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ON ANGIOGENESIS MODULATION

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ABSTRACT

During evolution, the transition from unicellular to multicellular organisms required the origin of a transport system capable of interconnecting specialized cells throughout the body of the organism. In higher animals, this route of transportation is the cardiovascular system, which allows gas exchange, and transport of immune cells, hormones, macromolecules, nutrients and waste products. Due to its central role, supporting other organs and tissues, the cardiovascular system forms early during the embryonic development and is the first functional organ of the body.

The construction of a vascular system can seem to be trivial (a circuit of patent tubes of various diameters, how complicated can it be?), but it is not. First, the system contains many different cell types that interact with one another. Endothelial cells constitute the actual tube in contact with the blood. Mural cells, either vascular smooth muscle cells around larger vessels, or pericytes in the case of the capillaries, "wrap" the endothelium and exert vasoactive control, provide it with structural support, and instructive molecular cues. Second, many cellular processes including oxygen sensing, proliferation, differentiation, apoptosis, and adhesion are at work when a vascular system is formed, all requiring tight regulation and coordination. Third, different vascular beds have different properties, which need to be established and regulated via cell signaling. For example, compare the difference in permeability of the kidney endothelium with that of the blood-brain barrier.

The phenomenon of blood vessel formation from pre-existing ones – angiogenesis – has been known for at least 100 years, and has been implicated in the pathology of many diseases, which in turn has sparked intensive research in the field in recent years. However, despite a tremendous effort to map and master this biological process, it is evident – given the somewhat meager results in the clinic – that more knowledge on how blood vessels are formed is required before effective drugs, inhibiting or stimulating angiogenesis, can be generated. For example, the identities of all genes involved are not known and more important, the principles of angiogenesis, according to which these genes effectuate their respective roles, are still very much in the dark.

Herein, I describe work aimed at identifying genes and chemical compounds previously not implicated in angiogenesis, as well as at characterizing the role of angiogenesis modulating genes. Included is: i) how the regulator of G-protein coupled signaling RGS5 was identified as a novel marker for pericytes; ii) how the role of Notch signaling in angiogenesis was characterized, and shown to regulate the number of endothelial tip cells, in turn affecting the density of the resulting vascular plexus; iii) how sixteen genes and twenty-eight compounds modulating angiogenesis were identified, and a role for the serine/threonine (S/T) phosphatases PPP1CA, PPP1CC, and PPP4C was uncovered using – for the first time in vertebrates – a combination of reverse and chemical genetics; and finally iv) how the drug Perhexiline maleate for the treatment of angina pectoris, was identified as an anti-angiogenic compound, using a functional cell-based chemical screen.

ORIGINAL ARTICLES

This thesis is based on the following articles hereinafter referred to by their Roman numerals.

I. Transcription Profiling of Platelet-Derived Growth Factor-B-Deficient Mouse Embryos Identifies RGS5 as a Novel Marker for Pericytes and Vascular Smooth Muscle Cells
Cecilia Bondjers, **Mattias Kalén**, Mats Hellström, Stefan J. Scheidl, Alexandra Abramsson, Oliver Renner, Per Lindahl, Hyeseon Cho, John Kehrl and Christer Betsholtz
American Journal of Pathology. 2003;162:721-729

II. Dll4 Signalling Through Notch1 Regulates Formation of Tip Cells During Angiogenesis
Mats Hellström, Li-Kun Phng, Jennifer J. Hofmann, Elisabet Wallgard, Leigh Coultas, Per Lindblom, Jackelyn Alva, Ann-Katrin Nilsson, Linda Karlsson, Nicholas Gaiano, Keejung Yoon, Janet Rossant, M. Luisa Iruela-Arispe, **Mattias Kalén***, Holger Gerhardt* and Christer Betsholtz*
Nature. 2007 Feb 15;445(7129):776-80.
*Equal contribution

III. Combination of Reverse and Chemical Genetic Screens Reveals Angiogenesis Inhibitors and Targets
Mattias Kalén*, Elisabet Wallgard*, Noomi Asker, Aidas Nasevicius, Lisa Athley, Erik Billgren, Jon D. Larson, Shannon A. Wadman, Elizabeth Norseng, Karl J. Clark, Liqun He, Linda Karlsson-Lindahl, Ann-Katrin Häger, Holger Weber, Hellmut Augustin, Tore Samuelsson, Chelsy K. Kemmet, Carly M. Utesch, Jeffrey J. Essner, Perry B. Hackett and Mats Hellström
Chemistry & Biology, 2009 Apr 24;16(4):432-41.
*Equal contribution

IV. The Angina Pectoris Drug Perhexiline is Anti-angiogenic and Enhances the Effect of VEGF Receptor Inhibition
Mattias Kalén, Holger Weber, Guillem Genové, Norbert Esser, Sina Koch, Hellmut Augustin and Mats Hellström
Manuscript, 2009

"We choose to go to the moon. We choose to go to the moon in this decade and do the other things, not only because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, because that challenge is one that we are willing to accept, one we are unwilling to postpone, and one which we intend to win. "
JFK Speech at Rice University Houston, Texas, September 12, 1962.

"It's better to burn out than to fade away."
Neil Young, Hey Hey, My My (Into the Black), 1979.

CONTENTS

LIST OF ABBREVIATIONS

ANG	Angiopoietin
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
COX 2	cyclooxygenase 2
CSL	centromer binding factor 1/suppressor of hairless/longevity assurance gene 1 (CBF1, Su(H), LAG1)
Dll4	delta-like 4
DNA	deoxyribonucleic acid
EC	endothelial cell
EGF	epidermal growth factor
EST	expressed sequence tag
FGF	fibroblast growth factor
FZD	frizzled
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
MAPK	mitogen-activated protein kinase
NICD	notch intracellular domain
NSAID	non steroidal anti-inflammatory drug
PC	pericyte
PDGF	platelet derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PlGF	placental growth factor
PP	protein phosphatase
Rβ	platelet derived growth factor receptor β
RGS5	regulator of G-protein coupled signaling 5
RIP-Tag	rat insulin promoter (SV40 large) T antigen
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
S/T	serine/threonine
TGFβ	transforming growth factor β
TIE	tyrosine kinase with immunoglobulin-like and EGF-like domains
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VSMC	vascular smooth muscle cell
VTA	vascular targeting agent

1 INTRODUCTION

"Science is always wrong. It never solves a problem without creating ten more."
George Bernard Shaw

1.1 WHY A VASCULAR SYSTEM?

In order to put this study in some historical perspective, let us start from the very beginning. Some two and a half billion years ago there was hardly any oxygen at all on Earth. Then the "great oxidation event" occurred and the level of oxygen in the atmosphere rose dramatically, likely due to a decline of methane-producing bacteria [1]. For life on earth, as we know it today, this was a seminal event since the use of oxygen for catabolization of carbohydrates and lipids is a key feature of animal life.

The simplest eukaryotic life forms, the protozoa, are unicellular organisms represented by, for example, the amoebae. They extract oxygen from the surroundings by diffusion, and thus no vascular system is required. However, then evolved the more complicated metazoans, or multicellular organisms, which have certain features that distinguish them from the protozoa. Their cells are organized into functional units, e.g. a tissue or an organ, each responsible for a specific function required to sustain life. The vascular system is one such specialized tissue, and this type of division of labor was required as animals became larger and thus distances became longer [2].

A vascular system is present in primitive invertebrate animals such as echinoderms (sea stars), insects and crustaceans. However, it is very different from the vertebrate vasculature, in most cases lacking a continuous interconnected layer of cells lining the lumen of the blood vessel [3]. For example, in the burrowing brittle star Hemipholis elongate, an Echinoderm, the water vascular system (basically a hydraulic system used to move its limbs) is also used to circulate hemoglobin-containing cells [4]. On the other side of the complexity spectrum we find the human vascular system which is a closed one, has a pump (the heart), an arterial and venous side, as well as a lymphatic system, which drains fluid and immune cells from the interstitial space back into the bloodstream. The main tasks of the vertebrate vascular system are to allow efficient transportation of not only oxygen, but also macromolecules, nutrients, and cells, to peripheral tissues and furthermore, to allow removal of carbon dioxide and waste products.

1.2 HOW DOES NATURE CREATE A VASCULAR SYSTEM?

The vascular system is precisely shaped to fit the anatomy of all organs it invests. This type of complex branching networks of linear structures are common in the multi-cellular anatomy of both plants and animals [5] and illustrated in **Figure 1** and article **II** (Fig. 1a). These structures are formed through a process termed branching morphogenesis, an early and key event essential for the success of metazoans [6]. Branching can occur in a single cell, such as a neuron when it forms dendrites and axons to establish communication with other neurons. Even branched tubes can be made by single cells, such as the terminal cell of the Drosophila trachea system [7]. Alternatively, branching can occur within a group of cells, such as in the vasculature, lung and exocrine glands.

One relevant question, given the striking structural similarity of these branched networks in species of both animals and plants, is whether the same mechanisms are

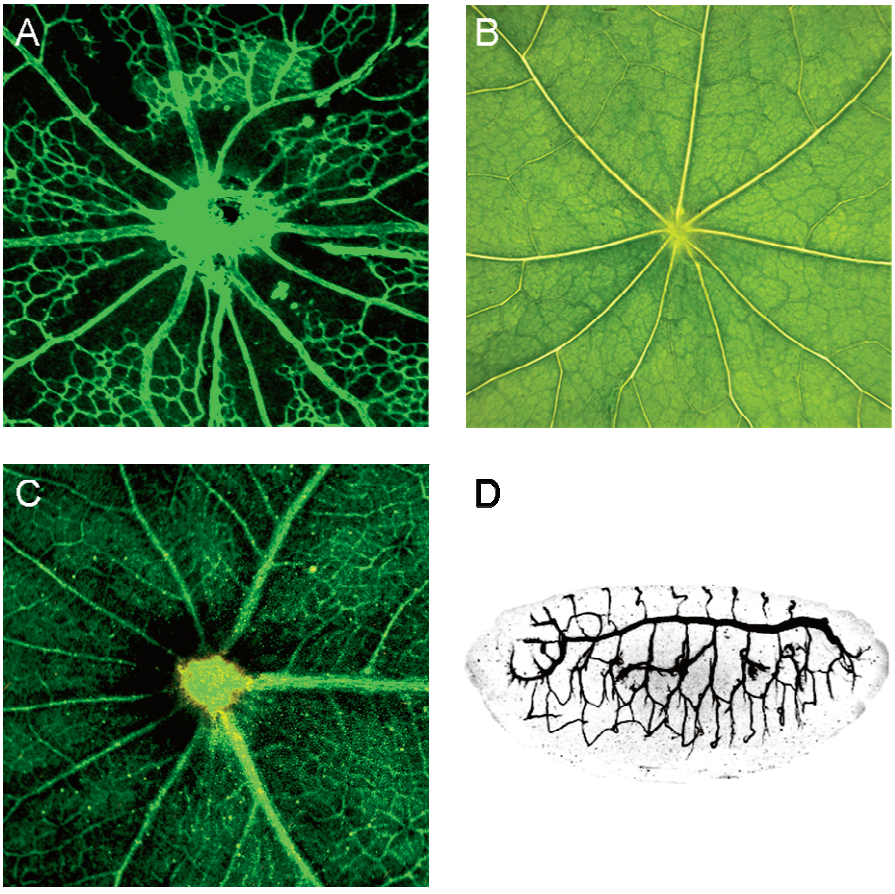

Figure 1. Similarities in branching morphogenesis throughout the kingdoms of animals and plants. (**A**) Arteries and veins spread from the optic nerve in the center towards the periphery during development of the mouse retina. The vasculature is stained using lectin, from the African bush Bandeiraea simplicifolia, which is conjugated to a fluorescent dye. (**B**) Photograph depicting a leaf from a Garden Nasturtium (Tropaeolum majus) with clearly visible veins (white/yellow). (**C**) The vascular system of the author's Chinese money plant (Pilea peperomioides) processed in Photoshop, displaying a remarkable similarity to the vasculature of a mouse retina. (**D**) The tracheal system of the fruit fly (Drosophila melanogaster) develops partly under the influence of the same genes as the vasculature of a mouse, and follows a recursive pattern common in nature. The retina is a courtesy of E. Wallgard. The fruit fly picture is adapted from [8].

implicated. Or with other words; has nature invented tube formation only once? The answer seems to be no. Vertebrates develop tubular structures using essentially the same main principles and employing the same set of genes. Even in invertebrates like the fruit fly, the trachea – a branched tubular system used for transportation of air into the animal – develops partly through the action of orthologs of genes involved in the development of the vascular system in humans and mice e.g. Wnt (Wingless in flies) [9], Notch [10], TGFβ (decapentaplegic) [11], and Robo [12]. However, plants and trees seem to be quite different from animals when it comes to tube formation. For example, Delta-Notch, a key signaling

pathway for vascular development in man, mouse, and fish is an "evolutionary invention" unique for multicellular animal life, hence not existing in plants [13]. In plants entirely different signaling molecules are at work when leaf venation takes place; mainly a plant hormone termed Auxin [14]. Thus, through evolution [15] different mechanisms for executing branching morphogenesis have evolved in plants and animals.

Finally, it is also quite intriguing that the molecular cues responsible for neuronal branching also play a role in branching of invertebrate and vertebrate vasculature [16, 17], implicating a evolutionary kinship between these two processes.

1.3 ANGIOGENESIS RESEARCH – A HISTORICAL PERSPECTIVE

Around year 1508, the always resourceful Leonardo da Vinci described the human vascular system in detailed drawings of dissected corpses, and speculated that the vasculature developed like a tree – from a seed (the heart), which develops roots (capillaries), and a trunk (the aorta) [18]. One century later, the English physician William Harvey was the first in the Western world to correctly describe the systemic circulation, including the role of the heart; to pump the blood around the body. Harvey's discovery was made in 1616, and published in 1628 in the book *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (An Anatomical Exercise on the Motion of the Heart and Blood in Animals) [19]. The capillaries, closing the circulatory loop between arteries and veins, were discovered by the Italian medical doctor Marcello Malphighi in 1661 [20] and in 1787, the British surgeon John Hunter first uses the term angiogenesis to describe blood vessels growing in the reindeer antler [21]. Furthermore, important discoveries on the origin of blood vessels, e.g. showing that they arise from the mesoderm, were done by the American embryologist Florence Sabin (the first woman elected to the National Academy of Sciences) in the 1920's [22]. Already in 1945, Algire and Chalkley could show that tumor growth is dependent on the development of vascular supply [23]. However, the modern history of angiogenesis starts with a landmark paper in New England Journal of Medicine in 1971 by the surgeon Judah Folkman, where he revived the idea by Algire and Chalkley that tumors depend on their capacity to recruit new blood vessels to survive and expand. Folkman also postulated that such tumor-associated angiogenesis is not a passive physiological response, but is regulated by specific factors, and that pharmacological intervention, targeting such factors, could be useful in the treatment of human cancers. In 2006, Avastin™, the first drug targeting one such factor (VEGF-A), was launched in the US market for first line treatment of metastasizing colorectal cancer [24].

For many years, the research field of angiogenesis has been associated with somewhat of a buzz, often bordering hype [25] (and my own observation), likely due to the promise of an entirely new approach to treat cancer – by starvation, cutting off the tumor's blood supply. The dust has now settled somewhat, due to the difficulty of anti-angiogenic drugs to deliver on the promise to cure cancer. Even so, over the years the development in the field has been dramatic, but then again so has the development of the entire field of biomedicine. A search for "angiogenesis" in the Pubmed database of the year 1998 generates 1156 original articles and 223 reviews. To generalize quite strongly, these articles were mainly occupied with dissecting the roles of the few key regulators known at the time, e.g. VEGFs (and PlGF)/VEGFRs, Angs/TIE1-2, Ephrins/Eph receptors, FGF2, TGFβ, integrins, VE-cadherin, and PDGF-B/Rβ. In 2008 the picture was entirely different: 5291 original articles and 1144 reviews describing what is now likely over 100

genes implicated in all aspects of angiogenesis, and several dozens of compounds affecting this process.

1.4 DEVELOPMENTAL ANGIOGENESIS

Given the need for nutrients and gas exchange, a cell is required not to be farther away than approximately 100 μm from a blood vessel [26]. Therefore, as it supports the development of the other organs, the cardiovascular system is the first functional organ of the embryo to be formed. In a process termed vasculogenesis (**Figure 2A**), the first vessels are assembled *de novo* from scattered mesodermal cells, which differentiate into endothelial precursor cells or angioblasts, which in turn form so-called blood islands. These primitive cellular structures fuse to form the future large vessels and the first rudimentary blood vessel network [27]. This network then grows and is remodeled through endothelial sprouting, splitting, growth and regression, collectively referred to as angiogenesis [28]. Early on the endothelial plexus acquires a coating of mural cells (pericytes or vascular smooth muscle cells, the latter around larger vessels) [29], a process essential for proper vascular morphogenesis and vessel stability [30-32] (**Figure 2B)**.

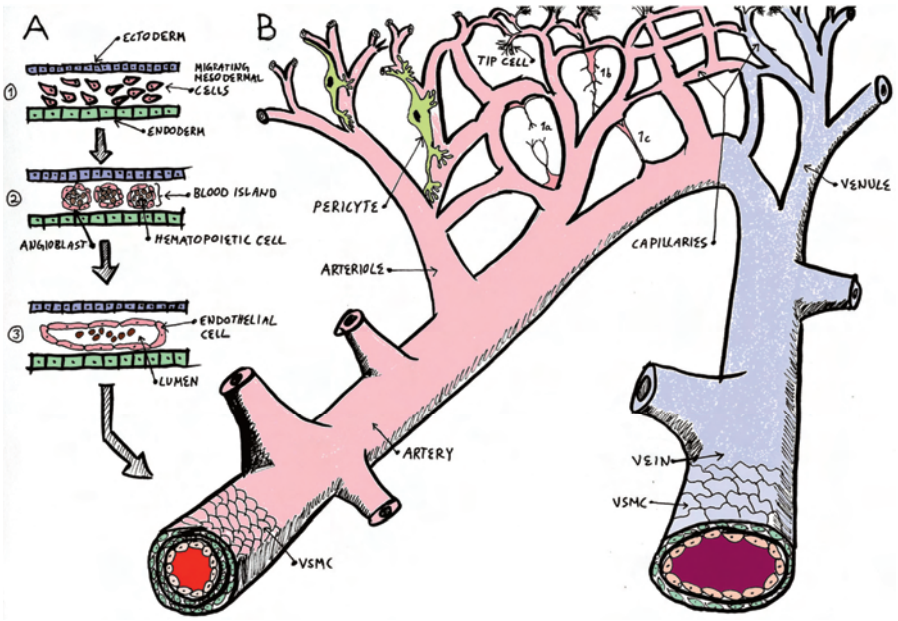

Figure 2. Schematic representation of vasculogenesis and angiogenesis. (**A**) Blood vessels, as well as blood cells, originate from the mesodermal germ layer (A1). Through differentiation, the formation of blood islands – comprised of hematopoietic cells enclosed by angioblasts – takes place (A2). Blood islands fuse and a primary capillary plexus is formed (A3). (**B**) The vascular system includes arteries, arterioles, venules, veins, and capillaries – bridging the arterial and venous sides (note the arteriovenous shunt!). Sprouting angiogenesis is initiated when endothelial tip cells extend long filopodia from pre-existing capillaries (1a). Filopodia make contact (1b) and form a solid strand (1c) which is subsequently lumenized. Adapted from [27, 28].

1.5 ANGIOGENESIS IN DISEASE

Blood vessels are present in almost all organs of the body (exceptions are the lens and cartilage). Therefore, blood vessels are (or become) implicated in numerous pathological conditions with different etiology, including cancer, age-related macula degeneration, rheumatoid arthritis, and ischemic heart disease. Estimates indicate that up to seventy different medical conditions might benefit from pro- or anti-angiogenic therapy [33].

The stage where a tumor starts recruiting blood vessels by releasing pro-angiogenic factors is often referred to as the 'angiogenic switch' [34]. Several different signals have been discovered to be capable of turning the switch 'on' including metabolic stress (low oxygen pressure or pH), mechanical stress, immune response and mutations. Furthermore, the pro-angiogenic factors can be provided, not only by tumor cells, but also by stromal cells, blood, and the extracellular matrix [35]. The recruited or co-opted tumor vasculature is often disorganized, tortuous, dilated and leaky [36] and hence, tumor blood flow is chaotic and variable, which leads to poor perfusion and hypoxic regions in the tumor [37] and **Figure 3**.

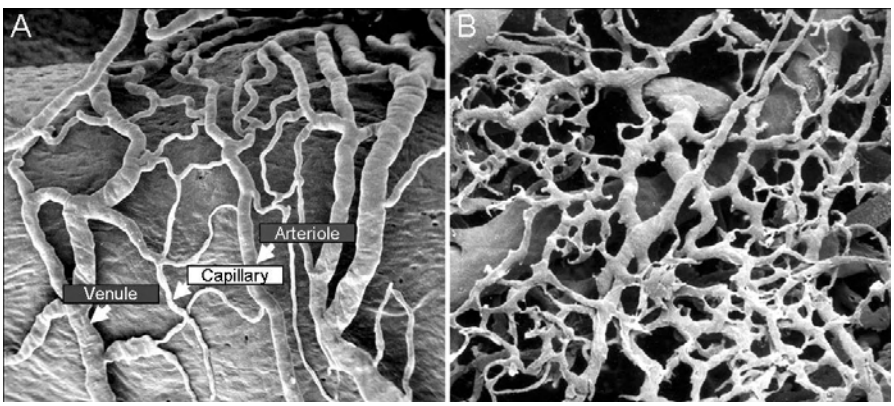

Figure 3. Tumor vasculature is abnormal. Scanning electron micrographs of polymer casts of different vascular beds. (**A**) Vasa vasorum of sinus caroticus in the rat, representing a normal, structured vasculature with identifiable venules, capillaries, and arterioles. (**B**) Highly disorganized vasculature of a xenografted human tumor in a nude mouse. Adapted from [38].

Pharmacological inhibition of tumor angiogenesis is attractive for several reasons. First, standard cancer drugs, cytotoxins, primarily target fast proliferating cancer cells whereas anti-angiogenic treatment targets the endothelium, and thus in theory attacks the tumor from another angle – through starvation. Second, angiogenesis is not active in the adult except during menstruation, wound healing, and pathological cellular growth, and thus anti-angiogenic treatment should be a quite selective therapy, leading to fewer side effects. Third, targeting of slowly proliferating endothelium (compared to the tumor cells) also potentially reduces the risk of drug resistance, although this has been observed [39]. Resistance to anti-angiogenic drugs is possibly caused by genetic alterations in the tumor endothelium due to fusion between ECs and malignant cells [40].

The realization that tumors require new blood vessels in order to grow beyond the size of a pinhead, or metastasize, has led to numerous efforts to develop anti-cancer

therapeutics by blocking tumor angiogenesis. So far, the best example of this approach is pharmacological inhibition of vascular endothelial growth factor A (VEGF-A). The anti-VEGF-A antibody Avastin™ was launched in 2006 and the improval in survival, attributable to its use, is similar or greater than that observed in any Phase III trial for the treatment of colorectal cancer – 3 months [24, 41]. However, Avastin™ and other recently approved anti-angiogenic cancer drugs, e.g. Sutent™ and Nexavar™, only prolong life in cancer patients, and do not constitute a cure. In addition, there are other drawbacks explaining why these drugs have not been the holy grail of cancer therapy everyone had hoped for: They are only efficient in combination with cytotoxic drugs, have major side effects, and, since different tumors may use alternate pathways to stimulate angiogenesis, certain tumor types are unresponsive to the drugs [39, 42, 43]. In addition, in experimental models, anti-angiogenic therapy has elicited malignant progression of tumors to increased local invasion and distant metastasis [44]. Moreover, as mentioned, current anti-angiogenic therapy of cancer requires combinatory treatment with cytotoxic drugs in order to have an effect on patient survival, which has led to speculation that the effect obtained can be attributed to the VEGF inhibitors' capacity to reduce leakage of poorly functional blood vessels and thereby allow for improved delivery of cytotoxins to the tumor mass [45]. Thus, the first generation of anti-angiogenic drugs is not optimal and recent insights need to be addressed when designing the second generation. For example, the pre-clinical cancer models that were used to evaluate anti-angiogenic treatment of cancer differ substantially from the human condition. In most mouse models (for an example, see **IV**) a tumor is implanted subcutaneously, which is rarely the site of spontaneously occurring tumors in humans. Interestingly, a novel mouse model for colorectal cancer was recently generated, potentially addressing the issue with questionable mouse models: Starr and co-workers crossed mice harboring mutagenic Sleeping Beauty (SB) transposons, with mice expressing SB transposase in the gastrointestinal tract epithelium, and most of the offspring developed intestinal tumors [46]. Second, it is likely that tumors can adapt to e.g. reduced levels of VEGF-A, due to Avastin™ treatment, by up-regulating the production of other angiogenic factors. Hence, a 'cocktail' of anti-angiogenic drugs is likely required in order to halt the growth of the tumor vasculature and achieve tumor starvation.

Other strategies for tumor eradication, implicating blood vessels, are vascular targeting agents (VTA), which are divided in two categories: i) small-molecule VTAs, where physiological or structural differences, such as proliferation rate, permeability or cytoskeletal differences between normal and tumor endothelial cells, are used to selectively occlude tumor vessels [47, 48]; ii) ligand-based VTA, where proteins, selectively expressed on blood vessels, are used to home antibodies or peptides linked with cytotoxins or radionuclides, to tumor vessels [49, 50]. Both approaches have been tested successfully in pre-clinical models but are still in clinical trials [51, 52].

One area where anti-angiogenic drugs *have* revolutionized treatment is within ophthalmology. The anti-angiogenic drug Ranibizumab (Lucentis™) has dramatically improved treatment of age-related macula degeneration. Not only does it halt the progress of the disease but also, in some cases, improves the patient's vision. Lucentis™ is a fragment of Avastin™, it is smaller and has a higher affinity for VEGF-A than Avastin™, and is delivered via intraocular injection every four weeks [53]. However, the first ocular anti-VEGF agent was Pegaptanib (Macugen™), approved by the FDA already in 2004. Macugen™ is an aptamer targeting $VEGF_{165}$ – the most abundant isoform of VEGF-A in

ocular disease. Intraocular injection of Macugen™ every six weeks demonstrated efficacy albeit not to the same extent as Lucentis™ [54, 55].

In therapeutic approaches where more blood vessels are desired, for example in ischemic heart disease and peripheral ischemic disease, success has been more elusive. Several randomized placebo-controlled clinical studies have been reported but without substantial clinical outcome [56, 57]. A banal explanation is that it is more complicated to build something functional than it is to disrupt or destroy it. Interestingly, very recently new functional blood vessels were generated, *in vivo,* in mouse models of myocardial heart infarction and hind limb ischemia, using antagomirs (single-stranded RNA oligonucleotides complementary to specific micro-RNAs) targeting a microRNA regulating several key genes in vascular function including integrin subunits $\alpha 5$ and α v, the sphingosine-1-phosphate receptor, and eNOS [58]. Perhaps the ability of a micro RNA to regulate several genes might be the answer to functional therapeutic blood vessel growth in the future.

2 AIMS

"Pipe installation must be carried out in accordance with current norms and directives."
Installation and maintenance instructions for ground source heat pump F1330.

In 1998, when I entered the research field of angiogenesis, very few genes implicated in angiogenesis were known and even less had been functionally characterized. A general hype – created by the enormous potential of angiogenesis revealed by striking pre-clinical findings – spurred premature clinical trials, initialized before mechanisms had been properly characterized, and biological processes fully understood (for examples, see [59, 60].

Given the above, we aimed to identify genes previously not implicated in blood vessel development and to characterize their role in the aforementioned process, with the intent to enable more efficient pharmacological treatment of diseases where angiogenesis is implicated.

3 RESULTS & DISCUSSION

"Die wichtigsten dinge durch röhren in der welt ausgerichtet werden."
Georg Christoph Lichtenberg 1742-1799, Sudelbücher C252.

3.1 IDENTIFICATION OF RGS5 AS A NOVEL PERICYTE-MARKER (ARTICLE I)

Gene targeting of platelet-derived growth factor B (PDGF-B) or its receptor (Rβ) in mice leads to aneurysms, hemorrhages and perinatal death due to a loss of vascular smooth muscle cells (VSMC) and pericytes (PC) [61-63]. In 1998, the analysis of the PDGF-B and Rβ knockout phenotypes had led to several observations: i) there was a variable loss of PC/VSMCs in mice with perturbed PDGF-B signaling, depending on tissue/organ; ii) the primary role of PDGF-B was to regulate proliferation and migration of PC/VSMC progenitors; iii) loss of PC/VSMCs affected gene expression in adjacent ECs [31, 32]. Having contemplated how to proceed with the analysis of the PDGF-B knockout mouse, we decided that identification of dysregulated genes – due to the loss of PC/VSMCs – would be of interest. The obvious experiment was to use a tissue where the loss was substantial, for example the brain, and compare endothelial gene expression between wildtype and PDGF-B knockout mice. Ideally, we reasoned, one would use pure samples of quickly *in vivo*-isolated blood vessels in order to maximize the likelihood that a "true", i.e. preserved, gene expression was being studied. Not only would the experiment potentially identify endothelial genes with dysregulated expression, due to the loss of pericytes, but it was also probable that novel PC-markers could be identified.

The question was which technology to use for the transcription profiling. We decided on the emerging DNA microarray technology, developed by Mark Schena, Joe deRisi and colleagues, in P.O. Brown's laboratory at Stanford, by which the gene expression levels of thousands of genes can be analyzed simultaneously [64-66], reviewed in [67]. We chose the spotted chip technology since it allowed us to print microarrays with selected EST-clones derived from the vasculature. Blood vessels are always under-represented relative to the main cell type of a particular organ, and thus EST-clones pertaining to blood vessel genes would be very few and far between in EST-libraries bought of the shelf. In order to create vascular-specific EST-libraries, for subsequent microarray fabrication, we developed a technique to purely *in vivo* isolate blood vessels from mice. This technique was also to be used when generating target material, i.e. RNA for the transcription profiling experiments. The isolation technology is based on the use of magnetic beads, coated by antibodies with affinity for EC-specific cell surface proteins (e.g. PECAM). This technology has later been successfully used in several studies to purely extract blood vessels from mouse models [68-71]. Importantly, at the time there were no whole-genome chips available from companies such as Affymetrix Inc. or Agilent Inc., and one reason for this was that the sequence of the mouse genome simply was not published until December 2002 [72].

During the process of establishing the DNA microarray technology, we did a proof of principle study using a small set of EST-clones from a commercial provider. Since the blood vessel isolation technique was not yet optimized, we used entire heads from wildtype and PDGF-B mice at embryonic (E) day E15.5 as our source of target RNA. On top of the gene list, over-expressed in wildtype compared to PDGF-B mice, we found *Rgs5*, a regulator of G-protein coupled signaling.

RGS5 is part of a family of intracellular proteins including thirty known members involved in regulation of G-protein coupled receptors (GPCRs) – the largest known superfamily of cell surface signaling receptors encoded in the human genome with approximately 900 members, of which several hundreds are involved in regulating features of the cardiovascular system including vascular tone, heart rate, and blood brain barrier function [73-75]. GPCR signaling is also involved in developmental angiogenesis. For example, Edg-1 receptor knockout mice display vascular abnormalities [76], which is also the case for disruption of adrenomedulin signaling via the GPCR Calcr and its co-receptor Ramp2 [77]. Coupled to GPCRs are heterotrimeric G-proteins ($G\alpha\beta\gamma$) that act as signal transducers, in turn activating for example adenylyl cyclase, phospholipase C, Rho GTPase or MAPKs, depending on type of $G\alpha$ subunit involved. Subsequent G-protein deactivation occurs through the intrinsic GTPase activity of the $G\alpha$ subunit, and partly by the GTPase activity provided by the RGS molecules, reviewed in [74, 78]. RGS proteins were originally identified as negative regulators of G-protein signaling, but it has now been established that they are multifunctional proteins that generally modulate G-protein signaling [78].

Having identified *Rgs5* as over-expressed in wildtype mice compared to PDGF-B knockouts, the gene expression was validated using mouse mRNA *in situ* hybridization, which confirmed that the gene was expressed in pericytes (**I**). Furthermore, *Rgs5*-deficient mice develop normally, have a slightly reduced body weight, and lack obvious defects in cardiovascular development or function apart from low blood pressure [79, 80]. It has, however, recently been reported that loss of RGS5 in the RIP-Tag mouse tumor model results in pericyte maturation, vascular normalization, and reductions in tumor hypoxia and vessel leakiness, and an enhanced influx of immune cells leading to prolonged life [81].

The effort described in **I** reflects a wish to better understand the vascular transcriptome, including differences between various vascular beds. For example, it is reasonable to believe that differences in permeability between the kidney glomeruli endothelium and the brain endothelium are reflected at the transcriptional level. Moreover, we know that differences between for example arteries and veins are manifested in gene expression but only a handful of selective markers have so far been identified, among them the arterial markers EphrinB2 and Connexin 37, and the venous markers Eph4 and Flt4 [82-86]. In an effort to gain knowledge about the vascular transcriptome Wallgard et al. recently identified fifty-one novel vascular markers using a combination of Affymetrix technology and the Gene Expression Atlas (SymAtlas) – an *in silico* repository of microarray gene expression profiles from mouse and human tissue samples [71, 87]. Additional efforts to identify the vascular transcriptome, or parts thereof, have been undertaken by our laboratory (**III**, [69, 70]) and by the groups of Bicknell and Kinzler/Fogelstein [88-91].

Today methods for mapping the entire vascular transcriptome are available. Affymetrix Inc., Agilent Inc. and others provide DNA arrays where the entire mouse

genome is present. The SymAtlas is being expanded and in addition, due to the enormous progress in technology development, DNA sequencing can now be used at a reasonable cost to 'deep sequence' vascular EST-libraries. Thus we are, after ten years, somewhat closer to the goal of an explored vascular transcriptome from which selective, well validated genes can be used as drug targets.

3.2 ELUCIDATION OF THE ROLE OF NOTCH SIGNALING IN ANGIOGENESIS (ARTICLE II)

As mentioned in chapter **1.2** above, several signaling pathways involved in branching morphogenesis, are conserved between species. One such pathway is that mediated by Notch, a protein encoded by a gene discovered in fruit flies in 1919, by virtue of the fact that partial loss of function (haploinsufficiency) results in notches at the margins of the wings [92]. The Notch protein, a 300-kD single-pass transmembrane receptor [93], is a critical regulator of many cellular processes, so many that the expression "all scientists are working on Notch, they just do not know about it yet", has been coined. Notch signaling is an evolutionarily conserved mechanism that is used by metazoans (from sea urchins to humans) [94, 95] to control cell fates through local cell interactions, e.g. to sort out the distance between hairs or feather primordia [96, 97] or to determine precursor cell fates in the sensory organs of the developing fruit fly via asymmetrical cell divisions, reviewed in [98]. In flies the ligands for the Notch receptor are termed Delta and Serrate. Mammals display a more complicated line-up with five ligands: Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged-1 (Jag1), and Jagged-2 (Jag2). Among these components of the Notch pathway, Notch1 and Notch4, as well as Dll1, Dll4, Jag1, and Jag2 are expressed by endothelial cells [99-101]. The Notch ligands are cell-surface proteins with multiple tandem epidermal growth factor (EGF) repeats in their extracellular domains. Ligand binding to the Notch receptor of a neighboring cell triggers several proteolytic cleavages of Notch, including one by the γ-secretase, residing in the transmembrane domain, releasing the Notch intracellular domain (NICD) from the cell membrane, which in turn translocates to the nucleus and directly interacts with the transcription factor CSL, initiating transcription of target genes such as *Hes*, *Hey* and *Nrarp*, reviewed in [102, 103]. With four receptors and five ligands (all with tissue specific and partly overlapping expression), at least three steps of proteolytic cleavage to release the NICD, sugar modifications of Notch – which affect ligand-binding properties [104], the picture of Notch signaling becomes almost overwhelmingly complex. Furthermore, endocytosis of Notch ligands in the signal-sending cell is important for signaling [105, 106] and recently, it was shown that endocytosis of Notch itself, and subsequent processing in early endosomes and multivesicular bodies, affects Notch signaling [107]. In addition, there are several intracellular regulators of Notch activity including Numb, Numb-like and Lethal giant larvae 1, enhancing or inhibiting Notch activity, reviewed in [103, 108]. As if this was not enough – and it apparently is not – there is evidence that the Notch signal might be bi-directional, i.e. that the Delta-expressing cell might receive instructions as well [109]. Finally, very recently, additional twenty-three Notch regulators were identified through a genome wide screen, using a transgenic RNAi library targeting 88% of the protein-coding genes in fruit flies [110].

In order to form a functional vascular network, stable yet adaptive, regulation of proliferation, differentiation, apoptosis and polarity is required. One key regulator of this

process is VEGF which drives proliferation and migration of endothelial cells to sites of hypoxia. However, one question quickly arises; how does the network become optimally dense? We and others found that Notch signaling is instrumental in this process (**II**, [111-115]). Using transcription profiling of mouse blood vessels, we identified a cluster of genes implicated in Alzheimer's disease, including components of the γ-secretase protein complex, responsible for the processing of presenilin but also cleavage of the NICD. Given the role of Notch in tracheal development [10], we tested the effect of γ-secretase inhibitors *in vivo* and found a dramatic increase in blood vessel sprouting, branching, and fusion (Figure 1 in **II**). This effect was due to a perturbed balance between endothelial tip and stalk cells, where reduced Notch signaling leads to increased number of tip cells and vice versa (**II),** and reviewed in[102]. Via modulation of the Notch signal using various genetic models, including mice heterozygous for Dll4, and a tamoxifen-inducible EC-specific Notch1 knockout, we could show that Notch signaling plays a critical role in sprouting angiogenesis in the mouse. Others have shown that inhibition of Notch signaling drives tumor angiogenesis, which, quite counter-intuitively, leads to decreased tumor growth, likely due to impaired perfusion of immature vessels [116, 117]. Recently it was also shown, in a loss- and gain-of-function study, that Jagged1 is a pro-angiogenic factor, which likely competes with Dll4 for binding the Notch 1 receptor [118].

From these studies we have learned that Dll4, Notch1 and Jag1 are used by endothelial cells to sort out which ones will become tip cells – spearheading the nascent angiogenic sprout – and which ones will become trailing, lumen forming, stalk cells. However, we do lack an understanding of the dynamics of this process. For example, judging from our studies (**II**) and those of others [99, 113], where RNA *in situ* hybridization and transgenic GFP markers have been used, Dll4 is expressed in ECs in the front of the forming vascular plexus, but also more proximally. Furthermore, it is expressed in both tip and stalk cells. There are however quantitative differences in Dll4 expression, it being high in arteries and in the front of the plexus. From this expression pattern it is hard to understand exactly *which* cells, at any particular time point, that are signaling, and we are left to interpret the effects of our experiments partly blindfolded due to technical limitations. For example, the GFP protein has a half-life of twelve hours and is therefore not suitable for tracking cellular events with a fast course.

As mentioned previously, in order for blood vessels to form lumen, migrate, branch and fuse in a three-dimensional tissue, a tight control of cell polarity is likely required. The key cell polarity regulator Lethal giant larvae (Lgl1) is known to affect Notch signaling, and Lgl1 knockout mice display hemorrhages in the brain possibly indicating defective angiogenesis [119]. However, these effects could be secondary to the severe hyperplasia of neural progenitor cells seen in these mice. In order to investigate the role of polarity in angiogenesis, and any effect Lgl1 might exert – in part by modulation of the Delta-Notch signaling pathway – I have generated mice with EC-specific targeting of Lgl1, and they display vascular abnormalities (unpublished data).

Finally, Notch mutations have been shown to cause human vascular disease. For example, mutations in the Notch pathway lead to the late onset inheritable genetic disease; cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which is caused by mutations in the Notch3 gene [120]. Furthermore, Alagilles syndrome, a dominantly inherited multisystem disorder, has been

linked to mutations in the *JAG1* gene. Patients with Alagille syndrome display i.a. pulmonary artery stenosis, and a predisposition to intracranial bleeding [121].

3.3 REVERSE AND CHEMICAL GENETIC SCREENS IDENTIFY ANGIOGENESIS REGULATORS (ARTICLE III)

The major hurdle in the era of whole genome approaches is not a lack of interesting genes, but rather how to quickly and reliably validate their function, and thereby transform sequence information and preliminary observations into knowledge. The ultimate tool in terms of generating qualitative validation data is the knockout mouse. However, this tool is not apt to handle the vast number of genes generated from your average microarray experiment. Thus, a semi-high throughput validation system would be valuable as an intermediate step before initiating the time consuming, and costly plain old lab-work required for making and analyzing a genetically tailored mouse model. Possibly it is the lack of such a tool, or just an inability to pose unique biological questions, that is reflected in the angiogenesis research field's enthusiasm for digging in the same spot, a spot constituted of, in chronological order; VEGF and TGFβ biology, Ang-Tie proteins, followed by Semaphorins, Netrins and other neurobiology related molecules, in turn followed by Notch, and lately the Wnts.

We wanted to identify novel regulators of angiogenesis and quickly, with sufficient degree of biological relevance, validate their function. Therefore, we combined reverse genetic (RG) and chemical genetic (CG) screens, using several vertebrate models including mouse and zebrafish. Transcript profiling in mice was used to identify 150 potentially druggable microvessel-enriched gene products. Orthologs of fifty of these were knocked down in a RG screen in zebrafish, demonstrating that sixteen were necessary for developmental angiogenesis. In parallel, 1280 drugs – or drug-like compounds – were screened in a human cell-based assay, identifying twenty-eight compounds selectively inhibiting sprouting angiogenesis (**III**). The use of a chemical library adds a dimension in this discovery attempt, namely the power to identify: i) a role for already known drug targets in angiogenesis; and ii) compounds with off-target effects on angiogenesis. Semi-high throughput validation systems have previously been utilized in various areas including angiogenesis [122-125], and the combination of reverse and chemical genetics has been performed previously in both worms and flies [126, 127], but as far as I know this was the first attempt in vertebrates.

When analyzing the resulting list of genes and compounds, the obvious finding was the identification, in both the RG and the CG screen, of genes encoding serine/threonine (S/T) protein phosphatases (PPs): *ppp1ca*, *ppp1cc* and *ppp4a*. This class of molecules catalyzes dephosphorylation on phospho-serine and phospho-threonine protein residues and reverses the effect of the approximately 400 S/T kinases present in the genome [128]. In the field of angiogenesis much attention has been paid to the role of kinases, in particular RTKs. Therefore, it was interesting to find a role for phosphatases, albeit the phosphatases were *S/T* PPs. There are five known gene families of S/T PPs: PP1 (PPP1), PP2A (PPP2)[sometimes PP4 and PP6 are also included in the PP2A family], PP2B (PPP3), PP5 (PPP5), and PP7 (PPP7) [129, 130]. PP1 consists of four isoforms, derived from three genes; *PPP1CA*, *PPP1CB*, *PPP1CC*, which are differentially expressed in

mammalian tissues. These few PP1 catalytic subunits are highly abundant, do not exist as free monomers in eukaryotic cells, and each perform multiple cellular functions. Furthermore, the catalytic subunits are fairly nondiscriminating as to their substrates. S/T PPs, being multimeric enzymes, are assembled from a small number of catalytic subunits combining hundreds of regulatory subunits. It is these regulators, rather than the catalytic subunits, that provide the essential determinants for cellular localization, substrate specificity, and degree of phosphatase activity, reviewed in [131]. Among the regulating subunits for PP1 we find the retinoblastoma protein, CDC25, SRp38, and SEN1. Currently there are over 100 known mammalian proteins that interact with PP1, reviewed in [132]. Interestingly, regulating subunits expressed in the vasculature have been identified, and include PHI-1 (PPP1R14B), which is highly expressed in ECs and smooth muscle [133], TGF-beta-inhibited membrane-associated protein (TIMAP or PPP1R16B), which is expressed at high levels in ECs, and capable of interacting with moesin, a protein important for actin rearrangement [134, 135]. Furthermore, EC permeability is controlled by activation of the myosin light chain phophatase (MLCP or PPP1R12B) through recruitment of PP1δ and the myosin phosphatase targeting subunit (MYPT1 or PPP1R12A) [136]. Regarding cellular effects of S/T PPs signaling – relevant for angiogenesis – PP1 has been implicated in regulation of the duration of the TGFβ signal [137], but also, via its interactions with moesin, of filopodia extension [138, 139]. In order to characterize the mechanism behind the effect of PP1 on angiogenesis, Jeff Essner co-author of **III**, is currently performing in-depth studies of zebrafish vasculature in his laboratory. He has observed that knockdown of *ppp1ca* and *ppp4c* in zebrafish leads to impaired perfusion and dilated or enlarged vessel diameter (J. J. Essner, personal communication).

An interesting finding in **III,** relating to phosphatases, is the *kia1274* gene, currently predicted as a tyrosine phosphatase. This gene was selected for further validation based on the results in **III** and has now been knocked out in mice resulting in a very strong vascular defect (Wallgard and Hellström, personal communication). As previously mentioned, the roles of many RTKs in angiogenesis have been characterized, but so far there has been less focus on phosphatases.

Besides the PPs, another overlap between the RG and CG screens was the identification of regulators of prostaglandin and leukotriene biosynthesis. Knockdown of either of the genes *alox5ap* and *ppap2a* resulted in vascular defects. Furthermore, the drug Nimesulide, inhibiting cyclooxygenase 2 (COX 2) – an enzyme that catalyzes synthesis of prostaglandin from arachidonic acid, and the drug Budesonide, which suppresses the synthesis of both prostaglandin and leukotrienes via activation of the glucocorticoid receptor, were identified as anti-angiogenic. COX 2 has previously been shown to regulate angiogenesis *in vitro* [140], and *in vivo* [141]. Moreover, COX 2 is regulated by VEGF stimulation [142]. Interestingly, continuous use of non steroidal anti-inflammatory drugs (NSAID) has been shown to decrease the risk for e.g. colon cancer [143], and this reduction could in part be due to the anti-angiogenic properties of NSAIDs.

In the RG screen we also identified several GTPases, including Rab5c and Rab11a, both of which are implicated in regulation of endocytosis [144]. This process is important for cell fate, motility, and signal propagation in many different types of membrane-bound receptors including Notch [145], RTKs (including VEGF receptors) [146, 147], Wnts

[148], and GPCRs [149]. As described previously, Notch, VEGFR and GPCRs have all been implicated in angiogenesis, and it is thus not far-fetched that regulators of endocytosis could affect angiogenesis. Moreover, Rab5 has been implicated in lumen formation in Drosophila [150].

We also identified *frizzled6* (*fzd6*) in the RG screen, a gene which has been knocked out in mice with a resulting hair patterning defect [151], but for which no vascular defect has been reported. However, *FZD6* gene expression in endothelial cells has been confirmed by others [152]. One possible explanation for the lack of a vascular defect in the *Fzd6* knockout mice is that there is a redundancy of Fzd-receptors in ECs. Wnt-signaling over FZD-receptors has been implicated in axon guidance and planar cell polarity – mechanisms which might be at play in angiogenesis. In fact, manipulation of Wnt signaling has been shown to affect vascular formation. For example, gene targeting of *Fzd4* in mice, leads to defects in retinal angiogenesis [153], and FZD4 has been linked to familial exudative vitreoretinopathy (FEVR), a hereditary ocular disorder characterized by a failure of peripheral retinal vascularization [154]. Wnt-signaling has also been implicated in blood-brain barrier formation [155].

In **3.2**, I briefly mentioned a tentative role for polarity regulation in angiogenesis. Polarity complexes like Scribble (Scribble, Discs large, Lethal giant larvae), Par (Par 3, Par 6 and aPKC), Crumbs (Crb/Pals/Patj) and core PCP complexes (i.a. Frizzled, Dishevelled, Flamingo, Diego), reviewed in [156-159], communicate intracellular and extracellular cues through reorganization of the cytoskeleton, and through trafficking of membrane and protein. Important for establishment and maintenance of polarity are also GTPases, such as Cdc42 and Rac1, reviewed in [160-162]. The GTPases identified in the RG screen could either be involved in regulation of polarity, or simply reflect the need of ECs for cytoskeletal rearrangements when extending filopodia during angiogenesis.

3.4 CHEMICAL SCREEN FOR ANTI-ANGIOGENIC DRUGS IDENTIFIES PERHEXILINE (ARTICLE IV)

There are many examples where a drug developed for one indication shows efficacy for another indication, e.g. beta-blockers – originally intended for treatment of arrhythmia – reduce blood pressure, Minoxidil – originally intended for high blood pressure – is effective against hair loss [163], Thalidomide – originally a sedative drug – is used to treat cutaneous manifestation of leprosy [164], and the combination of NSAIDs and Imitrex for migraine [165] (NSAIDs enhance the beneficial effects of Imitrex on migraine). In the future, our increased understanding of human biology will likely allow more 'indication switching', a process that is very cost-efficient from a socio-economic perspective. For example, the cost of the new anti-angiogenic drug Avastin™ is between USD 4000-9000 per month and patient, which is obviously more than old off-patent drugs are sold for (typically USD 10 per month and patient).

In an attempt to identify already existing drugs or drug-like compounds with anti-angiogenic properties, we screened 880 compounds in a three-dimensional cellular assay deploying human ECs, and identified Perhexiline maleate as an anti-angiogenic drug *in vitro* and *in vivo*, with a capacity to complement the inhibitory effect of VEGFR TKIs on tumor

angiogenesis (**IV**). Perhexiline was launched by the American company Richardson-Merrell in the 1970s for the treatment of angina pectoris, but later withdrawn due to severe hepatotoxicity and neurotoxicity in some patients. It was later shown that affected individuals were poor metabolizers of Perhexiline, due to mutations in their cytochrome *P450 2D6* gene, which led to accumulation of toxic levels of the drug. Perhexiline is still in use in New Zeeland and Australia, where patients undergo genetic screening before treatment and plasma levels of Perhexiline are continuously monitored [166].

There are several possible explanations for the effects observed in **IV**. Perhexiline is a lipophilic agent which may lead to interactions with the cell membrane, in turn interfering with ligand binding and internalization of signaling receptors important for angiogenesis. Perhexiline has also lysosomotropic properties, i.e. it enters lysosome where conditions are acidic, gets protonated and trapped. Perhexiline has been shown to affect endocytosis in liver cells [167], and one can speculate that by targeting the lysosome, it perturbs the endocytic machinery of the ECs or prevents ligand dissociation from key angiogenesis receptors, which ultimately affects intracellular signaling and vascular growth.

In terms of molecular targets, Perhexiline is known to affect several proteins including the enzymes Carnitine palmitoyltransferase 1 and 2 (CPT1/2) – responsible for fatty acid transport into the mitochondrion, L-type Ca^{2+} ion channels, and K^{+} ion channels. We have been able to exclude that the anti-angiogenic effects are due to inhibition of CPT1/2 since other CPT1/2-inhibitors, tested in our *in vitro* system, do not affect angiogenic sprouting. Furthermore, given the fact that Perhexiline has an effect on endocytosis, and that endocytic trafficking is critical for many cell signaling systems including Notch, RTKs (including VEGFRs), Wnts, and GPCRs (see 3.3 for references), the observed effects might stem from interference with these signaling pathways – all implicated in angiogenesis. Furthermore, endocytosis has been implicated in cell motility [168], obviously a key process in angiogenesis. For instance, in the fruit fly ovary, highly migratory border cells use two RTKs; EGFR and PVR (PDGF/VEGF receptor) to read guidance cues, and the spatial localization of RTK-signaling, within these migrating cells, is actively controlled by endocytosis, shuttling the receptors that interact with guidance cues to specific regions of the plasma membrane [169]. Out of the signaling pathways known to affect angiogenesis we have so far only assessed the effect of Perhexiline on the VEGFR2, and found that pre-treatment of cells with Perhexiline reduces phosphorylation of the receptor (**IV**).

In addition to an inhibitory effect on the tumor vasculature, Perhexiline might also have a direct inhibitory effect on the tumor cells themselves. In fact, Perhexiline has been shown to have an inhibitory effect on human colon tumor cell proliferation *in vitro* [170], and in the Lewis lung carcinoma model we did observe an anti-tumor effect of Perhexiline as a single agent (**IV**). Interestingly, lysomotropic compounds are known to permeabilize the lysosomal membrane, which releases cathepsins (lysosomal proteases) into the cytoplasm. Once in the cytoplasm, the cathepsins digest vital proteins, and this might induce cell death, reviewed in [171]. Thus, perhexiline might have a double effect on tumor growth; through its anti-angiogenic properties, as well as via its cytotoxicity.

4 CONCLUSIONS

"There is always an easy solution to every human problem – neat, plausible and wrong."
H.L. Mencken, "the divine afflatus" A Mencken Chrestomathy (Knopf, NY, 1949).

The work described herein was aimed at further increasing our understanding on how angiogenesis is modulated. To summarize: i) RGS5, a novel marker for pericytes, was discovered; ii) the mechanism through which Notch signaling affects angiogenesis was elucidated; iii) Sixteen genes and thirty-one chemical compounds modulating angiogenesis were identified, and a role for S/T PPs in angiogenesis was pin-pointed; iv) the angina pectoris drug Perhexiline was identified as an anti-angiogenic, and anti-tumoral drug, inhibiting VEGFR2 signaling.

In conclusion, it is clear to me from these studies of angiogenesis, a process ultimately aimed at generating a functional vascular system (which is much more than just a collection of tubes of various diameter for the transport of fluids), that this is an almost incomprehensibly complex process, requiring both temporal and spatial control of matter such as proliferation, differentiation, polarity, junction formation and adherence to neighboring cells and the matrix, regulation of permeability, cell fate decisions and division of labor between different cell types. Moreover, this is all orchestrated via thousands of genes; expressed, spliced, translated, glycosylated, exported, degraded, and regulated by yet other genes, and we learned recently, short interfering RNA embedded in the genetic code (as if it was not complex enough). Nonetheless, blood vessels and their formation is complex but the real marvel is obviously the brain – *le grand Chef du spectacle* – of which complexity I dare not to ponder in order to remain sane.

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Bertrand Russell

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