis is initiated by generation of a gradient of vascular endothelial growth factor (VEGF)-A[10-12]. VEGF-A is primarily expressed as three different isoforms with different affinities to the extracellular matrix. VEGF-A expression is often triggered by hypoxia inducible factor α (HIF1-α) that is induced by low oxygen pressure (hypoxia) in a growing tissue[11, 12]. When a nearby vessel is subjected to the gradient, specialized ECs called tip cells are formed, which extend filopodial protrusions and lead the growth of the sprouts towards the source of VEGF-A[11, 12]. Following the tip cells are the stalk cells, which are morphologically different from the tip cells. Tip cells are responsive to VEGF-A via its receptors, vascular endothelial growth factor receptors 1 and 2 (VEGFR1, or Flt1; and VEGFR2, or Flk1, or Kdr)[13, 14]. VSMCs/pericytes are recruited to the newly formed vessel through the *platelet-derived growth factor B* (PDGF-B) pathway[7]. ECs, and in particular tip cells[15], express PDGF-B, which attracts the pericytes that express the PDGF beta receptors (PDGFR-β)[7]. For proper formation of angiogenic sprouts, Delta-like 4 (DLL4) signaling via its receptor Notch1 is essential[15-20], which will be discussed below and in Papers I-II. The process of VEGF-A driven angiogenesis is schematically summarized in **Figure 2**.
Several other proteins have important roles in angiogenesis, such as TIE1, TIE2, Cadherin-5 (CDH5 or VE-cadherin), Claudin-5 (CLDN5), and Endoglin (ENG) that upon knock-out in mice display severe vascular defects and are all lethal during embryonic development[21-26].
Vascular endothelial growth factor receptor 3 (VEGFR-3, or Flt4) is activated by VEGF-C and VEGF-D (reviewed in[27]). VEGFR-3 is expressed in both lymphatic and vascular ECs during development, but becomes restricted to lymphatic ECs in the adult[28]. VEGFR-3 is also upregulated in the microvasculature of tumors and wounds[29, 30]. Recent discoveries about VEGF-A driven formation of tip cells and the role of Notch in angiogenesis led us to take a closer look at VEGFR-3 signaling, as its precise role in endothelial sprouting has been unclear (Paper II).

1.3 DELTA/NOTCH SIGNALING AND ANGIOGENESIS

The members of the Notch family of receptors (Notch1-4), as well as their ligands, Delta-like 1, 3, and 4 (DLL1, 3, and 4), and Jagged 1 and -2 (JAG1 and JAG2), are transmembrane proteins. Notch signaling has been studied extensively during development in *Drosophila melanogaster*, and has been found to control tracheal sprouting, boundary formation and cell fate determination[31-34]. The Notch pathway is evolutionary well conserved, and it controls several aspects of vertebrate developmental as well. One of these is axonal guidance during neuronal development, a process very similar to vascular guidance[35]. Particularly Notch1 has been stated to be involved in vascular development[36] (reviewed in[37]), such as remodeling of the primitive vascular plexus, endothelial-to-mesenchymal transition during development of the heart

valves, and arterio-venous differentiation. Both the full knock-out as well as the EC-specific knock-out of Notch1 lead to severe vascular abnormalities and embryonic lethality[36, 38]. The Notch ligand DLL4 is highly and selectively expressed in ECs, and is known to be required for normal vascular development[38-40]. However, the mechanism of action of the Notch pathway in angiogenesis was not clearly understood until recently, which was elucidated by us (Paper I) and others[16-20].

Notch requires processing by multiple enzymatic complexes to become activated. The Notch is protein processed by a furin-like convertase in the trans-Golgi before it is transported to the plasma membrane[41, 42]. Then, upon ligand binding, the transmembrane Notch is cleaved by the metalloprotease tumor necrosis factor-α converting enzyme TACE/ADAM17[43]. This is followed by proteolytic cleavage by the enzymatic complex γ-secretase, consisting of presenilin 1, presenilin 2, PEN-2, APH-1, and nicastrin[44-47]. The γ-secretase cleavage of Notch results in the release of an intracellular part of the protein, the Notch intracellular domain (NICD)[44, 48]. NICD is translocated to the nucleus, where it in conjunction with the transcription factor CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1 (CSL) activates the transcription of Notch target genes, such as the families of Hairy and enhancer of split (*Hes*), Hairy/enhancer-of-split related with YRPW motif protein (*Hey*), and Notch-regulated ankyrin repeat protein (*Nrarp*)[49-52]. Notch target genes act as transcriptional repressors (reviewed in[53]).

The γ-secretase was initially investigated due to its involvement in the formation of β-amyloid plaques in Alzheimer's disease (reviewed in[54, 55]). In Alzheimer's disease, plaques mainly consisting of the amyloid-β peptide are deposited in certain affected areas of the brain[56]. The peptide is cleaved off from a transmembrane protein, Aβ protein precursor (APP), by a series of proteolytic cleavages by γ-secretase and another enzymatic complex, β-secretase[57]. Thus the γ-secretase complex is implicated in the progression of Alzheimer's disease, and the therapeutic potential of compounds targeting γ-secretase, so called *gamma-secretase inhibitors* (GSI) has been investigated. Certain side effect of GSIs occur as a result of Notch inhibition[58, 59] (reviewed in[54]). This particular effect of the GSIs allowed us to study the function and cellular mechanisms of Notch signaling during angiogenesis in Paper I. Tip cells are induced by VEGF-A[12], and we discovered that tip cells inhibit neighboring stalk cells via DLL4/Notch1, thus inhibiting them to become tip cells (**Figure 2** and **3**).

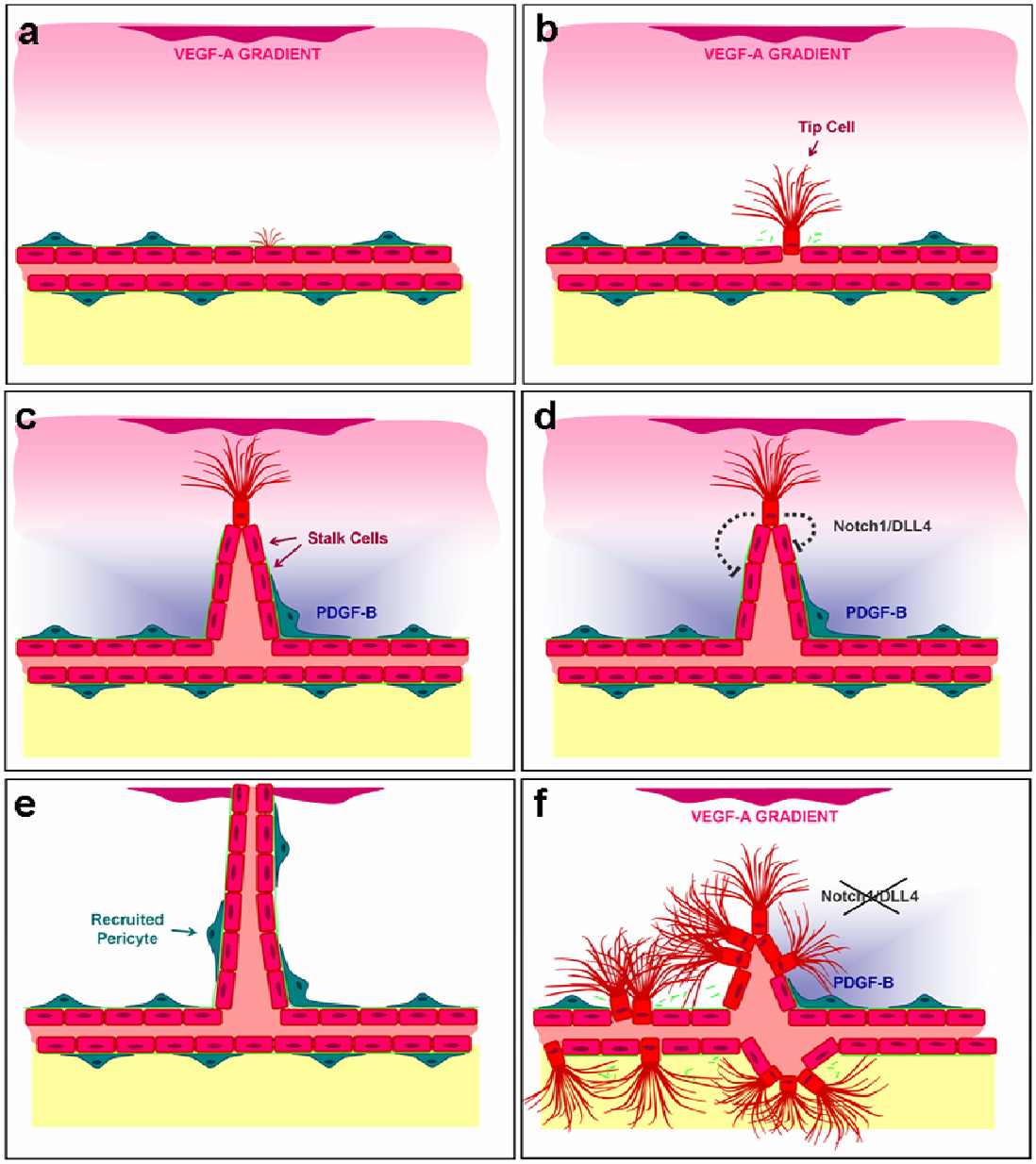

Figure 2. Cartoon describing the process of VEGF-A driven angiogenesis.
When a tissue is deprived of nutrients and oxygen, it releases three isoforms of VEGF-A with different matrix-binding properties. This leads to the generation of a VEGF-A gradient (**a**), resulting in the formation of tip cells on a nearby vessel (**b**). Tip cells extend filopodial protrusions and lead the angiogenic sprout towards the source of VEGF-A expression (**c**). Following the tip cell are the stalk cells, which are morphologically and functionally different from the tip cells (**c**). The tip cells express DLL4 that via Notch1 inhibit the stalk cells to acquire a tip cell phenotype (**d**). PDGF-B is also released by the tip cells, leading to recruitment of pericytes to the newly formed vessel (**c** and **e**). If the Notch signaling pathway is chemically or genetically disrupted, multiple ECs acquire the tip cell phenotype (**f**). This leads to excessive sprouting, and development of a malformed and dysfunctional vascular network (**f**).

1.4 PATHOLOGICAL ANGIOGENESIS

Angiogenesis is involved in many pathological processes, such as cancer, ischemic heart disease, wound healing, and retinal complications. In cancer, angiogenesis is needed for tumor growth and progression of disease. According to Judah Folkman and co-workers (reviewed in[60]), tumors need to turn on the "angiogenic switch" by shifting the local balance of proangiogenic and antiangiogenic factors towards a proangiogenic state, in order to grow in size and develop metastatic potential. Targeting angiogenesis would therefore be a strategy to treat solid tumors, a field that has relatively recently become of great interest, and is under both pre-clinical and clinical investigation. Angiogenesis is currently targeted therapeutically mainly by VEGF-A inhibitors in cancer and age-dependent macula degeneration (AMD)[61-63]. One of these inhibitors is a monoclonal antibody that neutralizes VEGF-A[62], Bevacizumab (Avastin™). However, treatment with Bevacizumab is not straight forward as major side effects can be induced, it is efficient only in combination with cytotoxic drugs, and some tumor types are quite insensitive[17, 64, 65]. Other antiangiogenic targets have been tested, but human trials have so far been negative in spite of encouraging preclinical data. DLL4 has been identified as a promising new antiangiogenic target in this study (Paper I) and by others[15-20]. Both VEGF-A and DLL4 exert important and dose-dependent functions during angiogenesis via receptors on ECs. Several other proteins with EC-specific expression have been discovered, but their usefulness as antiangiogenic targets are yet to be demonstrated. Hence, the therapeutic potential of alterations in VEGF-A- or DLL4 signaling demonstrate that further microvascular targets should be sought in pathways that are EC-specific. However, few novel angiogenic drug targets have been identified and there is a need to develop improved angiogenesis-inhibiting drugs.

1.5 GENOMICS AND TRANSCRIPTIONAL PROFILING

In genomics experiments, the expression levels of thousands of genes, or whole genomes, are studied simultaneously. This leads to the generation of transcriptional (or expression-) profiles of the tissue or cell type under investigation, which in combination with bioinformatic tools can give lots of valuable information. The term for this process is *transcriptional profiling*, which is sometimes also called expression profiling. Several different platforms are used, which often also give differing results on the same material. The three most common platforms, which were also used and/or discussed in this study, are briefly summarized below.

1.5.1 Spotted cDNA microarrays

The first step is to construct a cDNA library, which is generated by reverse transcription of mRNA transcripts into cDNA:s. The cDNA:s must be sequenced in order to be annotated with biological information. The cDNA library is printed, spotted, onto a glass slide with special surface chemistry to enhance binding. One spot correspond to one gene or gene transcript. The spots are called probes. For the hybridization experiment, mRNA labeled with fluorescent dye (the target) is incubated on microarrays resulting in complementary annealing of the target to the probe when sequences are identical. Hybridized microarrays are scanned, and spots where fluorescently dyed targets have annealed will give rise to a signal. Often two sources of

mRNA are labeled with two different fluorescent dyes and hybridized simultaneously, so that ratios of signals can be calculated upon scanning.
This method is less expensive than the SAGE method described below, if the microarrays are obtained from a vendor. However, if the cDNA library and microarrays are fabricated in-house, also this methodology may become quite a costly venture. The great benefit of manufacturing own cDNA libraries and subsequent microarrays, is the specificity and novelty of the product that may serve as competitive advantages. In our case (Paper IV), we generated transcriptional profiles from in-house produced microarrays derived from a cDNA library representing *in vivo* isolated microvasculature. The tissue specificity of the cDNA library allowed us to investigate the vascular transcriptome directly, an approach that had never been done before to our knowledge. This dataset will be discussed in the background section for Paper I, and cDNA libraries will be discussed in chapter 7.

1.5.2 Oligonucleotide microarrays – Affymetrix GeneChips™

These microarrays consist of oligonucleotide probes, chemically synthesized at specific locations on a coated quartz surface. The precise location where each probe is synthesized is called a feature, and millions of features can be contained on one array. Similarly to target hybridization to a cDNA microarray, a labeled mRNA sample is hybridized to this type of microarray, followed by scanning and data analysis. This method is less expensive than the SAGE method described below, and a great advantage is that when many labs use the same microarrays there will be increasing deposits of data, which can be directly compared.
Transcriptional profiles generated from Affymetrix GeneChip™ microarrays were used in Paper III, and will be discussed in chapter 7 of this study.

1.5.3 Serial analysis of gene expression – SAGE

SAGE is a sequencing-based sampling method, rather than a hybridization/detection based technique as the two mentioned above. mRNA is extracted from a sample, in vitro transcribed into cDNA, and digested into to short sequence tags (10-14bp). A number of short sequence tags are linked together, forming a concatamer, which eventually is sequenced in a high throughput manner. After data processing the number of short sequence tags is counted, thereby giving a transcriptional profile of that particular sample in terms of amount of transcripts. This method is rather expensive, but the results are more qualitative than those obtained using hybridization-based techniques since the result is based on counting the number of different transcripts. Furthermore, there is no need for existing clones or known sequences beforehand to be able to perform experiments.
This platform was discussed in Paper III, and in the chapter 7 of this study.

1.5.4 Functional Genomics

In order to make sense out of the often very large datasets generated in a genomics experiment, biological function has eventually to be assigned to the gene/genes of interest. This is usually done by gene inactivation and subsequent study of resulting phenotypes. Genes are usually targeted in cell lines, zebrafish, or mice. The higher the

species or organism, the more complex processes can be studied, and thus more intricate phenotypes can develop.
We have applied this approach in Paper IV, using morpholino gene knock-down in zebrafish. In the discussion regarding Paper III, we are describing our current experiments using gene knock-out in mice.

2 AIMS OF THIS STUDY

Paper I: Determine of the role of endothelial Notch signaling in angiogenesis.

Paper II: Determine of the role of VEGFR-3 in angiogenesis and its relation to the Notch pathway.

Paper III: Identify novel genes and proteins involved in vascular biology by combining transcriptional profiles of mouse microvasculature with publicly available microarray data.

Paper IV: Identify novel genes and proteins involved in angiogenesis and compounds targeting these by combining reverse- and chemical genetic screens.

3 PAPER I: DLL4 SIGNALLING THROUGH NOTCH1 REGULATES FORMATION OF TIP CELLS DURING ANGIOGENESIS.

3.1 BACKGROUND

The idea to investigate the role of Notch signaling in ECs originated from the microarray screen performed in Paper IV, which aimed to find novel genes involved in angiogenesis and vascular biology. We discovered an enrichment of genes involved in the Notch pathway, as well as genes encoding subunits of the γ-secretase complex in the transcriptional profiles of different vascular beds in the mouse. We also found that not only were GSIs commercially available, they were also readily absorbed when subcutaneously administered to mice, resulting in a very distinct, abnormal and potent angiogenic response in a dose-responsive manner. We used the GSIs DAPT (*N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester*), Compound X (*S-3-[N9-(3,5-difluorophenyl-a-hydroxy-acetyl)-L-alanilyl]amino-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one*)[58, 59], and JLK-6 (*7-amino-4-chloro-3-methoxyisocoumarin*) for the investigations described in Paper I.

To study the Notch pathway, including the effects of GSIs and Notch agonists, on angiogenesis in vivo we used the developing postnatal mouse retina as a model system. During the first postnatal week of the developing retina, angiogenic sprouting, vascular plexus formation, and remodeling can be easily studied in the retina, which has been described in detail before[12].

3.2 RESULTS AND DISCUSSION

We used both chemical and genetic loss-of-function experiments to determine the involvement of Notch in retinal angiogenesis. For chemical inhibition we administered the GSIs DAPT, and Compound X, which inhibit Notch, as well as JLK-6 that does not inhibit Notch, to postnatal mice and analyzed their retinas. Treatment with DAPT or Compound X, but not JLK-6, resulted in increased vascular density and vessel diameter associated with elevated numbers of ECs and vascular sprouts in the developing retinal vascular plexus, leading to excessive sprouting and fusion (**Figure 3 a-d**, and data not shown). Upon DAPT-treatment, retinal mRNA levels of the Notch target gene *Nrarp* were reduced, confirming a direct inhibitory effect on Notch signaling.

Mouse mutants of the Notch ligand *Dll4* display severe vascular defects[38-40], and this was one of the genetic models we studied. DLL4 is expressed in tip cells, as well as in arterial ECs. The phenotype of retinas from *Dll4* heterozygous mice was similar to that of long-term GSI-treated mice, displaying increased filopodial protrusions, vessel branching and numbers of tip cells (**Figure 3 e-h**).

We used VEcad-CreER^{T2}/Notch1$^{floxed/floxed}$ mice to study the role of EC-expressed Notch1, a genetic model in which tamoxifen-inducible Cre enzyme in ECs[66] deletes a critical Notch1 sequence flanked by loxP sites[67]. The offspring from crossing with R26R (VEcad-CreER^{T2}/R26R/Notch1$^{floxed/floxed}$) was administered tamoxifen and postnatal retinas were analyzed by staining of the LacZ reporter. These retinas partly displayed increased sprouting and number of filopodia extending from the tip cells.

Many of the LacZ-positive cells also displayed tip cell characteristics, while LacZ-negative cells were distributed evenly among tip and stalk cells. The observation that Notch1 inactivation leads to the acquirement of a tip cell phenotype suggests that Notch1 activation suppresses the tip cell phenotype in the stalk cells via cell-to-cell communication. Our data show that inhibition of Notch signaling using either chemical- or genetic strategies led to increased numbers of tip cells (**Figure 3 a-h**).

We also used a synthetic peptide analogous to the d/serrate/Lag-2 domain of Jagged1 (JAG1), a known Notch agonist[68] to investigate whether a gain-of-function experiment, i.e. activation of the Notch pathway, would give the opposite effect as seen upon inhibition. The peptide was administered to postnatal mice, and their retinas were analyzed, showing activation of the Notch pathway, i.e. reduced filopodial density at the vascular front, and decreased vessel density (**Figure 3 i-l**). Clearly, and as expected, activation of Notch lead to the opposite effects compared to the situation occurring after Notch inhibition.

The overall conclusion in Paper I is that neighboring ECs signal via DLL4/Notch1 during sprouting angiogenesis in the postnatal mouse retina. This ensures formation of the appropriate number of tip cells, sprouts and branches during angiogenesis.

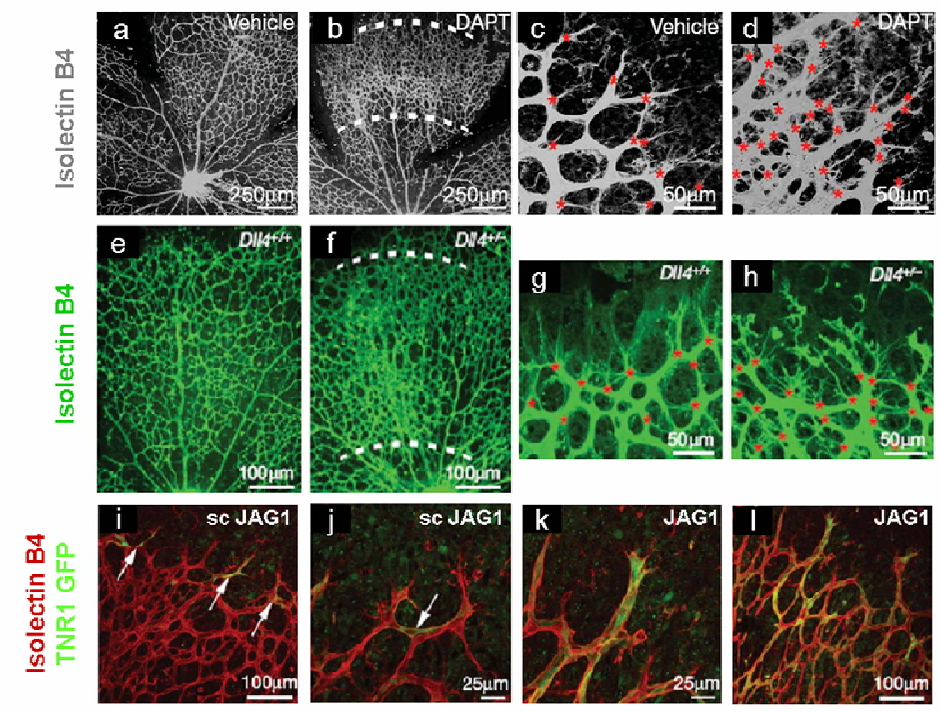

Figure 3. Inhibition or activation of the Notch signaling pathway affects angiogenic sprouting.

From Hellström et al. 2007 (Paper I). (**a-l**) Depicted are whole mounted postnatal retinas, stained with Isolectin B4 (white in **a-d**, green in **e-h**, red in **i-l**). Disruption of the Notch signaling pathway, using DAPT (**a-d),** or mice with only one *Dll4* allele (**e-h**), led to increased number of filopodial protrusions (red asterisks in **c**, **d, g,** and **h**), and a denser vascular network (**a-h**). On the contrary, administration of the Notch agonist JAG1 led to reduced number of filopodial protrusion and decreased vascular density (**i-l**). Broad induction of Notch reporter in TNR1 mouse (GFP; green/yellow) was also seen upon JAG1 treatment. Arrows indicate scattered Notch-reporter activity at the vascular front (**i-l**).

3.3 CONCLUSIONS

- DLL4/Notch1 signaling between ECs restricts tip cell formation in response to VEGF, thereby regulating the formation of appropriate numbers of tip cells to control vessel sprouting and branching.

- Inhibition of Notch-signaling leads to excessive tip cells formation and increased vascular density.

- Activation of Notch-signaling leads to fewer tip cells and reduced vessel density.

3.4 FUTURE PERSPECTIVES

Compounds targeting DLL4/Notch signaling, such as the GSIs and their derivatives that were originally developed for Alzheimer's disease, are putative pharmacological regulators of angiogenesis. The logical continuation of this project is therefore to further assess the therapeutic potential of targeting the Notch pathway. It was recently reported that administration of blocking antibodies targeting DLL4 resulted in reduced growth rate of various tumors grown on mice[17]. These are indeed promising results, and the next step would be to generate sufficient pre-clinical evidence as a basis for clinical testing interesting humans. The ultimate goal would be to conclude a phase III clinical trial with positive results, leading to approval of a new anti-cancer drug targeting the Notch pathway.

4 PAPER II: BLOCKING VEGFR-3 SUPPRESSES ANGIOGENIC SPROUTING AND VASCULAR NETWORK FORMATION.

4.1 BACKGROUND

Vegfr3 knock-out mice display severe vascular defects leading to embryonic lethality[69], and zebrafish knock-down of the *Vegfr3* gene orthologue results in disturbed intersegmental vessel (ISV) formation[70]. VEGFR-3 is known to be upregulated in the microvasculature of tumors and wounds[29, 30]. Recently it was reported that blocking antibodies against VEGFR-3 inhibit tumor growth due to disturbed angiogenesis [71]. Interestingly, these reports suggest that VEGFR-3 signaling is important in both developmental and tumor angiogenesis, and in Paper II we investigated the involvement of VEGFR-3 in these processes. Recently it was reported that zebrafish knock-down of a Notch signaling component resulted in increased sprouting and induced VEGFR-3 expression[19]. Therefore we also wanted to investigate if Notch is involved in regulation of VEGFR-3 in Paper II.

Several tumor models (B16 melanomas, Lewis lung carcinomas, metastasizing human colon carcinomas, LNM35, and transgenic Rip1Tag2 mouse insulinomas) grown on *Vegfr3*/LacZ mice were used to study tumor angiogenesis. This mouse model allows for visualization by staining of the LacZ reporter, expressed under the *Vegfr3* promoter. To study the functional roles of VEGFR-3 and Notch signaling in angiogenesis, the postnatal mouse retina was used as a model system.

4.2 RESULTS AND DISCUSSION

LacZ staining indicative of Vegfr3 promoter activity was positive in tumor blood vessels and particularly strong in angiogenic sprouts. This provided a clear indication that VEGFR-3 is expressed in angiogenic tumor vessels.

We concluded that VEGFR-3 was highly expressed in the angiogenic vessel front in the early postnatal retina (representing normal angiogenesis), but not in mature vessels at later time points. In addition, immunofluorescent staining of VEGFR-3 showed that the protein was mainly localized to the filopodial protrusions of the tip cells. VEGF-C expression was detected in the vicinity of the vascular plexus, and the protein was also found to bind to ECs.

Blocking antibodies targeting VEGFR-3 was administered to newborn mice, and subsequently the postnatal retina was analyzed. These retinas displayed reduced vascular density, decreased number of sprouts, and less branch points compared to the control (**Figure 4 a, d**). As a positive control VEGFR-2 blocking antibodies were administered, which led to a drastic inhibition of angiogenesis as expected (**Figure 4 b**). The effects were even more apparent when the VEGFR-2 and VEGFR-3 antibodies were administered together in combination, which led to a reduction in vascular plexus size and decreased number of endothelial sprouts (**Figure 4 c**). Blockage of VEGFR-3 signaling in the tumor model of lung carcinoma resulted in a marked reduction in endothelial sprouts as well. These results imply that VEGFR-3 inhibitors can block angiogenesis by suppressing endothelial sprouting. When LNM35 and B16 tumors

were treated with the combination of VEGFR-3 and VEGFR-2 antibodies, the vascular density was decreased compared to administration of either antibody alone. The combination of antibodies proved to be more effective compared to blockade of VEGFR-2 alone, as it led to a more significant decrease in vascular surface area, viable tumor area, and tumor growth.

Overexpression of both VEGF-A and VEGF-C in ear skin led to a substantially increased angiogenic response compared to overexpression of one factor at the time. The vasculature was normalized when both VEGFR-3 and VEGFR-2 were blocked with monoclonal antibodies in this model. However, animals treated with either antibody alone still exhibited excessive vessel formation. This indicates that VEGFR-3 can maintain some degree of angiogenesis even when VEGFR-2 signaling is inhibited.

To investigate if Notch regulates VEGFR-3 expression we used the same basic strategy as in Paper I, i.e. by studying the effects of DAPT (inhibits Notch signaling) or synthetic JAG1 (activates Notch signaling) on postnatal retinal angiogenesis. After DAPT treatment, VEGFR-3 expression was increased in (but not restricted to) the angiogenic front (**Figure 4 e-g, k-m**), and retinal *Vegfr3* transcription was induced. JAG1 treatment led to reduced vascular VEGFR-3 expression (**Figure 4 h-j**) as well as suppressed *Vegfr3* transcription. These results suggest that Notch signaling negatively regulates VEGFR-3 expression *in vivo*. Upon DAPT treatment, retinal *Vegfr3* mRNA levels did not change until after 12 h, while *Pdgfb* and *Nrarp* (used as reporters of Notch activity) responded already at the 6 h time point. This suggests that Notch negatively regulates *Vegfr3* transcription via intermediate modulators, such as the Notch target genes HEY1, HEY2 or HES1[72], which act as transcriptional repressors. The Notch target genes were downregulated at the same time point (12 h) as when the *Vegfr3* transcription was increased. Administration of VEGFR-3 antibodies during DAPT treatment partly reduced the hyperactive sprouting/branching phenotype.

We could also show that DAPT treatment induced VEGFR-3 expression in tumor vasculature. Administration of antibodies blocking either VEGFR-3 or VEGFR-2 suppressed vascular hyperplasia, but no additive effect was gained when combining the two antibodies

In *Dll4* heterozygous mice, one of the studied genetic models of reduced Notch signaling, we found increased VEGFR-3 protein and mRNA levels in the postnatal retinas This suggests that DLL4, via Notch, negatively regulates *Vegfr3* expression. Similar to the effects on DAPT treated retinas, administration VEGFR-3 antibodies to the *Dll4* heterozygous mice led to a reduction in phenotype severity, as seen by decreased numbers of filopodia and reduced filopodial length.

Our results implicate VEGFR-3 as a regulator of vascular network formation, and also as a key player in the acquirement of the vascular phenotype resulting from Notch inhibition.

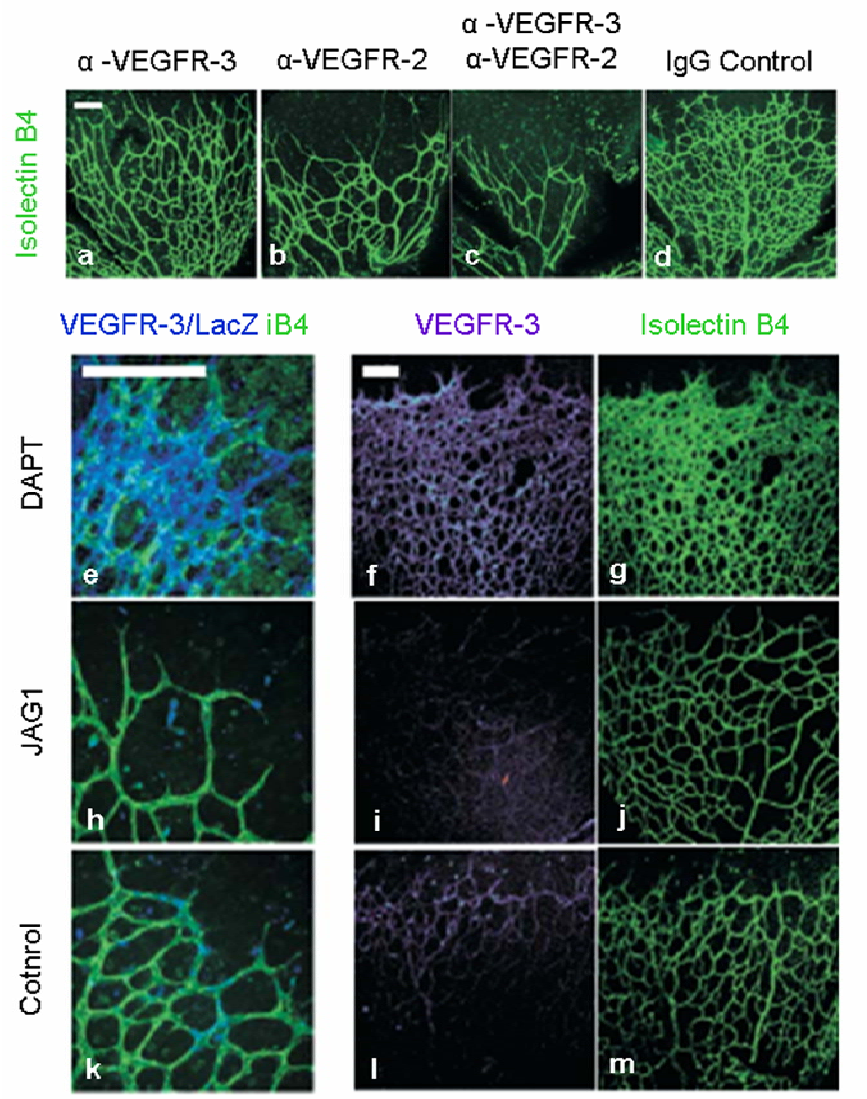

Figure 4. VEGFR-3 is involved in angiogenic sprouting.
From Tammela *et al.* 2008 (Paper II). **a-c**) Blocking antibodies targeting VEGFR-3 inhibit angiogenic sprouting in the postnatal mouse retina. Mice were administered anti-VEGFR-3 antibodies (**a**, α-VEGFR-2; **b**, α-VEGFR-3; and **c**, α-VEGFR-3 and α-VEGFR-2). **d-m**) Notch signaling downregulates VEGFR-3 in ECs. In **e-g,** retinas from *Vegfr3*/LacZ heterozygous mice treated with DAPT are shown, displaying *Vegfr3* promoter activity (blue pseudo-color). JAG1 treated retinas are shown in **h-j**, VEGFR-3 (purple rainbow color), and wild type or vehicle control retinas are displayed in **k-m**. Isolectin B4 staining (green). Scale bars, 100 mm.

4.3 CONCLUSIONS

- VEGFR-3 is highly expressed in angiogenic sprouts.

- Genetic targeting of VEGFR-3 or blocking of VEGFR-3 signaling with monoclonal antibodies result in decreased sprouting, vascular density, vessel branching, and endothelial cell proliferation.

- Administration of antibodies against VEGFR-3 and VEGFR-2 in combination results in additive inhibition of angiogenesis and tumor growth.

- Stimulation of VEGFR-3 augments VEGF-A induced angiogenesis and sustains angiogenesis even in the presence of VEGFR-2 inhibitors.

- Genetic or pharmacological disruption of the Notch signaling pathway leads to widespread endothelial VEGFR-3 expression and excessive sprouting, which can be inhibited by blocking VEGFR-3 signals.

4.4 FUTURE PERSPECTIVES

Antiangiogenic therapies targeting the VEGF pathway, e.g. Bevacizumab, have drawbacks regarding adverse effect and development of resistance as already discussed[17, 64, 65]. Targeting VEGFR-3 may therefore provide additional efficacy for current antiangiogenic therapies, particularly in conditions that are unresponsive to Bevacizumab. This should be further investigated, and the potentially synergistic effect when combining antibodies targeting VEGFR-2 and VEGFR-3 should be assessed.

5 PAPER III: IDENTIFICATION OF A CORE SET OF 58 GENE TRANSCRIPTS WITH BROAD AND SPECIFIC EXPRESSION IN THE MICROVASCULATURE.

5.1 BACKGROUND

Only a handful of attempts to determine EC-specific transcriptomes have been published, e.g. the transcriptomes of cultured ECs[73-75], ECs from normal or pathological tissues acquired by laser capture[76, 77] or cell sorting[78-81], but only two studies investigated endothelial profiles in normal tissues in comparison with corresponding nonvascular cells[79, 82]. Therefore, in Paper III, we attempted to determine the microvascular transcriptome with the aim to identify new putative vascular drug targets. Our approach was to combine publicly available microarray data with our own transcriptional profiles of mouse microvasculature.

5.2 RESULTS AND DISCUSSION

The rationale for the investigation in Paper III is outlined in **Figure 5**. For assessment of public microarray data we downloaded Affymetrix transcriptional profiles from a wide range of mouse tissues from GNF SymAtlas, an extensive collection of transcriptional profiles provided by the Novartis Foundation[78]. We aimed at finding candidate transcripts with expression profiles similar to those of 3 genes known to have highly EC-selective expression, namely *Kdr* (*Vegfr2*), *Cdh5* (VE-cadherin), and *Pecam1* (*Cd31*), which were used as baits. Similarity was measured as Pearson correlation between the expression profiles of baits and candidates.

The profiles of known vascular markers, such as *Tie1*, *Robo4*, *Eng*, *Epas1*, *Notch4*, *Esam1*, and *EphB4*, correlated well to the profiles of the baits as expected. However, the profiles of known lung epithelial markers, such as Surfactant protein (*Sftp*) b, -c, and –d, also displayed high similarity to the baits. This is most likely due to the much higher proportion of ECs in lung tissue (~50%) than in other organs (≤5%). Thus, we identified a mixed vascular/lung cluster, consisting of 132 genes that correlated with the baits. Filtering against kidney glomerular/non-glomerular and brain vascular/non-vascular microarray profiles separated contaminating lung markers, leaving 58 genes with broad and specific microvascular expression (**Table 1**).

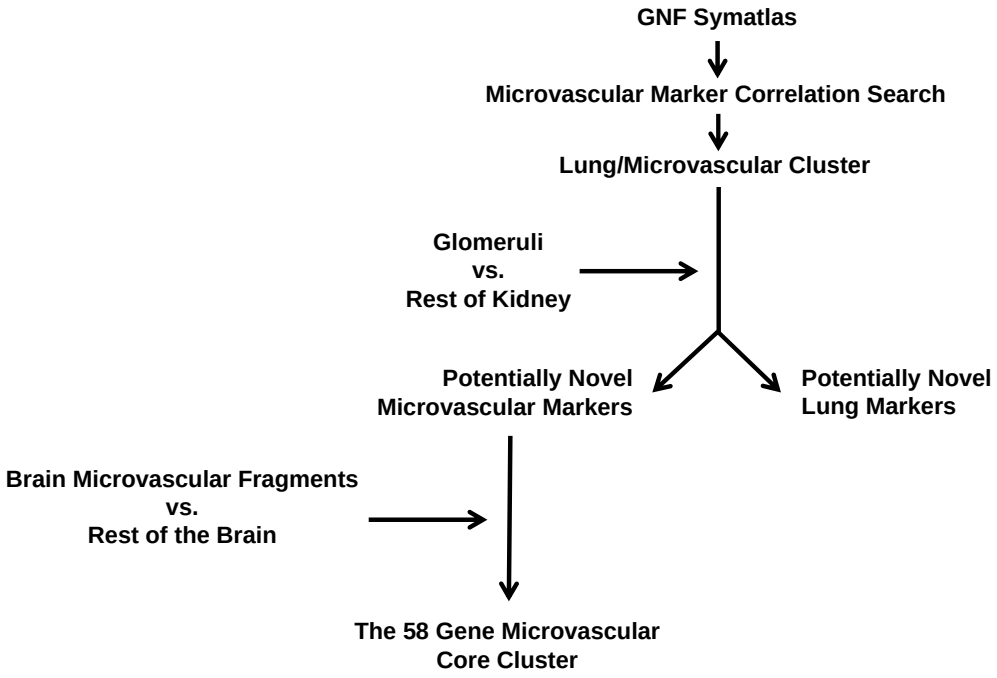

Figure 5. A flow-chart presenting the rationale behind identification of the microvascular core cluster.

Of these 58 genes, 26 were already known to be involved in vascular biology to various extent, leaving 32 gene products that have not been studied in detail or linked to endothelial functions before. 7 out of the 32 genes were further expression validated using real-time quantitative polymerase chain reaction (RT Q-PCR), and were all found to be highly enriched in the microvasculature compared to the corresponding nonvascular tissue. The 7 genes consisted of the putative G protein–coupled receptors (GPCRs) EGF latrophilin 7 transmembrane domain containing 1 (Eltd1; also called ETL or EGF-TM7-latrophilin–related protein) and GPCR 116 (Gpr116), the receptor activity modifying protein 2 (Ramp2), the putative transporter regulatory protein Slc9a3r2, the putative transporter Slc43a3, the Ras interacting protein 1 (Rasip1), and the hypoxia induced gene 2 (Hig2; NM_023516), which are described below.

Both ELTD1 and GPR116 are predicted to belong to the adhesion-type subfamily of GPCRs[84], but no functional data regarding either gene has been published. It has been implied that ELTD1 is involved in cardiomyocyte differentiation and coronary angiogenesis based on reported expression in cardiomyocytes, bronchiolar smooth muscle cells (SMCs), and vascular SMCs in heart and lung [85]. In contrast, we found ELTD1 to be highly, broadly, and selectively expressed in vascular ECs. GPR116 has been reported to be expressed in lung, kidney, and placenta [86], which are all tissues with high vascular content. Gpr116 mRNA was recently localized to ECs or mesangial cells in developing mouse glomeruli[87]. We found that GPR116 is highly expressed in vascular ECs (Paper III), which may suggests that the previously reported expression patterns in well vascularized tissues actually reflect a high content of ECs in these tissues.

In heterodimeric complex with the GPCR calcitonin-like receptor (CALCRL), RAMP2 forms a functional adrenomedullin (AM) receptor. Homozygous AM or *Calcrl* gene knock-out mice phenocopy each other and are embryonic lethal mainly due to a severe vascular phenotype[88, 89]. We found both *Calcrl* and *Ramp2* to be present in the microvascular cluster (**Table 1**), and we confirmed high expression of *Ramp2* in vascular ECs. During the preparation of Paper III, the phenotype of *Ramp2* knock-out mice was reported, demonstrating similarities to the AM and *Calcrl* knock-outs, and an essential role for Ramp2 as a co-receptor in the formation of the embryonic vasculature[90, 91].

SLC9A3R2 (Na+/H+ exchanger 3 protein kinase A regulatory protein, NHERF2, or E3KARP), is a member of the NHERF family of PDZ-containing proteins. Studies have shown that NHERFs regulate trafficking of ion transporters and other membrane proteins and transduce physiological and pathological signals that regulate ion homeostasis in mammals[92]. We confirmed high expression of Slc9a3r2 in vascular ECs, which implies a functional role in vascular biology. Also, by using immunohistochemistry we clearly demonstrated enrichment of SLC9A3R2 protein in various subsets of vascular ECs in several different human tissues.

Ras is a signaling molecule that interacts with several diverse downstream effectors and stimulates multiple signaling cascades, thereby regulating cellular proliferation, differentiation, and apoptosis. Mitin *et al*[93] identified a novel Ras-interacting protein, RAIN (RASIP1), which functions as an effector for Ras in ER and Golgi. RASIP1 interacts with Ras in a GTP-dependent manner and is localized to perinuclear, juxta-Golgi vesicles and is recruited to the Golgi by activated Ras. It has also been reported that RASIP1 was expressed in ECs using microarrays, and that RASIP1 is "a pan-endothelium marker"[80]. We confirmed enriched expression of RASIP1 in vascular ECs, suggesting that *Rasip1* is highly expressed and functions in normal ECs *in vivo* as well.

SLC43A3 (EEG1; embryonic epithelia gene 1) was found to be a putative transporter expressed during epithelial organogenesis by Stuart *et al.*[94]. They showed by Northern blot analysis and *in situ* hybridization on mouse tissues that *Slc43a3* was highly expressed in the liver, lung and kidney during embryogenesis, which are all tissues with a high vascular content. In this study, we confirmed high mRNA levels of *Slc43a3* in vascular ECs. These results imply that the tissue-selective expression described in Stuart *et al.*[94] could in part reflect a signal from the vasculature in those tissues, and that SLC43A3 may also play a role in vascular biology.

Little is known about NM_02356 (mouse orthologue of the human gene Hig2; hypoxia inducible gene 2), but Kenny *et al.*[95] showed that HIG2 represents a non-cell autonomous target of the Wnt/β-catenin pathway that might be involved in human cancer, and that HIG2 is a secreted protein. In this study, we confirmed enriched expression of NM_023516 in vascular ECs.

The relation between the 58 gene microvascular cluster identified in Paper III and recently published datasets, i.e. from Seaman *et al.*[76] and Herbert *et al.*[77], will be further discussed in chapter 7; Recent studies on the vascular transcriptome.

Table 1. The 58 gene microvascular cluster.

From Wallgard *et al.* 2008 (Paper III).

Gene	Description
1200002N14Rik	RIKEN cDNA 1200002N14 gene
2310016C08Rik	RIKEN cDNA 2310016C08 gene
Acvrl1	activin A receptor, type II-like 1
Adcy4	adenylate cyclase 4
AI173486	expressed sequence AI173486
Ankrd47	ankyrin repeat domain 47
BC028528	cDNA sequence BC028528
Calcrl	calcitonin receptor-like
Caskin2	cask-interacting protein 2
Ccbp2	chemokine binding protein 2
Cdh5	cadherin 5
Centd3	centaurin, delta 3
Cldn5	claudin 5
Col4a3	procollagen, type IV, alpha 3
Cttnbp2nl	CTTNBP2 N-terminal like
Egfl7	EGF-like domain 7
Ehd4	EH-domain containing 4
Eltd1	EGF, latrophilin seven transmembrane domain containing 1
Eng	endoglin
Entpd1	ectonucleoside triphosphate diphosphohydrolase 1
Epas1	endothelial PAS domain protein 1
Ephb4	Eph receptor B4
Erg	avian erythroblastosis virus E-26 (v-ets) oncogene related
Esam1	endothelial cell-specific adhesion molecule
Fgd5	FYVE, RhoGEF and PH domain containing 5
Gpr116	G protein-coupled receptor 116
Grrp1	glycine/arginine rich protein 1
Hbegf	heparin-binding EGF-like growth factor
Hspa12b	heat shock protein 12B
Icam1	intercellular adhesion molecule
Icam2	intercellular adhesion molecule 2
Kdr	kinase insert domain protein receptor (VEGFR-2)
Lats2	large tumor suppressor 2
LOC670044	similar to Mothers against decapentaplegic homolog 6 (SMAD 6)
Lrrk1	leucine-rich repeat kinase 1
Mmrn2	multimerin 2
Myo1b	myosin IB
Notch4	Notch gene homolog 4 (Drosophila)
Npnt	Nephronectin
Npr3	natriuretic peptide receptor 3
Nrp1	neuropilin 1
Pecam1	platelet/endothelial cell adhesion molecule 1
Pltp	phospholipid transfer protein
Ptprb	Protein tyrosine phosphatase, receptor type, B
Ptprm	protein tyrosine phosphatase, receptor type, M
Ramp2	receptor (calcitonin) activity modifying protein 2
Rasip1	Ras interacting protein 1
Robo4	roundabout homolog 4 (Drosophila)
Sdpr	serum deprivation response
Slc43a3	solute carrier family 43, member 3
Slc9a3r2	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 2
Slco2a1	solute carrier organic anion transporter family, member 2a1
Sox13	SRY-box containing gene 13
Sox7	SRY-box containing gene 7
Stard9	START domain containing 9
Tenc1	tensin like C1 domain-containing phosphatase
Tie1	tyrosine kinase receptor 1
X99384	cDNA sequence X99384

5.3 CONCLUSIONS

- By combining publicly available and own microarray data we have identified 58 gene transcripts with broad and specific expression in microvascular endothelium.

- 26 out of the 58 genes have previously been implicated in vascular biology.

- 32 genes out of the 58 do not have known functions in angiogenesis or vascular biology.

- *Gpr116*, *Ramp2*, *Slc9a3r2*, *Slc43a3*, and *NM_023516* were further validated and show considerably enriched and selective expression in microvasculature.

- Many of 32 gene products are predicted to be cell surface expressed or implicated in cell signaling processes and should therefore be explored as putative microvascular drug targets.

5.4 FUTURE PERSPECTIVES

Gene discovery is an adventurous but descriptive form of research that eventually needs to be moved forward to the analysis of gene function. Based on the microvascular core cluster identified in this paper, we have selected GPR116, ELTD1, RAMP2 and X99384 for further functional studies in mice.

We selected GPR116 and ELTD since they are predicted to be transmembrane G-protein coupled receptors, and thereby potential drug targets. GPCR signaling can often be studied using agonists or antagonists, and the potential localization of these proteins facing the blood makes them straightforward to target pharmacologically, at least in theory. Additionally, these receptors may reveal new pathways that have not been studied in EC biology before.

Regarding RAMP2, we will continue to investigate this phenotype in closer detail even though the phenotype of the knock-out mice was been published in the course of our work [90, 91].

There are no articles published on X99384, but according to Gene Ontology (GO) this protein has been predicted to have protein tyrosine phosphatase activity. This is interesting from a drug targeting perspective, as there are many compounds targeting this class of enzymes.

These knock-outs are currently analyzed by us and quite some work remains to unravel what roles these proteins may play in vascular biology. Thorough phenotypic analyses will be performed, especially with regards to possible vascular defects. The first goal is to assess is if the knock-outs are embryonic lethal or not. If yes; the specific time and cause of lethality will be determined, and phenotype severity in heterozyogous animals will be studied. If not; knock-out individuals will be studied from embryonic stages to adulthood to see if there are progressive phenotypes. If there are no obvious phenotypes, knock-outs will be challenge the in various ways, e.g. OIR/ROP (Oxygen

Induced Retinopathy/Retinopathy Of Prematurity) where pathological angiogenesis in the retina is induced and studied, tumor implantation and/or ischemia models.

6 PAPER IV: COMBINATION OF REVERSE- AND CHEMICAL GENETIC SCREENS REVEALS NOVEL ANGIOGENESIS INHIBITORS AND TARGETS.

6.1 BACKGROUND

As discussed above, there is a need to discover additional drug targets involved in angiogenesis and vascular biology, as well as compounds affecting these targets. We aimed to do this by performing parallel reverse genetic (RG) and chemical genetic (CG) screens.

In a RG screen, genes are (individually) inactivated, e.g. using gene knock-out in mice or morpholino knock-down in zebrafish in a high-throughput/semi high-throughput fashion, followed by analyses of any phenotypes that may develop. In a CG screen, drugs/drug-like compounds are tested in a high-throughput manner, for example in cellular assays, and phenotypic changes of interest are assessed.

6.2 RESULTS AND DISCUSSION

The rationale for performing the RG- and the CG screens are described in **Figure 6** below.

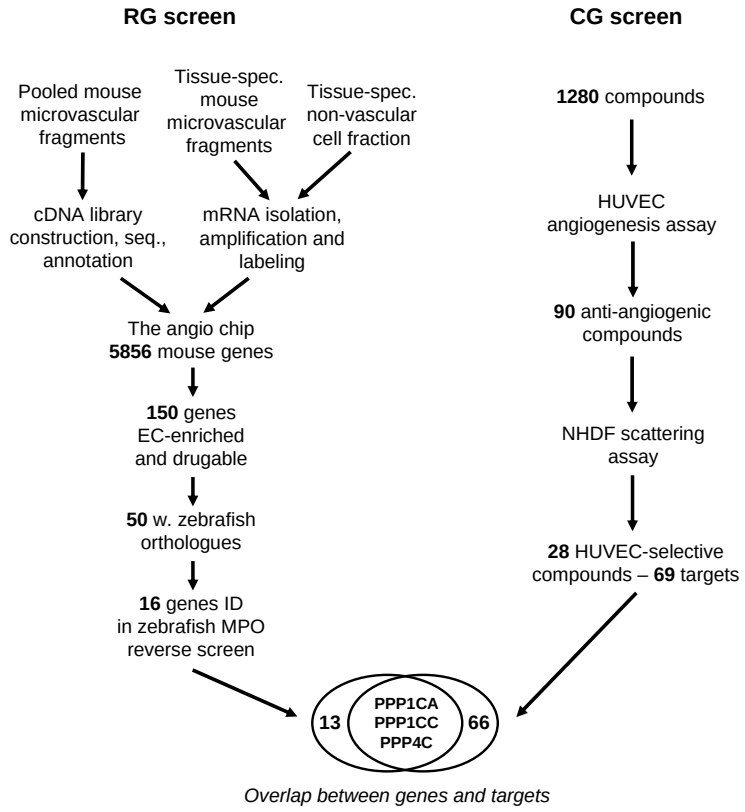

Figure 6. A flow-chart describing the RG- and CG screens that were carried out in parallel.

From Kalén and Wallgard *et al.* 2008 (Paper IV).

We constructed cDNA microarrays derived from RNA from microvasculature isolated from various mouse tissue since we wanted to identify genes selectively expressed in the vasculature (**Figure 6**). Transcriptional profiles on microvasculature from several different tissues were generated using these microarrays. We selected 150 genes that were enriched in the microvasculature, and which were also considered interesting based on a set of criteria. These criteria consisted of presence of signal peptides and/or transmembrane motif according to Ensembl annotation, and drugability as predicted by Gene Ontology annotation. In the subsequent RG screen, 50 out of the 150 genes were knocked down in zebrafish, and 16 resulted in severe vascular phenotypes. The vascular phenotype consisted of defective ISV sprouting, and/or disturbed circulation, as measured by FITC- or Rhodamine-Dextran microangiography (**Figure 7 a-l, Table 2**). These results suggest that the 16 genes identified here sustain important roles during vascular development.

Table 2. The 16 genes identified in the RG screen.
From Kalén and Wallgard *et al.* 2008 (Paper IV):

		Transcriptional Profiling Results			ZF knock-down results	
Gene	**GO Molecular Function**	**Vasc. sel.**	**Pref. vasc. bed**	**Pref. stage**	***cdh5* ISH**	**FITC-Dextr.**
Alox5ap	enzyme activator activity	Yes	Skin	-	0%	
Ctsz	cysteine-type peptidase activity	Yes	-	-	12%	19%
Fzd6	non-G-protein coupled 7TM receptor activity	Yes	-	-	21%	
Gnb2l1	GTPase activity	Yes	-	Embryo	12%	
Hexb	beta-N-acetylhexosaminidase activity	Yes	Brain	Adult	28%	
Kiaa1274	protein tyrosine phosphatase activity	Yes	Brain	Embryo	10%	
Pp2ry5	purinergic receptor activity, G-protein coupled	Yes	Skin	Adult	20%	24%
Ppap2a	phosphatidate phosphatase activity	Yes	-	Adult		
Ppih	cyclosporin A binding	Yes	Heart	Embryo	3%	
Ppp1ca	protein ser/thr phosphatase activity	Yes	-	Embryo	0%	
Ppp1cc	protein ser/thr phosphatase activity	No	Brain	-	32%	
Ppp4c	protein ser/thr phosphatase activity	Yes	Heart	Embryo	18%	28%
Rab11a	GTPase activity	Yes	Heart	Adult	0%	32%
Rab5c	GTPase activity	Yes	Skin	-	0%	
Ralb	GTP binding	Yes	-	Embryo	12%	
Sat1	diamine N-acetyltransferase activity	Yes	Heart	Embryo	0%	

In the CG screen, we tested compounds from the chemical library *List of Pharmacologically Active Compounds* ($LOPAC_{1280}$; Sigma-Adrich Inc.), consisting of 1280 compounds. These 1280 compounds were tested in an assay based on human umbilical vein endothelial cells (HUVECs)[96], which resulted in identification of 90 compounds that inhibited endothelial sprouting. These compounds were further tested in a fibroblast assay to assess cell type selectivity, which resulting in the selection of 28 compounds that displayed a selective inhibitory effect on endothelial sprouting. These 28 compounds are so far known to target 69 proteins, which may represent novel vascular drug targets (**Table 3**).

Table 3. The 28 compounds identified in the CG screen.
From Kalén and Wallgard *et al.* 2008 (Paper IV).

Compound	Description	Targets
3-Phenylpropargylamine hydrochloride	Potent, time-dependent inhibitor of dopamine beta-hydroxylase (DBH)	DBH
6-Hydroxy-DL-DOPA	Precursor of the catecholaminergic neurotoxin, 6-hydroxydopamine; converted to 6-hydroxydopamine by L-aromatic amino acid decarboxylase	ADRA1A, ADRA1B, ADRA1D, ADRA2A, ADRB1, ADRB2, ADRB3
7-Chloro-4-hydroxy-2-phenyl-1,8-naphthyridine	A1 adenosine receptor antagonist	ADORA1
Ammonium pyrrolidinedithiocarbamate	Prevents induction of nitric oxide synthase (NOS) by inhibiting translation of NOS mRNA	NOS1, NOS2A, NOS2B, NOS2C, NOS3
beta-Lapachone	Induces apoptosis in HL-60 cells; anticancer agent	TOP1
Bromoacetyl alprenolol menthane	Alkylating beta adrenoceptor antagonist	ADRB1, ADRB2, ADRB3
Bromoacetylcholine bromide	Affinity alkylating agent of nicotinic acetylcholine receptors	CHRNA1-7, 9-10
Budesonide	Anti-inflammatory glucocorticoid	NR3C1
Dequalinium dichloride	Selective blocker of apamin-sensitive K+ channels	KCNN1-3
Emetine dihydrochloride hydrate	Apoptosis inducer; RNA-Protein translation inhibitor	CASP15, 7-10, 12 & 14
Endothall	Potent inhibitor of phosphatase 2A (PP2A)	PPP1C, PPP2CA
Indirubin-3'-oxime	Cyclin dependent kinase (CDK) inhibitor; competes with ATP for catalytic subunit binding	CDC2
LY-294,002 hydrochloride	Specific phosphatidylinositol 3-kinase (PI3K) inhibitor.	PIK3C3, PIK3CA, PIK3CB, PIK3CD, PIK3CG
Methapyrilene hydrochloride	H1 Histamine receptor antagonist	HRH1
Mevastatin	Antibiotic; inhibits post-translational prenylation of proteins such as Ras and geranylgeranylation of Rho	HMGCR
Morin	Flavonoid with anti-oxidant properties; oxyradical scavenger	XDH
NF 023	Potent, selective P2X1 receptor antagonist	P2RX1
Nimesulide	Highly selective COX-2 inhibitor	PTGS2
NS 2028	Specific soluble guanylyl cyclase inhibitor	GUCY1A2-3, GUCY1B2-3
ODQ	Potent and selective NO-sensitive guanylyl cyclase inhibitor	GUCY1A2-3, GUCY1B2-3
Oxatomide	Suppresses PAF-induced bronchoconstriction; inhibits the release and actions of leukotrienes and other mediators	HRH1
PD 404,182	KDO-8-P synthase inhibitor	*Acts on bacterial proteins*
Phenylephrine hydrochloride	alpha1 Adrenoceptor agonist; mydriatic; decongestant	ADRA1A, ADRA1B, ADRA1D
Piceatannol	Non-receptor kinase Syk and Lck inhibitor	LCK, SYK
Rauwolscine hydrochloride	alpha2 Adrenoceptor antagonist	ADRA2A
SKF 89145 hydrobromide	Dopamine agonist	DRD1
SP600125	Selective c-Jun N-terminal kinase (c-JNK) inhibitor.	MAPK8-10
Tetraethylthiuram disulfide	Alcohol dehydrogenase inhibitor	ADH1A, ADH4-6, ADHFE1, AKR1A1, RDH14, ZADH1-2

The overlap between the RG- and the CG screen consisted of three members of a superfamily of serine/threonine (S/T) protein phosphatases, *Ppp1ca*, *Ppp1cc* and *Ppp4c*, and one compound, Endothall that targets this family. *Ppp1ca* and *Ppp1cc* belong to a subfamily called PPP1, while *Ppp4c* belong to the PPP2 family[97, 98].

Endothall is known to inhibit members of the PPP1 and -2 families[99, 100]. Treatment of zebrafish with Endothall led to a dose-dependent effect on lumen formation, similar to that seen in the zebrafish knock-downs of *Ppp1ca*, *Ppp1cc* and *Ppp4c* (**Figure 7**).

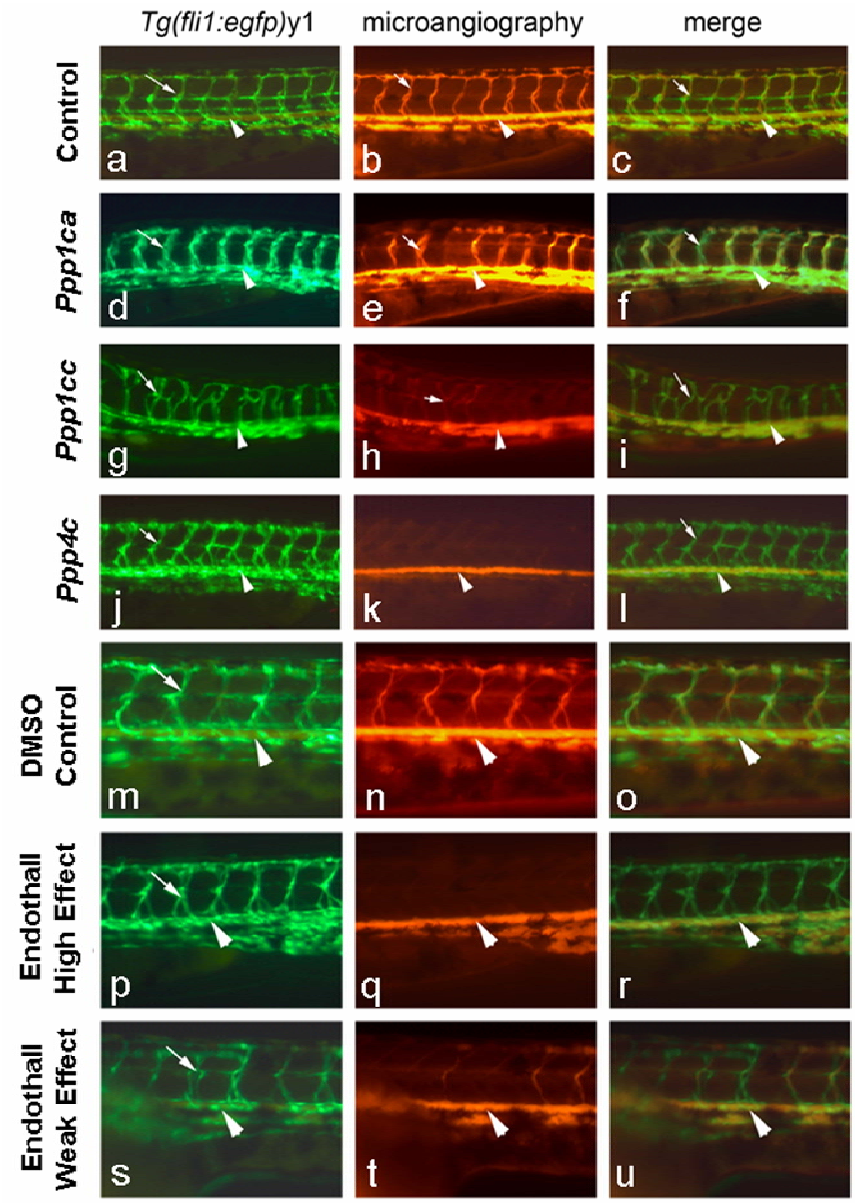

Figure 7. Zebrafish phenotypes.
From Kalén and Wallgard *et al.* 2008 (Paper IV). The phenotypes resulting from zebrafish knock-downs or Endothall treatment were analyzed in the same way. ECs in the trunk were assessed for defects in migration in the Tg(fli1:egfp)y1 line (green fluorescence in **d**, **f**, **g**, **i**, **j**, **l**, **m**, **p**, and **s**), and in circulation using microangiography (Rhodamine dextran imaging in **n**, **q**, and **s**) at 2 dpf. All panels display lateral views of the trunk. Dorsal aorta is marked with an arrowhead and the ISVs are marked with arrows. **a-l**) Knock-down of *Ppp1ca*, *Ppp1cc*, and *Ppp4c* resulted in defects in EC pathfinding and tubulogenesis. Images are all lateral views of the trunk vasculature at 2 days post fertilization (dpf). Control embryos were injected with a mixed base morpholino **(a-c)**, and knock-down embryos were injected with morpholinos against *Ppp1ca* (**d-f**), *Ppp1cc* (**g-i**), and *Ppp4c* (**j-l**). The *Ppp1ca* and *Ppp4c* knock-downs resulted in enlarged ISVs (**d**, **j**) while knock-down of *Ppp1cc* resulted in excessive branching of

the ISVs (**g**). At 2 dpf, circulation as observed by microangiography was observed in control injected embryos (mixed-base MO) in the dorsal aorta, cardinal vein, and ISVs (**b**). Rhodamine dextran dye often entered the ventral aspect of the ISVs in the *Ppp1ca* and *Ppp4c* knock-downs (a more severely affected embryo is shown for ppp4c – **e** and **k**), but a circulatory loop was not established. The *Ppp1cc* knock-down embryos showed either an absence of circulation or thin vessels with reduced circulation (**h**)**. (c, f, i,** and **l)** are merged images of the embryos shown the previous two panels. Treatment with either carrier (DMSO control in **a**, **b**, and **c**) or Endothall did not alter endothelial migration (**a**, **d**, and **g**), but microangiography (**b**, **e**, and **h**) revealed defects is circulation consistent with defects in tubulogenesis. Two classes of affected embryos are shown. In weakly affected embryos (low effect) some of the ISVs would fail to circulate Rhodamine Dextran (**t** and **u**) In more severely affected embryos (high effect), most of the ISV failed to transfer dye (**q** and **r**).

The PPP1 and PPP2 gene families contain many members that have been implicated in numerous cellular functions (reviewed in[97-99]). It has been suggested that they may be functionally redundant as they are highly conserved and homologous. However, the severe vascular phenotype in the S/T protein phosphatase knock-downs indicates specific functions in the vasculature. It has previously been reported that the PPP1 family controls vascular permeability, cytoskeletal structure and filopodia extension. PPP2A has been implicated in endothelial migration[99, 101-103]. Our results in Paper IV suggest that the S/T protein phosphatases PPP1CA, PPP1CC and PPP4C are involved in blood vessel formation, as they were identified in two screens involving different species and models. These results also suggest that combination of reverse- and chemical genetic screens is an efficient strategy for identification of new drug targets.

6.3 CONCLUSIONS

- By combining reverse- and chemical genetics we have identified new candidate drug targets with roles during normal blood vessel development.
- In the RG screen, 50 genes were knocked down in zebrafish and 16 of these emerged to be necessary for developmental angiogenesis.
- In the CG screen, 28 compounds targeting 69 proteins were found to selectively inhibit endothelial sprouting.
- The RG and CG screens identified an overlap of three members of a superfamily of serine/threonine (S/T) protein phosphatases, P*pp1ca*, *Ppp1cc* and *Ppp4c*, and one compound, Endothall, targeting that family.
- Treatment of zebrafish with Endothall leads to a dose-dependent effect on lumen formation, similar to that seen in zebrafish knock-downs of the identified S/T protein phosphatases.

6.4 FUTURE PERSPECTIVES

Future studies will address further the roles of PPP1CA, PPP1CC and PPP4C in angiogenesis and vascular biology. Dr. Jeffrey J. Essner and co-workers at Iowa State University, Ames, IA, USA have developed a genetic model in zebrafish to examine tube formation during the development of the ISVs. The effects of Endothall will also be assessed in these studies.

The remaining 27 compounds and their respective drug targets would also be very interesting to investigate further, as some of them are currently targeted pharmacologically for treating indications others than those involving angiogenesis. Example of this is the enrichment of compounds targeting the beta-adrenergic pathway, involved in blood pressure regulation, which are used to treat high blood pressure.

7 RECENT STUDIES ON THE VASCULAR TRANSCRIPTOME

7.1 COMPARISON BETWEEN THREE DATASETS - BACKGROUND

In addition to Paper III, two reports on the vascular transcriptome have recently been published by Seaman *et al.*[81] and Herbert *et al.*[82].

Seaman and co-workers[81] aimed at defining the normal vascular transcriptome and to study how it is altered by neighboring malignant cells. To do this, they generated SAGE transcriptional profiles from isolated ECs derived from *in vivo* mouse vasculature from normal resting tissues, regenerating liver, and different tumors. They claim to have identified 27 brain endothelial markers (BEMs), 15 liver endothelial markers (LEMs), 12 angiogenic endothelial markers (AEMs), and 25 tumor endothelial markers (TEMs).

Herbert and co-workers[82] described an improved way of generating transcriptional profiles from cDNA libraries by employing new bioinformatic- and statistical methods to score differential gene expression between two pools of cDNA. These methods were applied to the latest available cell line- and tissue cDNA libraries, including SAGE data, and cDNA libraries derived from vascular ECs, such as HUVECs, human aortic- and umbilical vein- ECs. Depending on which libraries and statistical methods were combined, three datasets of 104, 27, and 58 genes containing EC-specific genes were generated.

It was of great interest to determine how the different vascular datasets from Paper III[104], Seaman *et al.*[81], and Herbert *et al.*[82] related to each other, in terms of overlap and non-overlap, and this was our goal. Although Seaman *et al.*[81] and Herbert *et al.*[82] aimed to identify novel tumor endothelial markers; they generated a substantial amount of data derived from normal endothelium. As the 58 gene microvascular cluster identified in Paper III reflects expression in normal vasculature, we compared it to transcriptional profiles derived only from normal vasculature, and excluded any data on tumor endothelium in the datasets from Seaman *et al.*[81] and Herbert *et al.*[82].

Seaman *et al.*[81] presented a total of 54 genes claimed to be selectively expressed in normal ECs (27 BEMs, 15 LEMs, and 12 AEMs), which were used for this comparison. All 54 genes were present on the Affymtrix microarrays on which we based our analysis in Paper III.

In the paper from Herbert *et al.*[82] a total of 189 genes selectively expressed in normal ECs were presented (104 + 27 + 58 genes), of which 124 were present on the Affymetrix microarrays.

Surprisingly, there was no overlap between our 58 gene microvascular cluster and the 54 genes from Seaman *et al.*[81]; nor did any of the 124 genes from the Herbert *et al.*[82] paper match the 54 genes from Seaman *et al.*[81] (**Figure 8**). However, there was an overlap of 15 genes between our 58 gene microvascular cluster the 124 genes from Herbert *et al.*[82] (**Figure 8, Table 4**). The overlap consisted mostly of genes with already known vascular expression and function.

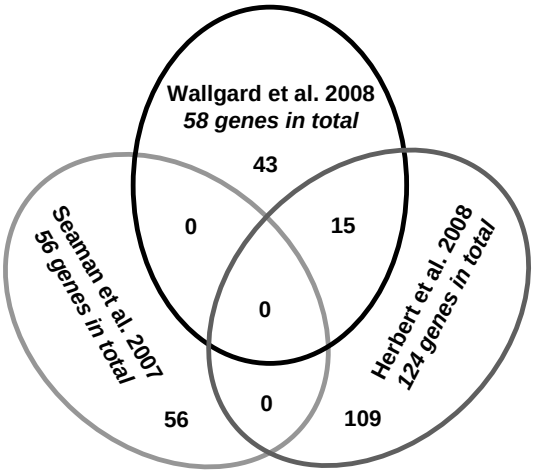

Figure 8. The relationship between three different vascular datasets.

Table 4. The 15 gene overlap.

Gene	Description
Acvrl1	activin A receptor, type II-like 1
Calcrl	calcitonin receptor-like
Cdh5	cadherin 5
Egfl7	EGF-like domain 7
Eltd1	EGF, latrophilin seven transmembrane domain containing 1
Eng	endoglin
Erg	avian erythroblastosis virus E-26 (v-ets) oncogene related
Fgd5	FYVE, RhoGEF and PH domain containing 5
Icam2	intercellular adhesion molecule 2
Mmrn2	multimerin 2
Pecam1	platelet/endothelial cell adhesion molecule 1
Robo4	roundabout homolog 4 (Drosophila)
Sdpr	serum deprivation response
Sox7	SRY-box containing gene 7
Tie1	tyrosine kinase receptor 1

7.2 DISCUSSION AND CONCLUSIONS

Why is the overlap between the three datasets not greater, as all studies claim to describe "the vascular transcriptome"? Firstly, we suggest that the 58 gene microvascular cluster identified in Paper III, reflects on a pan-endothelial core transcriptome, whereas Seaman *et al.*[81] and Herbert *et al.*[82] rather describe tissue- or developmental stage specific transcriptomes. The selection criteria used by us in a multi-step fashion to arrive at the final microvascular cluster were very strict (**Figure 5**), compared to the criteria used in other two studies. Other factors that may affect the resulting transcriptional profiles include the choice of species for investigation. Data from one species can not always be directly extrapolated to another species, meaning that it is not highly surprising that the expression data on human vasculature in Herbert *et al.*[82] differs from the mouse derived data in Paper III and Seaman *et al.*[81].

Although using the same method to obtain ECs (isolation of microvascular fragments with magnetic beads coated with EC-specific antibodies), we used beads coated with α-PECAM1-antibodies[105], while Seaman *et al.*[81] coated the beads with α-Endoglin-antibodies. The majority of ECs isolated with these two variants of the method are most likely the same. However, this could, at least in theory, result in two very distinct subsets of ECs being isolated, which subsequently would result in different transcriptional profiles.

Related to this issue is the matter of which actual type of ECs were used for the transcriptional profiling. We exclusively generated profiles from vascular fragments isolated directly from *in vivo* sources, including angiogenic vessels from the developing embryo, and compared those to their non-vascular counterparts. Seaman *et al.*[81] profiled the transcriptomes of microvasculature isolated from mainly brain, and, to represent angiogenic endothelium, regenerating adult liver. Thus the data generated by us and by Seaman *et al.*[81] differ in which vascular beds that were profiled. However, usage of isolated *in vivo* microvasculature in experiments like these gives is a considerable advantage over cells cultured *in vitro*, as they represent vessels in their natural surroundings, e.g. proper tubes that experience the shear stress of blood flow, and that are surrounded by mural cells. Herbert *et al.*[82] derived transcriptional profiles from ECs grown *in vitro* (HUVEC), isolated aortic- and umbilical vein ECs; meaning that neither microvasculature nor angiogenic ECs were represented among the profiles. These clear differences in types of starting material could affect the end result.

Also, we used Affymetrix microarrays to generate data, while Seaman *et al.*[81] used SAGE. Herbert *et al.*[82] also used SAGE to some extent, but in a combination with various cDNA libraries. Due to technical reasons intrinsic to the methods, the choice of platform clearly can affect the varying outcomes of these three papers.

Interestingly, 68 out of the 124 genes identified by Herbert *et al.*[82], were significantly upregulated ($P < 0.05$; log2 ratio < 2) in our dataset on embryonic brain microvasculature compared to the corresponding nonvascular brain tissue, meaning that these genes are highly and selectively expressed in brain microvasculature. This may suggest that Herbert *et al.*[82] have identified sets of genes that are selectively expressed in vascular beds corresponding to certain tissues, rather than genes that are expressed in a pan-endothelial manner.

7.3 FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Apparently there are numerous factors influencing the outcome in attempts to define the vascular transcriptome. In addition to exploring normal endothelium, there is also the task of defining transcriptomes of ECs in pathological conditions, such as in various forms of cancer, retinopathies, and diabetic complications. Both Seaman *et al.*[81] and Herbert *et al.*[82] made clever efforts in finding endothelial markers in solid tumors, and these kinds of experiments will probably be more extensively explored in the future. Another common family of pathologies involving ECs is arteriosclerosis, i.e. hardening and thickening of the arterial vessel wall as a result of macrophages infiltration, formation of lipid/cholesterol plaques, calcification, or other factors (reviewed in[106]). In addition to reduced lumen diameter, another characteristic of arteriosclerosis is endothelial dysfunction (reviewed in[106]) where ECs fail to synthesize sufficient amounts of nitric oxide (NO) leading to decreased vasodilatory ability. In addition to the other pathologies mentioned, the several different states and conditions in the

arteriosclerosis family of disease would be of great interest to define using transcriptional profiling on human samples.

To obtain the most comprehensive view on the vascular transcriptome, it is probably beneficial to combine all available datasets containing profiles on various ECs regardless of their origin, e.g. despite differences in species, endothelial subset, technical platform, or other variables, rather than excluding certain data sets. As it is almost impossible to expect only one or a few expression profiling experiments to reveal the full vascular transcriptome, a more inclusive approach would likely be most profitable. This type of meta-analysis of all endothelial-related transcriptional data in the public domain would require a substantial effort, but would likely provide a more comprehensive and accurate dataset. Our current understanding of the vascular transcriptome, both as a way of looking at thousands of genes at the same time as looking at individual genes involved in essential pathways, may be illustrated as a jigsaw puzzle in the process of being completed (**Figure 9**).

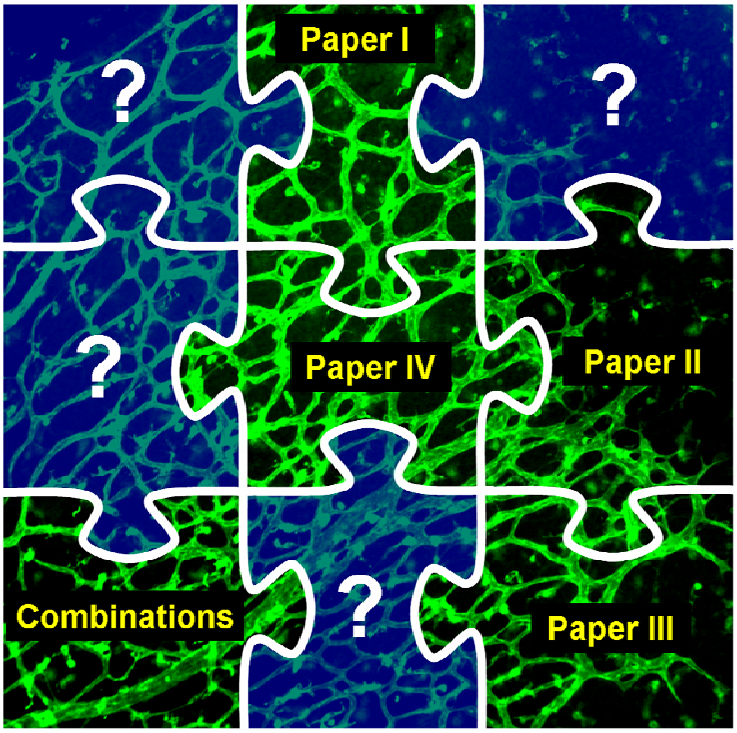

Figure 9. The vascular puzzle.

Further addition of other –omics types of data, such as proteomics and interactomics, could be imagined as an increase in the number of dimensions of the puzzle. Perhaps the final puzzle, containing all kinds of vascular data, would have far more dimensions than the three we can perceive.

The discoveries made in Papers I-II on individual genes and the angiogenesis-related pathways they are crucially involved in, are contributions to some aspects of the puzzle. Papers III-IV rather contributes with sets of genes important in vascular biology, thereby putting additional pieces of the puzzle together. However, regardless of how many pieces of the puzzle that will be correctly put together, there will probably always be missing, or mismatching, pieces. As annoying as this might be, these pieces are the constituents of the source of curiosity that will allow us all to keep continuing asking questions, and to ensure that there are always new discoveries to be made.

8 ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to everyone who in numerous different ways helped me put this thesis together; you are all such great teachers! I would especially like to acknowledge the following:

Christer Betsholtz, my supervisor and coach, multi-tasking magician and circus director, for sharing your enthusiasm, fighting spirit, and creating such an excellent scientific environment. Without your everlasting support, encouragement, and faith in me, I would not be the person I am today.

Mats Hellström, my co-supervisor, bilhandlare, mentor, and friend, for all the fantastic discussions, data-interpretation-marathons, and highly appreciated espresso breaks. With your sparkling intellect and great sense of humor, you really taught me how to do research.

Mattias Kalén, my dear friend, and great thinker, for all the interesting discussions ranging from life in general to detailed matters of science, your fearless and explorative way of living, and the laughs that could brighten any day.

Simin Rymo, my darling friend, for amazing support, all the great talks with so much depth and humor, teaching me how to live the life I want, your strength, and wonderful friendship.

Ying Sun, my great friend, for all the lovely chats, your great sense of humor, and always being brave.

The current members of the Betsholtz team: **Maya Nisancioglu**, for the enjoyable (and sometimes a bit too adventurous...), trips around the world, and help to create a comfortable work environment. **Liqun He**, my teacher in bioinformatics, for all the interesting discussions, and always helping me with great patience. **Guillem Genové**, for exciting discussions, for highlighting the importance of bulle, and bringing a Mediterranean flavor to the lab. **Karin Strittmatter**, for nice chats, and always helping me with the weirdest little things. **Konstantin Gaengel**, for always being interested in discussing science, and sharing your kindness. **Johanna Andrae**, wild life expert, for always lending a helping hand when needed, and your good mood. **Annika Armulik**, for intellectual discussions, and introducing me to the Estonian riviera. **Kazuhiro Hagikur**a, for your knowledge on Japanese brands of whiskey and their origins, and your nice way of being. **Jenny Norlin**, for being a fellow old-school microarrayer. **Radiosa Galini**, for bringing an Italian vibe to the lab. **Sara Kamph**, for always having a minute to talk. **Long Long**, for your enthusiasm.

The following former members of the Betsholtz group: **Holger Gerhardt**, my friend, for your continuous support, many laughs, and your positive outlook on life and people. **Minoru Takemoto**, for the great development of the Microarray Factory, and creation of an intellectual yet friendly atmosphere. **Desiree von Tell**, for nice dinners, and your excellent interior design skills. **Cecilia Bondjers**, for taking isolation of microvasculature to a higher level. **Alexandra Abrahamsson**, for inventing the best IHC protocols. **Johan Dixelius**, for bringing tasty fruit to the lab. **Maria Enge** and **Mattias Bjarnegård**, for helping me out when being new at almost everything.

Karl Tryggvason and the members of his group, for creating a good scientific environment in the Matrix Biology division, both in the labs and in the lunch room. **Ann-Charlotte Andersson, Ann-Sofie Nilsson, Anna Domogatskaya, Anne-May Österholm,** for nice chats, **Asmundur Oddsson, Berit Rydlander, Bing He, Dadi Niu,** for always being friendly**, Elisabeth Raschperger, Elisabeth Åman, Eyrun Hjorleifsdottir, Gunvor Alvelius, Jaakko Patrakka, Juha Ojala, Kan Katayama, Ljubica Perisic, Mark Lal, Masatoshi Nukui,** for your sense of humor, **Sergey Rodin, Susan Warner, Timo Pikkarainen,** for nice talks, **Xiangjun Xu, Yi Sun**, and **Zhijie Xiao**. **Kerstin Bengtson**, for all the help during my first year. Former member **Stefania Cotta-Done**, for your bright smiles, and all your fabulous shoes.

Arindam Majumdar, for exciting discussions, always with a friendly attitude. **Lwaki Ebarasi**, for your good mood, and enjoyable talks every now and then. **Sajila Kisana** and **Sunil Udumala.**

Karin Julenius.

Group Lindahl, including: **Per Lindahl**, for creatively bringing interdisciplinary science into focus, and being such a nice person. **Erik Larsson**, for interesting and hilarious discussions regarding genomics, as well as the

habitat and behavior of the bergstunga, your great sense of humor, and crucial help on bioinformatic matters. **Henrik Lindskog**, fellow BVLP:are, for friendship, and nice talks. Former member **Stefan Scheidl**, for being a microarray pioneer, and your efforts in trying to teach me how to waltz.

The former AngioGenetics crew, including: **Noomi Asker**, my friend and office mate, for all talks, laughs, and your way of making things better. **Linda Karlsson**, unchallenged queen of ISH, for your lovely sense of humor, and teaching me so many things. **Elizabeth Norseng,** bioinformatics- and yoga expert, for a lot of fun, and great work. **Lisa Athley**, for introducing me to Josef-yoga, and the unforgettable trip to Santa Fe. **Tore Samuelsson**, for showing me the bioinformatics-world with great patience and friendliness, and being a co-inventor of the most delicious fish stew/soup/bouillabaisse ever made at a company kick-off. **Ann-Christin Häger**, for always coming to work with a smile. **Mattias Karlsson**, for nice chit-chats.

The Uffe-squad, including: **Ulf Eriksson**, my bonus-supervisor, for your energetic and open minded way of approaching any discussion, and introducing me to VEGF-B biology. **Carolina Rosenlew**, my friend and colleague, for your undeniably positive attitude, all the support during easy and difficult times, and making any conference enjoyable. **Monika Ehnman**, for the nice talks, and room sharing in Toronto. The Pietras division, including: **Kristian Pietras**, for fun and creative discussions, and your nice personality. **Charlotte Hägnan**, BJJ queen, for great company, and your good mood.

The group of **Kari Alitalo**, especially **Tuåmas Tammela** for exciting collaborations, interesting and enjoyable correspondence, and showing me what a perfect thesis should look like.

Jeffrey Essner, for fun and interesting collaborations, and your grand fishing expertise.

Bo Olsson, my former high-school science teacher, for always believing in me.

Helena Ohlsson, darling friend and former BVLP:are, for great friendship, your fantastic sense of humor, for always being there, and since you always know the answer to "Hur vet man att man är i Götteborrg?".

Erik Norvell, my friend, for all the laughs, and talks about everything.

Daniel Liothe, for friendship, training, and philosophy. **Tina Liothe**, for friendship, and shared affection for Saltholmen. **Fredrik Simu**, for being who you are.

My darling gang of Muppet friends that makes my life so much brighter: **Caroline Améen**, fitness queen, for great friendship, numerous Zenit breakfasts with Sandra, the absolutely craziest trip to London ever known to mankind, and shared påskäggsfrosseri,. **Sandra Rodrigo Blomqvist**, martial arts- and cocktail expert, for great friendship, your and Caroline's many visits to Stockholm that cheered me up immensely, shopping Glasgow to bits, and all the nice talks. **Kajsa Améen**, teaching master, for joint jumping into jacuzzis with surprisingly few bubbles, your wonderful laughter, and being the world champion in the nynnatävling. **Ulla and Torkel Améen**, for all the enjoyable stays in Hunnebostrand.

Cathrine Waldenvik, for the great friendship that goes back so far, endless summer nights at the balcony of my parent's house, introducing me to the Sundays and all the wonderful music that followed, sharing of the darker moments, and teaching me how to live a true life.

All the fantastic yoga teachers, especially **Örjan Abrahamsson** and **Sabina Derneby**, for guidance in the art of breathing, and helping me bring back the light within. **Ashtanga-Camilla**, for showing me how to re-discover the bliss of ashtanga yoga. Om shanti, my friends.

Carola Fredstrand, fellow yogini and dear friend, for lovely tea moments after classes, helping me unravel the mysteries of life and relationships, and being so generous.

Susanne Schwalbe, for you warm and friendly nature, and being such an excellent training companion.

Åsa Wallentin, friend and sister-in-law, for introducing me to the magnificent, fluffy, hero Canis, and being such a nice person.

My dear family, for love and endless support: Mamma **Kerstin,** for all the fun shopping sprees, and making the best food in the world. Pappa **Gunnar**, for always believing that I can do anything I set my mind to. My sister **Monika**, for backing me up no matter what, all the fun things we have done, and loving care. My sister **Annika**, for always having a positive attitude. **Juliana**, for never giving up. My brother **Jerker**, for fun hikes in the woods of Småland. **Gunilla** and **Erik**, for happy reunions in Huskvarna.

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