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IMAGING VASCULAR DEVELOPMENT IN ZEBRAFISH (DANIO RERIO)

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Cover: Front: Mesodermal Cell Migration Tracks During Vasculogenesis.
Back: Cross Section of Rendered DA and PCV with Cell Tracks.

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PANTA RHEI

SO LONG, AND THANKS FOR ALL THE FISH
(D. ADAMS)

TO MY FAMILY

ABSTRACT

Formation of a functional vascular network is a prerequisite for physiological organ function in vertebrates. Pathological vascularization furthermore plays a role in several human diseases, such as diabetic retinopathy, cardiovascular disease and cancer. Understanding the mechanisms governing embryonic vascular development may give more insight into pathological vascularization. Vascular development has classically been subdivided into two subcategories: (1) vasculogenesis - the *de novo* creation of vessels from angioblast precursors and (2) angiogenesis - the creation of blood vessels from preexisting vessels. Although a large number of studies have been performed on vascular development, several biological mechanisms behind both angio- and vasculogenesis remain unresolved.

The aim of Paper I was to analyze the role of Angiomotin-like protein 1 (AmotL1) in developmental angiogenesis. We showed that AmotL1 is an important mediator of endothelial cell junction formation *in vivo*.

In Paper II, we show (*in vivo, ex vivo* and *in vitro)* that Shingosine-1-phosphate receptor 1 (S1PR1) is a critical negative regulator of angiogenic sprouting. S1PR1 loss of function causes endothelial hypersprouting both in mouse and zebrafish, whereas activation inhibits sprouting and enhances cellular adhesion. This article shows how a blood borne signal (S1P) induces vascular stabilization via S1PR1, junctional VE-cadherin and inhibition of VEGFR2 signaling.

In Paper III, individual cell tracking of precursors originating in the LPM confirmed that precursors migrate to the midline in two waves in accordance with cell identity. Arterial-venous specification appears to occur in the lateral plate mesoderm (LPM), whereupon sprinting (fast migrating) precursors migrate to dorsal positions at the midline – forming the dorsal aorta (DA). Although the bulk of asymmetrical divisions presumably occurs in the LPM, some hemangioblasts continue to divide asymmetrically at least once during axial vessel formation.

LIST OF PUBLICATIONS

I. **Angiomotin-like protein 1 controls endothelial polarity and junction stability during sprouting angiogenesis.** Zheng Y, Vertuani S, Nyström S, Audebert S, Meijer I, Tegnebratt T, Borg JP,Uhlén P, Majumdar A, Holmgren L.. Circ Res. 2009 Jul 31;105(3):260-70.

II. **The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2.** Gaengel K, Niaudet C, Hagikura K, Laviña B, Muhl L, Hofmann JJ, Ebarasi L, Nyström S, Rymo S, Chen LL, Pang MF, Jin Y, Raschperger E, Roswall P, Schulte D, Benedito R, Larsson J, Hellström M, Fuxe J, Uhlén P, Adams R, Jakobsson L, Majumdar A, Vestweber D, Uv A, Betsholtz C. Dev Cell. 2012 Sep 11;23(3):587-99.

III. **Arterial-venous and hematopoietic specification during zebrafish vasculogenesis - differences in precursor migration speed, guidance and potency.** Nyström S, Majumdar A, Uhlén P, Holmgren L. 2014. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

2plsm	Two photon laser scanning microscopy
A/V	arterial-venous
AGM	Aorta–gonad–mesonephros
AJ	Adherens junction
ALM	Anterior Lateral Mesoderm
AMOT	Angiomotin
AmotL1	Angiomotin Like 1
AmotL2	Angiomotin Like 2
DA	Dorsal Aorta
Dll4	Delta-like 4
EC	Endothelial Cell
Etsrp/Etv2/ER71	Ets-related factor (Etv2/ER71 orthologue)
Fli	Friend of Leukemia
Flt1	Vegfr1
Flt4	Vegfr3
Hey	Hairy-and-enhancer-of-split related
Hpf	Hours Post Fertilization
ICM	Inner Cell Mass
Kd	Knock down
Kdr	Vegfr2/kdrb
Kdrl	Vegfr4/ Flk1/kdra
KO	Knock out
LPM	Lateral Plate Mesoderm
MO	Morpholino
NA	Numerical Apperture
PCV	Posterior Cardinal Vein
PDGF-B	Platelet Derived Growth Factor-B
PDGFRβ	Platelet derived Growth Factor receptor β
PLM	Posterior Lateral Mesoderm
PTU	1-phenyl-2-thiourea
ROI	Region of interest
S1PR1	Shingosine-1-phosphate receptor 1
Som	Somite (stage)
Sphk	Sphingosine Kinase
Ti:Sapphire	Titanium Sapphire
TJ	Tight Junction
Tnnt2	Cardiac Troponin II (*Silent Heart* mutation)
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor

1 INTRODUCTION

1.1 VASCULAR DEVELOPMENT

The development of an organism is a highly regulated affair involving numerous steps that ensure the final formation of cells, supporting tissue and organs that make up the fully grown individual. The formation of a vascular system is crucial for ensuring that the growing embryo reaches its oxygen and nutrient requirements and thus, in mammals the cardiovascular system is the first functional organ system to form [1]. As organisms became larger and more complex during evolution, the need arose for a nutrient and oxygen delivery system capable of supplying cells located far away from the surface oxygen source [2]. Invertebrate arthropods and mollusks have a circulatory system lined with cardioblasts which is open to the coelom and interstitium but closed towards the outside and separated from the gastrointestinal tract. In other invertebrates like the amphioxus and annelids, the mesothelially lined circulatory system is anatomically separated (closed) from the coelomic cavity. The closed circulatory system of vertebrates is composed of a hierarchical network of tubes lined with endothelial cells ([3] and reviewed in [4]). Lining the walls of the vascular tubes are endothelial cells (ECs) resting on a basal membrane that recruits supportive mural cells to the tube periphery. These mural cell - vascular smooth muscle cells (vSMCs) or pericytes - regulate EC function and stabilize vessel integrity [5]. Common to the aforementioned organisms is the basement membrane and that blood flow is generated through contraction of muscle cells (myoepithelial or cardiac muscle cells).

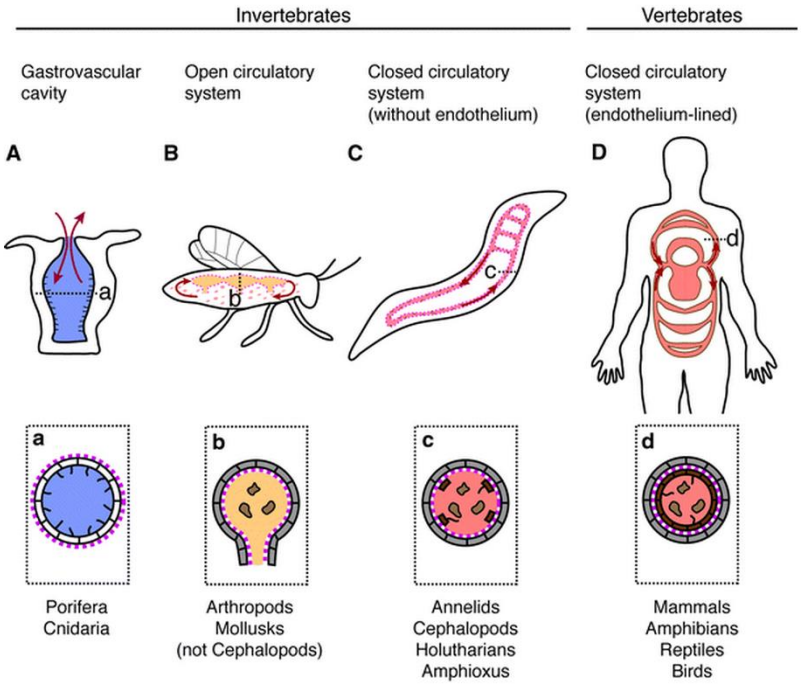

Figure 1. Circulatory systems in multicellular organisms. [4] Cellular and Molecular Life Sciences © Springer Basel AG 2010 ; 10.1007/s00018-010-0400-0

During vertebrate development, blood vessels are initially formed through vasculogenesis (Figure 2A) – the *de novo* formation of vessels from precursor cells. Subsequent vascular formation occurs via so called angiogenesis (Figure 2B), whereby endothelial tip cells migrate towards an extracellular gradient of vascular endothelial growth factor (VEGF).

Experiments in chicken performed in the first half of the 20th century showed that blood cells and vessels are derived from common progenitors. These progenitor cells were dubbed 'angioblasts' by Sabin (Sabin, 1917 reprinted as [6]) and later 'hemangioblasts' by Murray [7]. In mice, the cells of the vascular system originate from mesodermal precursors initially appearing in the extraembryonic yolk sac. These form blood islands – cell aggregates where the outer cells differentiate into endothelial cells and the inner cells differentiate into primitive hematopoietic cells. Blood islands subsequently fuse, forming a vascular plexus [8–11]. Intraembryonic angioblasts form the ventral and dorsal aortae, vitelline arteries (surrounded by multiple layers of smooth muscle) and veins (surrounded by a thinner layer of smooth muscle) [8]. After initial vasculogenesis, angiogenic endothelial cell sprouts branch out from the larger vessels, anastomosing with other vessels and form the branched network that is the vascular system. The lumen of capillaries and venules are associated with mesoderm derived pericytes (rather than vSMCs) that regulate permeability and contractility [8,12,13].

The formation of branched tubular networks is a genetically controlled process involving initial tube formation, elongation, branching and fusion. Although not exactly identical, several aspects of vascular development are highly conserved between zebrafish and higher vertebrates (mice and humans). Blood vessel pathology lies at the 'heart' of several human diseases – such as cardiovascular disease and cancer. Whereas *de* novo formation of blood vessels is uncommon in healthy adults, the growth and maintenance of solid tumors relies on the formation of a tumor associated blood supply – thus providing a target for anti-cancer therapy. Understanding the mechanisms governing vascular development and biology will inevitably provide the basis for novel therapeutic considerations in a range of diseases involving blood vessel pathology.

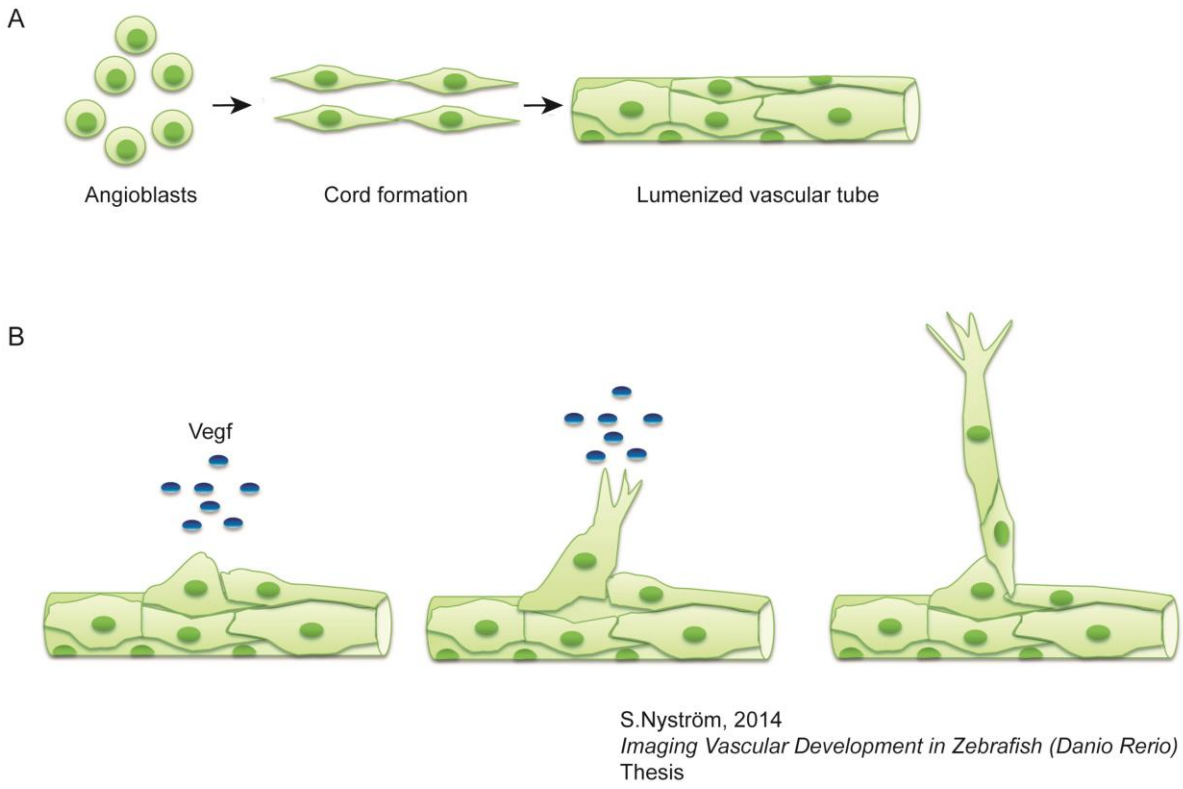

Figure 2. (A) Vasculogenesis – the formation of vascular tubes from precursor cells. **(B)** Sprouting angiogenesis. A tip cell leaves the larger vessel, migrating towards a chemokine gradient. The tip cell is followed by stalk cells that maintain connections to the vessel of origin.

1.1.1 Vascular Endothelial Growth Factor (VEGF)

1.1.1.1 VEGF ligands

During angiogenesis, a gradient of soluble VEGF guide sprouting vessels through binding to VEGF receptors expressed on leading branch cells. VEGF stimulates EC migration through local actin polymerization in the direction of the VEGF gradient [14]. There are several subtypes and isoforms of VEGF and VEGF receptors. Humans have 5 VEGF family members - VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF.

In zebrafish, there are two VEGF-A orthologues and as of yet no VEGF-B orthologue has been identified. The importance of VEGF family members in blood vessel formation is supported by evidence from mice VEGF gene knockouts. Both VEGF-A ($^{+/-}$)([15],[16]) and VEGF-C ($^{-/-}$) [17] deletions in mouse are embryonic lethal. Heterozygous Vegfa ($^{+/-}$) causes severe EC differentiation and tubulogenesis defects. Homozygous VEGF-B ($^{-/-}$) deletion is viable but have cardiac and coronary vessel abnormalities [18]. Finally, VEGF-D ($^{-/-}$) knockouts only exhibit minor pulmonary lymphatic aberrations [19]. Not only do these studies show that developmental blood vessel formation is dependent on functional VEGF expression, but also that embryonic vascular development is governed by genetically conserved control mechanisms. VEGF-A has several human isoforms (Vegf121, Vegf145, Vegf165, Vegf189, and Vegf206) [20–22]. Zebrafish express VEGF-A orthologues from two separate genetic loci that each express two separate isoforms. VegfAa (chromosome 16) give rise to VegfAa121 and VegfAa165 [23,24]isoforms, and the

VegfAb locus (chromosome 4) produces the isoforms VegfAb171 and VegfAb210 [25]. Vegf121 and vegf165 overexpression is involved in EC differentiation and hematopoiesis. Maternal VegfAa mRNA is present at embryonic 1-4 cell stage and the endogenous VegfAa expression subsequently varies depending on developmental stage - adjacent to the yolk at 12 hpf and in the hypochord starting at 15-16 hpf ([23,26]). Hypochord VegfAa expression is furthermore dependent on the presence of the notochord, indicating an early interplay between neuronal and vascular developmental circuits. In the brain, VegfAa is expressed in cells flanking the anterior forebrain and hindbrain [23]. Overexpression of both VegfAa isoforms induces ectopic vessel formation [23], indicating that VegfAa functions as pro-angiogenic factor through action on zebrafish endothelial cells. Vegfa overexpression also results in higher arterial marker expression in the PCV and downregulation of venous markers in the DA [27].

VegfAa is furthermore thought to stimulate both endothelial and hematopoietic lineages, since overexpression of both VegfAa121 and -165 isoforms increases expression of endothelial (Kdrl/Vegfr4 and tie1) and hematopoietic (scl and gata1) markers [23]. According to Nasevicius et al, although proper axial vessel formation is requires VegfAa - initial axial vessel patterning is independent of VegfAa signaling [28]. VegfAb knock-down affects ISV formation and induces vessel leakiness at around 2-3 dpf [25]. VegfAa might be the predominant form acting on ISV formation, since VegfAb knockdown predominantly affects anterior ISV formation and since simultaneous knock-down of VegfAa and –Ab phenocopies VegfAa morphants ([25,28]).

In summary, the zebrafish VegfA orthologues (VegfAa and VegfAb) control sprouting angiogenesis and vascular integrity. Only VegfAa modulates vasculo- and hematopoiesis in addition to angiogenesis. In addition to its role during lymphangiogenesis, VegfA and –C acts synergistically on angiogenesis *in vitro*. Mammalian VegfC was classically believed to signal through VegfR3 (Flt4), but is now believed to also act on VegfR2 (Kdr) possibly through VegfR2 and -3 dimerization ([29,30]). The zebrafish VegfC orthologue, located on chromosome 1, is expressed in the hypochord at 18 hpf and the lateral DA and at the mid/hindbrain boundary at 24hpf. Intersegmental vessel formation is mediated in part by VegfC, possibly via VegfC – kdrl/vegf4 binding [31]. Vegfc - Flt4/Vegfr3 mediated angiogenesis (dorsal sprout formation) is modulated by the notch ligand dll4, possibly by suppressing VegfC - Flt4/Vegfr3 driven intersegmental artery (but not vein) formation [32]. To avoid confusion, VegfAa will henceforth be referred to as VegfA.

1.1.1.2 VEGF receptors

VEGF binds to type III tyrosine kinase receptors (VEGFR1-3).

In mammals, VEGF-A binds to VEGFR1 (Flt1) and VEGFR2 (Kdr/flk1). VEGF-B binds to VEGFR-1. Both VEGF-C and –D binds to VEGFR-3 (Flt-4). Of the three receptors, VEGFR2 and VEGFR1 are considered to be the most important for blood vessel formation and VEGFR3 signaling is primarily responsible for lymphangiogesis. Neuropilins furthermore function as VEGF cofactors. Zebrafish have one VEGFR1 (Flt1), two VEGFR2 (Kdra and Kdrb) and one VEGFR3 (Flt-4) orthologue. In mammals, VEGFR1 is the receptor for its cognate ligand VEGFC. Mammalian VEGFR1 is considered to inhibit angiogenesis by acting as a VEGF sink - thus competing with pro-angiogenic VEGFA-VEGFR2 binding. VEGFR-1 knockout mice exhibit hyperproliferation, an effect of VEGFA/R2 disinhibition.

In the past, there has been some confusion on the classification of zebrafish VEGF receptor orthologues. Initially, only one VEGFR2 orthologue was found (Flk-1/Kdra) on chromosome 4. It later became apparent that there were two VEGFR2 orthologues in zebrafish – kdra (Chr4) and kdrb (Chr 20). Covassin argued that kdra is in reality more closely related to VEGFR1 phylogenetically and may have evolved as a functional orthologue of mammalian VEGFR2 [30]. Others argue that kdra actually represent a fourth class (Vegfr4) of vertebrate VEGF receptor and should therefore be annotated as vegfr4/kdr-like(kdrl) rather than flk1/kdra. According to the present nomenclature, there are four zebrafish VEGF receptors – Flt1/Vegfr1, Kdr/Vegfr2 (kdrb), Flt4/Vegfr3 and Kdrl/Vegfr4 (kdra) ([33–35]). Both Kdrl/Vegfr4 and Kdr/Vegfr2 are expressed in all endothelial cells and are activated downstream of VEGF [25]. During early vascular development – Kdr/Vegfr2, Flt4/Vegfr3 and Kdrl/Vegfr4 are co-expressed in most blood vessels.

The zebrafish Flt1/Vegfr1 receptor is located on chromosome 24 and gives rise to a membrane bound (mFlt1) and a soluble (sFlt1) isoform. Krueger et al characterized Flt1 expression and knockdown phenotype [34]. The Flt1 isoforms are differentially expressed during development. Expression of membrane bound (mFlt1) variant does not change significantly from 24-48 hpf, whereas sFlt1 expression increases to peak around 30hpf. At 30hpf, mFlt1 is expressed in DA, CV and segmental sprouts whereas sFlt1 is not expressed in the cardinal vein. Knockdown of Flt1/Vegfr1 affects angiogenic dorsal sprout formation but does not perturb vasculogenic axial vessel formation. Translational blocking of both isoforms results in excessive dorsal sprout branching past the parachordal level, abnormal inter-sprout connections, increased numbers of sprout stalks/tip cells as well as increased filopodial activity. Flt1/Vegfr1 overexpression accordingly inhibits intersegmental vessel sprouting. Flt1/Vegfr1 negatively regulates tip cell differentiation and branching in part via Notch signaling. It is also possible that as in mice, Flt1/Vegfr1 expressed at the base of the stalk cells inhibits lateral or retrograde branching independently of notch.

Of the three mammalian receptors, VEGFR-2 has been most extensively studied in relation to vascular development. VEGFR-2 signals through proteins with Src domains, such as PIP3 and PLCγ1 and downstream signaling elicits survival, proliferation, migration and increased permeability. PLCγ1 / DAG / IP3R / Ca^{2+} signaling induces EC proliferation via PKC and the MAPK pathway ([36], [37]). PI3K and Src regulate EC permeability, actin remodeling as well as acting in a pro-survival fashion via inhibition of the pro-apoptotic proteins Caspase-9 and Bad. Directional endothelial cell migration along the soluble VEGF gradient may be regulated through secondary messengers downstream of VEGFR2 converging on actin remodeling (PI3K/Src/PIP3/Rac), EC permeability (Rac and NO) and focal adhesion turnover (FAK/Paxillin) pathways.

Kdrl/Vegfr4 is expressed from 12 hpf in the lateral mesoderm, is found in the forming DA at 15-29 hpf and is expressed in the axial vessels as well as inter-somitic sprouts by 24 hpf. Kdrl is required for sprouting angiogenesis during the formation of intersegmental vessels. Although Kdrl is expressed in the axial vessels, isolated Kdrl knockdown affects angiogenesis - but not vasculogenesis or hematopoiesis [38]. Cytoplasmic or kinase domain mutations in Kdrl causes vascular development defects [38]. Kinase domain mutation (kinase dead mutant) causes partial dorsal sprout formation and abolishes DLAV formation [30].

Kdr/VegfR2 has been shown to be expressed in dorsal sprouts, in the DA but not in the PCV. Although Kdr can partially rescue Kdrl/Vegfr4 loss of function, Kdr may not be required for vascular development [30]. Kdr knockdown leaves hematopoiesis unaffected. Combined knockdown of Kdr and Kdrl caused incomplete DA / CV segregation, increases EC apoptosis and blocks formation of dorsal sprouts [39].

The zebrafish VEGFR3 orthologue Flt4 is located on chromosome 14 and produces one known transcript. At 24 hpf, the Flt4/Vegfr3 receptor is expressed in intersegmental sprouts and DA. DA expression subsequently becomes down-regulated ([30,32]). Signaling through Flt4 does not seem to contribute to arterial sprout formation, but morpholino knock down of Flt4 or Vegfc perturbs cranial vein (PHBC) formation [30] which indicates that Vegfc - Flt4 signaling modulates venous rather than arterial angiogenesis. VEGFC knockdown in Kdrl/Vegfr4 mutants reduces intersegmental vessel expression of the arterial marker efnb2a, whereas loss of Flt4 in the same background does not affect efnb2a expression. Although VEGFC and VEGFR3 are thought to primarily control lymphangiogenesis rather than angio/vasculogenesis, VEGFC has also been shown to signal through VEGFR2 [31] which could be explained by the fact that VEGFR1 can heterodimerize with VEGFR2 [29]. Such pathway cross-talk has been demonstrated also in Zebrafish [30].

In summary, VEGF ligands and receptors are crucial for proper morphogenesis of the vascular tree during zebrafish embryogenesis. During angiogenesis, migration of dorsal intersegmental vessels is positively regulated by attracting VegfA (predominantly VegfAa) binding to Kdrl/Vegfr4 and Kdr/Vegfr2 receptors but may also be regulated through VegfC - Flt4/Vegfr3 signaling. Although VegfC/Flt4 signaling is classically associated with lymphangiogenesis, VegfC and Flt4/Vegfr3 are also involved in angiogenic sprouting. At the moment, VEGF/R signaling remains the main target for anti-angiogenic therapy [40].

1.1.2 Cell migration and guidance

Cell migration and guidance are developmentally conserved program present in organisms as remote as humans, zebrafish (Danio rerio) and the fruitfly (Drosophila). Secreted and membrane-bound guidance cues (such as VEGF) bind to transmembrane receptors (e.g. VEGFR) on target cells that induce an intracellular signalling cascade - resulting in either migration in the direction of the chemokine gradient, or away from it in the case of repulsive signalling (e.g. Slit-Robo during neurogenesis). On a cellular level, migration occurs through the activity of Rho GTPases that modulate local myosin activity and actin polymerization / depolymerisation. RhoGTPase activity is regulated by RhoGEFs (guanine nucleotide exchange factors), RhoGAPs (GTPase-activating proteins) and RhoGDIs (Guanine Nucleotide Dissociation Inhibitors). RhoGTPases exist in either an inactive GDP-bound state or in an active GTP-bound state. When activate, RhoGTPases interact with downstream effectors. RhoGEFs activate the RhoGTPase by loading GTP. RhoGAPs catalyse hydrolysis of GTP to GDP inactivating the GTPase. RhoGDIs regulate local RhoGTPase activity through the sequestering of inactive RhoGTPases. Of the 20 known mammalian Rho proteins RhoA, Rac and Cdc42 have been characterized in detail [41].

1.1.3 Endothelial Cell Junctions

As described above, blood vessels are tubes with the inner lining consisting of endothelial cells facing the lumen of the vessel. Defects in vessel permeability can arise from aberrant mural cell coverage or faulty formation of inter-cellular junctions. Junctional permeability, stability, cell shape and polarity is regulated by several junction type specific proteins and the actin cytoskeleton [42]. Additionally fully functional cell junctions are critical during vascular lumen formation. Endothelial specific junctional proteins include VE-cadherin and VE-PTP located in adherens junctions and Claudin 5 which is found in tight junctions [43]. I will briefly describe the two junctional types prevalent in ECs, tight junctions (TJs) and adherens junctions (AJs).

1.1.3.1 Tight Junctions

TJs are located apically in EC - sealing the junctional cleft from the vascular lumen. TJs promote vascular maturation and maintenance. The main function of TJs is to restrict paracellular permeability between the lumen and the basolateral surface. TJ adhesion is maintained by e.g. Claudins, occludin, JAM family members and ESAM. Claudin 5 is the most important cell adhesive component of TJs. Intracellular proteins associating with claudins and JAM include Zo-1 and -2. TJs are especially important in controlling paracellular permeability in vessels located in the brain [44].

1.1.3.2 Adherens Junctions and VE-cadherin

The more basally located AJs also control paracellular permeability, with the addendum that they control blood vessel morphogenesis and stability. AJ adhesion is mediated by cadherins (VE-cadherin and N-cadherin) that bind to β-catenin, p120 and plakoglobin. Associated to the AJs are kinases, phosphatases (VE-PTP, SHP2 amongst others), actin binding proteins (such as α-catenin) and growth factor receptors such as VEGFR2/KDR). In mice, VE-cadherin KO is embryonic lethal at E9.5 due to vascular remodeling/maturation defects and VE-cadherin is important for maintaining vascular integrity [45,46]. VE-cadherin stabilization of growing vessels is thought to be achieved through antagonism of pro-migratory (i.e. destabilizing) VEGFR2 signaling [47]. Paracellular AJ permeability can be regulated through VE-cadherin phosphorylation (induced by VEGF, VE-PTP etc), VE-cadherin internalization or cleavage. In mice, VE-cadherin has furthermore been implicated in EC polarization maintenance and lumen formation [4849].Interestingly VE-cadherin is responsive to transcription factors important for vasculogenesis (such as TAL-1 and Ets-1), suggesting that VE-cadherin may modulate vasculogenesis. Murine VE-cadherin ablation does however not affect initial plexus formation.

1.2 ZEBRAFISH AXIAL VESSEL FORMATION AND PRIMITIVE HEMATOPOIESIS

Similar to chicken and the extraembryonic murine blood islands, zebrafish angioblasts (endothelial precursors) and primitive blood cells (primitive erythro- and myeloblasts) are derived from the lateral plate mesoderm (LPM) [50–54]

During the first 10 hours of zebrafish embryo development, the basic body plan is established through the formation of the antero-posterior and dorsal-ventral body axes, development of the three germ layers (ectoderm, endoderm and mesoderm) and progenitor specification (**Figure 3**). These processes are controlled by factors such as BMP, Nodal, FGF and Wnt-signaling [55–57].The outer ectodermal layer gives rise to the epidermis, nervous and sensory organs. The inner endodermal layer produces the GI tract and associated structures (reviewed in [56]). A soluble gradient of secreted Bmp family members is required for the induction of ventral mesoderm (high concentration) and dorsal ectoderm (low concentration) [58,59] as well as for the intra-mesodermal patterning of cells into notochord (dorsal), paraxial mesoderm (ventral) (forms the somites) and lateral plate mesoderm (ventral). BMP signaling furthermore controls the specification of mesoderm into anterior lateral plate mesoderm (ALM) (that forms the heart and pronephros) and posterior lateral mesoderm (PLM). At the end of this BMP controlled dorso-ventral patterning, the mesodermal precursors that will form the axial vessels and primitive hematopoietic cells are found as two bilateral stripes in the PLM [58,60].

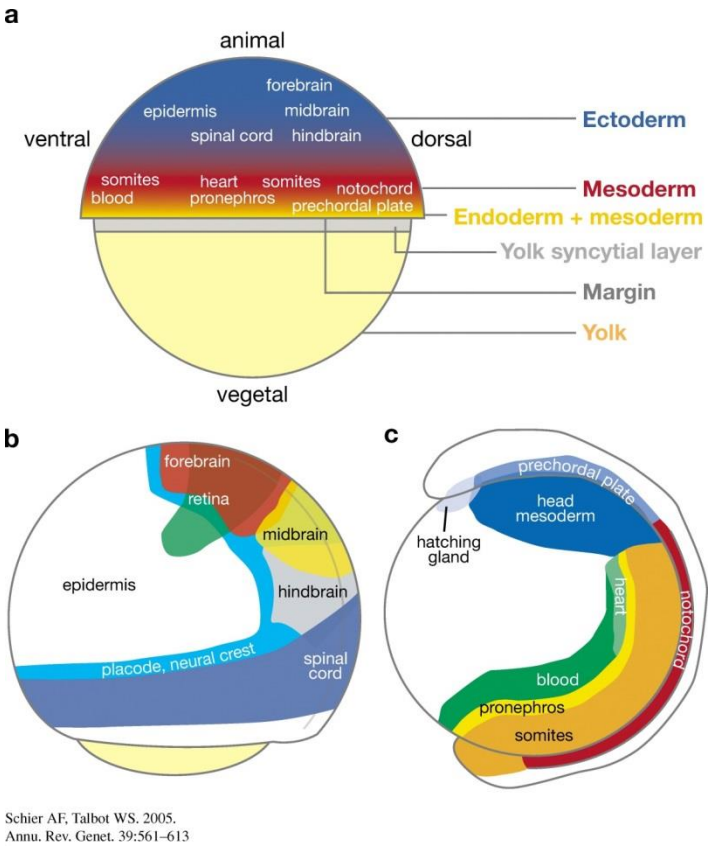

Figure 3. Zebrafish fate map (*Figure 3 in* [56]*).* **(A)** 50% epiboly (gastrulation onset). **(B)** Ectoderm fate map at 90% epipoly **(C)** Mesoderm fate map at early somite stage. (Schier AF, Talbot WS. Molecular genetics of axis formation in zebrafish. Annu. Rev. Genet. 2005;39:561–613.)

1.2.1 Vasculogenesis

Mesodermally derived angioblasts were shown to migrate medially from the LPM to the midline where they coalesce into a vascular cord called the inner cell mass (ICM). These angioblast precursors migrate medially as two temporo-spatially separated waves (see below) [61,62] ventral to the notochord and hypochord, dorsal of the endoderm and medial to the somites [63]. Angioblasts first form the DA, which is lumenized around 22 hpf, and subsequently the PCV at ventral positions to the DA [35,51,64]. When reaching their destinations – arterial and venous ECs elongate, flatten, form cell-cell contacts with neighboring ECs and subsequently the walls of the tubular lumen ensheathing mesodermally derived blood cells. In line with previously published results, we observe (data not shown) that the DA lumen forms prior to that of the PCV. At roughly 24 hpf, both axial vessels are formed and circulation initiates shortly thereafter [65].

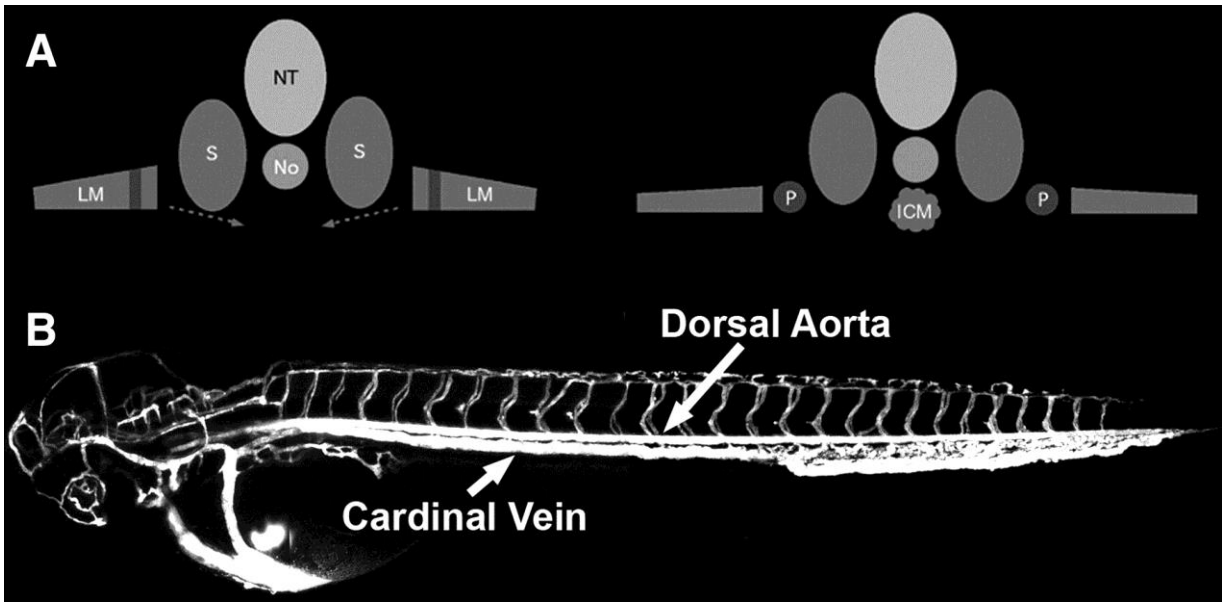

Figure 4. **(A)** Schematic cross section of the developing zebrafish trunk. LM=lateral mesoderm. S= Somite. No = Notochord. NT = neural tube**. (B)** 2.5 dpf (days post fertilization) embryo. (Swift MR, Weinstein BM. Arterial-venous specification during development. Circ. Res. 2009;104(5):576–88) [62].

1.2.2 Hematopoiesis

Similar to vasculogenesis, hematopoiesis has been described to occur in two waves. In the case of axial vessel formation these two waves describe the differential migration pattern of pre-arterial and pre-venous angioblasts towards the midline (see below). The hematopoietic waves on the other hand refers to primitive and definitive hematopoiesis, where primitive hematopoiesis occurs in the anterolateral mesoderm (ALM), the posterolateral mesoderm (PLM). Myeloid cells are derived from the ALM, whereas (nucleated) erythrocytes originate from the PLM [66–69]. In mice, the extraembryonic mesoderm has been shown to generate erythro/myeloid cells (in addition to ECs) at E7,5 [70,71]. The birthplace of definitive, second wave, hematopoiesis is the dorsal floor of the DA which is also referred to as the aorta–gonad–mesonephros (AGM) region [72–74]. There, hematopoietic stem cells (HSCs) are generated from

the so called haemogenic endothelium (arterial ECs) through a cell-division independent process [72–74]. HSCs enter circulation via the cardinal vein and proceed to populate the caudal hematopoietic tissue (CHT) (35-52 hpf), thymus (3 dpf) and kidney marrow (4-5 dpf) [75]. Interestingly, haemogenic endothelium is present also in mice – yielding HSCs that seed the fetal liver and bone marrow. Murine HSCs enter circulation via the arterial lumen (as opposed to the venous lumen in zebrafish) [76–79].

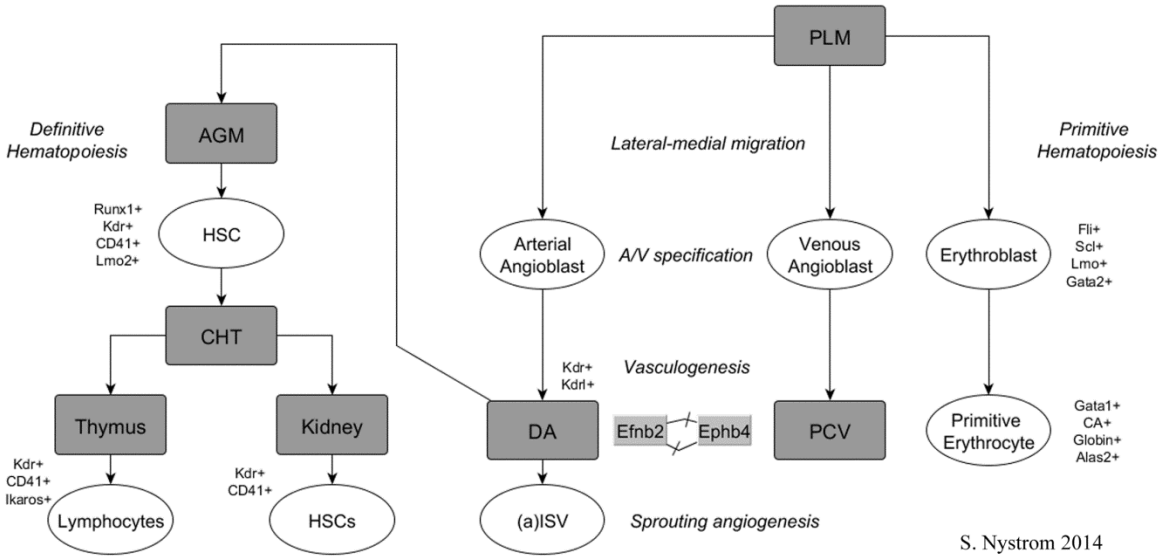

Figure 5. Vasculogenesis, angiogenesis and hematopoietic waves in zebrafish. Common origin for endothelial cells and hematopoietic cells.

1.2.3 Arterial-venous specification During Vasculogenesis

1.2.3.1 Pre-arterial and venous cells migrate as distinct populations to the midline

Lateral-medial angioblast migration from the LPM was previously described to occur in two waves. The first wave of angioblasts was observed to form the DA and the second wave was thought to construct the PCV (ventral to the DA) [80–82]. In 2009 conflicting data was published purporting that endothelial cells migrated ventrally from the DA prior to forming the PCV (rather than originating from the second wave of mesodermal precursors) and that arterial-venous specification occurs at the midline rather than in the LPM [83]. In a recently published article, the original observations were vindicated in that venous ECs originate from the second wave of angioblasts [84]. The authors concluded that angioblasts are organized in a medial and a lateral pool, where medial angioblasts become arterial (DA) and lateral venous (PCV) endothelial cells due to spatial proximity to VEGF (receiving a higher concentration of Vegf and Hh than the lateral blasts) [84]. It was however concluded that the actual A/V specification occurs at the midline. The exact timing of the arterial-venous fate decision is to date not clear and has been suggested to occur anywhere between 7 to 12 som (or about 13-15 hpf) [85] to 20 som [84].

In paper III, we confirm that PLM cells originating from the lateral plate migrate in two waves to the midline. First wave cells migrate to dorsal positions, whereas second wave cells migrate directly to ventral positions.

1.2.3.2 Angioblast migration and Arterial – Venous specification

1.2.3.2.1 Ephrin signaling vasculogenesis

Expression of the arterial marker EphrinB2 begins when precursor cells reach the midline. Both EphrinB2 and the Venous marker flt4 (VEGF3) can be detected as early as 25 som stage. The Ephrin receptor tyrosine kinases and their ligands (the ephrins) regulate morphogenesis and cell movements during development. Eph receptors and their ligands are transmembrane proteins that require cell-cell contacts in order to induce intracellular signalling events. Signalling can be bidirectional – i.e. can go through receptor to ligand and vice versa. Forward and reverse ephrin-Eph receptor signalling can in this way act both in a negative and positive fashion, eg. inhibiting or activating migration. EphrinB2 and EphB4 are differentially expressed in the arterial and venous endothelium in murine embryos prior to circulation onset [86,87]. Although EprhinB2^{+} (arterial) - EphB4^{+} (venous) signalling has been shown to modulate A/V differentiation [83], others have proposed that EphrinB2 and EphB4 expression may be thought of solely arterial/venous markers without a causal relationship with A/V differentiation[87].

1.2.3.2.2 Arterial Specification is Dependent on Shh, VEGF and Notch signaling

In sprouting angiogenesis, endothelial tip cells migrate towards an extracellular gradient of soluble VEGF [88] and has been shown to control arterial specification of angioblasts during vasculogenesis [62,84,89–92] First wave angioblasts express Kdrl/Vegfr4 and the transcription factors *fli1a* and *etsrp/etv2* [27,63,84].Vegfa expression from the somites is dependent on Sonic hedgehog (Shh) secreted from the notochord located in the ventral somites (ventral of the forming DA) [27]. Arterial marker expression in the Shh orthologue Sonic-you (*syu*) / Pinhead mutant is reduced and axial vessel formation is perturbed in *Syu* embryos - forming a single Flt4/Vegfr3^{+} vessel [60]. Through binding to Vegf receptors (Kdrl/Vegfr4 and Kdr/Vegfr2) on pre-endothelial cells (angioblasts), Vegfa activates Notch signaling which has been shown to induce arterial fate in angioblasts.[65,85,93,94] which has previously been shown to promote arterial specification [27,65,85,94]. First wave angioblasts express activated phosphorylated ERK (downstream of Vegf), which later becomes restricted to the arterial ECs [95]. Venous specification on the other hand is Vegfa independent [30,94].

From 10 to 20 som, both arterial (e.g. grl, efnb2a, cldn5b) [89,96,97] and venous (e.g. flt4)[98] markers have been reported to be expressed by first wave cells. According to Nasevicius et al, although proper axial vessel formation requires VegfAa - initial axial vessel patterning occurs independently of VegfAa signaling [28]. Isolated Kdr/Vegfr2 or Kdrl/Vegfr4 Kd is not sufficient to completely block vasculogenesis [38]. Only simultaneous knock down of Kdrl/Vegfr4 and Kdr/Vegfr2 results in incomplete DA/PCV segregation and the formation of a single axial vein [39].

Notch signaling, downstream of VEGF and Shh [27], is required for arterial specification both in mice and zebrafish [89,99]. The Notch transmembrane receptors interact with DSL (delta-serrate-lag2) ligands. Downstream targets of Notch in mammals include Jagged1, Jagged2, Hes and Hey [100]. Gridlock (grl) is the zebrafish orthologue of Hey2 (a transcriptional repressor). Grl is expressed in the PLM and expression later becomes restricted to the DA [85]. Mutants with

perturbed notch signalling such as mindbomb (*mib*) and gridlock (*grl*) exhibit perturbed DA formation and reduced expression of the arterial marker Ephrinb2a [89,97]which is expressed downstream of grl/hey2 [101]. Interestingly, even though midline Shh is absent in *syu* mutants – cells are recruited to the midline but fail to form two separated vessels. According to the current model, these embryos do not express Vegfa from the somites and Notch mediated arterial specification is absent. In addition to controlling Vegf expression, Shh induces somite and EC expression of Calcitonon receptor-like receptor alpha (Calcrla) that in turn upregulates Vegfa expression and can induce arterial differentiation cell-autonomously independent of VEGF [102,103].

Around 20 somite-stage, arterial markers are down-regulated in venous cells and Notch3 starts being expressed in the DA [89]. This has been suggested to be the time-point of A/V specification [84]. As mentioned above, [83] observed that a subpopulation of angioblasts migrate ventrally from the DA around 21 and 23 hpf where they form the PCV. These two findings contradict each other in that, according to [83] arterial and venous-fated angioblasts exist in a common vessel primordium whereas the evidence presented by [84] suggests that pre-arterial and pre-venous cells migrate as distinct populations towards the midline. In the ventral migration model, ephb4^{+} pre-venous cells migrate away (ventrally) from ephrinb2a^{+} arterial ECs.

1.2.3.2.3 Venous specification

Although arterial differentiation has been extensively characterized, not much is known about venous specification. In mice, the orphan nuclear receptor Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) promotes venous differentiation of ECs possibly via inhibition of Notch signaling [104] and COUP-TFII$^{-/-}$ mice are embryonic lethal at E9.5 exhibiting severe hemorrhaging, edema, enlarged blood vessels and aberrant cardinal vein formation [105]. Murine COUP-TFII additionally works together with the transcription factor Sox18 to induce Prox1 expression in cardinal vein ECs (E9.5) [106–108] that subsequently sprout dorsally in response to Vegfc/Flt4 signaling and form the first lymphatic structures of the embryo [17]. Zebrafish COUP-TFII (nuclear Receptor 2F2 or NR2F2) is reportedly restricted to venous EC in the axial vessels and MO mediated Kd was shown to exhibit vascular leakage, defective A/V segregation and ISV formation. Venous marker expression (flt4 and ephb4a) was down-regulated) in NR2F2 morphants and ECs were purportedly shifted to a venous fate (without an increase of arterial marker expression). In contrast to mice, NR2F2 may not inhibit notch signaling in zebrafish [109]. Recent data show that coup-TFII and Sox18 are not essential for lymphangiogenesis [108].

1.2.3.2.4 Summary

In summary, although VEGF is crucial for proper A/V specification it is unclear whether or not Vegfa induces a fate switch in the lateral plate or, conversely, if Shh – Vegfa – Notch mediated arterial differentiation occurs only at the midline. The exact temporospatial pattern of angioblast migration is still debated. We (Paper III) and [84] show that pre-arterial and pre-venous precursors migrate as distinct populations to the midline, whereas others [35] support the model of ventral migration of venous ECs from a common precursor pool [83]. In the case of ventral migration, bi-directional Ephrin signaling purportedly controls segregation of the artery and vein. If vein formation is not reliant on ventral migration, the role of Ephrins could be to maintain A/V

identity through repulsive signaling (rather than playing a regulatory role in the initial phase of A/V patterning). Since Vegf signaling does not affect medial migration, it is

It has been shown that lateral-medial migration of angioblasts occurs in the absence of Shh, Vegfa, kdrl and kdr - although these mutants / MO treated zebrafish all exhibit perturbed A/V specification. Exactly what factors control lateral-medial migration is currently unknown. In chicken, FGF signaling is involved in angioblast induction and Hedgehog (Hh) for vasculogenesis To date, it is unknown whether or not FGF is required for angioblast specification in zebrafish. In Paper III, we show that arterial and venous angioblast migration speed differs. Although the endoderm is not required for axial vessel formation, endoderm derived factors have been shown to affect migration speed [82].

1.2.4 The Common Origin

As mentioned above, a common mesodermal origin for endothelial and hematopoietic cells was suggested in the early 20th century based on rudimentary descriptive analysis of development in chicken. (Sabin, 1917 reprinted as [6] and [7]. Bi-potential cells capable of producing endothelial and hematopoietic cells are referred to as "hemangioblasts".

Multiple ETS binding sites are present in all endothelial enhancers and promoters [110] such as in kdrl, fli1, tie2, tal1, VE-cadherin, gata2, PDGFRβ as Notch4. The transcription factor Tal1 (Scl) is required for zebrafish hematopoiesis and, although not *required* is suspected of playing a role also in endothelial differentiation [111]. GATA2 is thought to regulate hematopoietic and endothelial development and has been suggested to be involved in progenitor specification [110]. The basic helix-loop-helix (bHLH) transcription factor Scl is required both for hematopoietic and endothelial development (DA formation) [111]. LMO, Tal1 and GATA2 (all three together) activate VE-cadherin.On top of the transcriptional network lies *fli1* (Friend of leukemia factor I), an Ets transcription factor. Fli1 is expressed in endothelial and hematopoietic precursors residing in the LPM. Fli1 is expressed in *cloche* mutants (in which differentiated ECs are abolished and primitive hematopoiesis perturbed) that lack expression of other EC markers such as Tal1, Gata2 and Tie2 [51,60,112], which implies that Fli1 functions early during vasculo- and primitive hematopoiesis. It has been shown that Fli1 acts upstream of Tal1 and Gata2 in hemangiopoiesis. Fli1 and Gata2 function upstream of other EC markers such as Tal1, Lmo2 and Kdrl [113]. Lack of Fli1 does not disrupt axial vessel formation. This may be due to functional redundancy between Fli1 and other factors such as Fli1b. It has been suggested that Fli1 might be more relevant for primitive hematopoiesis than for endothelial differentiation [110]. The transcription factor Etv2 (Ets-related factor) is expressed very early during murine vasculogenesis (completely absent at E10.5). Both vasculogenesis and hematopoiesis is severely perturbed in Etv2 $^{-/-}$ mice , lacking any detectable vessels, blood islands or angioblasts and lack expression of endothelial Flk1, Pecam and Tie-2 [114]. Etsrp is the zebrafish orthologue of Etv2. Etsrp mutant (*y11*) of which Kd embryos exhibit defective vascular development [115]. It is thought that Etv2/Etsrp is one of the more downstream genes controlling EC development and Etv2 is required for the expression of endothelial genes such as kdrl and seems to be required for angioblast specification [84].

In zebrafish - Fli1^{+}/Etv2(Etsrp)$^{+}$/Scl^{+}/Gata^{+}/Lmo2^{+} cells are found both in the ALM and the PLM [53,69,116,117]. LPM cells express markers common to both blood and endothelium corroborates the theory that endothelial and primitive hematopoietic cells originate from a common precursor pool of mesodermal cells in the lateral plate. Another strong argument is that mutants such as *cloche* [51,116] and *spadetail* affect both the endothelial and hematopoietic lineages [51]. Cultured mesodermal cell can furthermore produce both endothelial and hematopoietic cells [118]. One question is whether bi-potentiality is retained during vasculogenesis or not. A large degree of notch dependent proliferation has been observed in the LPM, implying that asymmetrically dividing cells may not be required once the precursors have left the lateral plate. In mouse and chicken, hemangioblasts occurrence was infrequent during vascular development [119,120]. In zebrafish, it has previously been shown that the majority of ventrolateral mesoderm cell divisions give rise to daughter cells of the same lineage [120]. In paper III we show that (albeit less frequent) asymmetrical division does occur during vasculogenesis.

1.3 ZEBRAFISH ANGIOGENESIS

1.3.1 From the Dorsal Aorta

After the axial vessels are initially formed - around the time the DA is lumenized (22 hpf) - endothelial cells break off from the cells forming the dorsal left/right aspects of the nascent tube and sprout dorsally forming the (arterial) intersomitic vessels (a)ISVs (also called segmental arteries(SAs)) [121,122]. The intersegmental (arterial) sprouts migrate dorsally, where they anastomose and form the dorsal longitudinal anastomotic vessels (DLAVs). Like in vasculogenesis and primitive hematopoiesis, sprouting angiogenesis from the axial vessels can be divided into two 'waves'. The first wave entails the formation of the SAs-/(a)ISVs, whereas during the second wave (32 hpf) - vessels sprout from the PCV that become either segmental veins (SVs / (v)ISVs) or form the parachordal lymphangioblasts [122] (that subsequently form lymphatic vasculature[32]). SAs/(a)ISVs consists of 3-4 cells during the sprouting process and later around 4-6 cells [123].

Dorsal migration is led by endothelial tip cells driven by Vegf (Vegfaa, -ab and –c) [25,27,28] followed by stalk cells that maintain connections to the DA. Tip and stalk cell selection is regulated by delta-like 4 (Dll4)–Notch signaling (reviewed in [124]). Vegf binds Vegf receptors that induces Dll4 expression in tip cells (higher than in stalk cells) [125], which in turn inhibits sprouting of adjacent stalk cells via Notch. Notch signaling increases expression of the decoy receptor Vegfr1/Flt1 in adjacent cells - limiting the Vegfr2 (kdr/kdrl in zebrafish) mediated pro-migratory effects of VEGF. Notch signaling is furthermore decreased in neighboring cells, further suppressing tip-cell behaviour. In Paper III, we argue that pre-arterial cells are specified in the LPM but show that pre-arterial cells are seemingly randomly dispersed in the LPM. It is possible that a similar mechanism selects precursor tip cells and (inhibits) neighboring blasts cells prior to lateral-medial migration (reviewed in [35]). Angiogenic sprouting is further antagonized via Sema-Plexin mediated upregulation of sflt1 [126]. Ablation of flt4/Vegfr3 has no effect on SA/aISV formation (modulated by high arterial Dll4 levels), but embryos fail to form SV/vISVs and lymphangiogenic sprouts.

1.3.2 Hindbrain angiogenesis

In paper II, 2 photon laser-scanning microscopy (2plsm) imaging was used to investigate the function of S1P/S1PR1 signaling during hindbrain angiogenesis. The hindbrain receives blood flow predominately from the basilar artery (BA) which also perfuses the brainstem and cerebellum. During early development (between 24 and 60 hpf), zebrafish hindbrain blood supply is provided by the basilar artery (BA), central arteries (CtAs), bilateral primordial hindbrain channels PHBCs and posterior communicating segments (PCSs). The PHBCs form through vasculogenesis from mesoderm derived angioblasts to the lateral dorsal aortae (LDAs) around 24 hpf [127]. The BA may form through a combination of angiogenesis and vasculogenesis from endothelial cells originating from the PHBCs [127] The CtAs on the other hand are formed in a stereotyped fashion via sprouting angiogenesis. CtAs sprout from the bilateral PHBCs and anastomose with the BA (caudal) and the PCSs (rostral). CtA sprouting begins around 32-36 hpf [128] The CtAs become fully formed around 60-72 hpf [127] [128]. BA, PHBC and CtA formation is VEGF signaling dependent [127] [128]. CtA patterning occurs in the absence of blood flow (silent heart mutant) although these vessels are thinner and may lack correctly formed lumen [127].

1.4 SPHINGOSINE-1-PHOSPHATE RECEPTOR S1PR1

Shingosine-1-phosphate receptor 1 (S1PR1) belongs to a family of G-protein coupled receptors that bind the phosphorylated bioactive form of sphingosine - Shingosine-1-phosphate (S1P). Sphingosine is phosphorylated by sphingosine kinase (SPHK). Plasma S1P produced by endothelial cells and platelets bind S1P receptors expressed in endothelial cells, of which S1PR1 is the most important during vascular development [129–131]. It has previously been shown that S1PR signaling is involved in the regulation of vascular permeability, recruitment of mural cells as well as lymphocyte trafficking [132,133]. S1P deficient mice (Sphk1/2 double KO) have previously been shown to be embryonic lethal, exhibiting defects in neurogenesis (neural tube closure) and vascular development [134]. Complete S1P ablation results in embryonic lethality around E11,5-13,5. The vascular phenotype seen in these mice was vessel dilatation, hemorrhaging and reduced cSMC coverage of the DA. Analysis of S1PR1-3 knockout mice showed a certain degree of functional redundancy between the receptors – as the vascular phenotype in double and triple KO mice is much more severe phenotype than in single KOs. It was suggested that S1PRs are required for vSMC coverage and that in the absence of S1PRs (especially S1PR1) embryos die as a result of deficient vSMCs coverage of large vessels [131]. Platelet derived growth factor (PDGF)-B binding to PDGF receptor beta (PDGFRβ) recruits stromal vSMCs/pericytes to brain capillaries. Correct pericyte and vSMC function is required during vascular development [135].

1.5 ANGIOMOTIN FAMILY

1.5.1 Angiomotin Family

Angiomotin (Amot) was originally discovered by the Holmgren group when conducting a yeast-two-hybrid screen for novel Angiostatin binding receptors [136]. Amot was expressed in vascular endothelial expression and had pro-migratory effects in transfected ECs [136] - hence the name. The membrane associated Amot protein is a member of the Angiomotin family of proteins which, in all, consists of angiomotin (Amot) (Troyanovsky, Levchenko, Månsson, Matvijenko, & Holmgren, 2001, angiomotin-like 1 (AmotL1) [137,138] and angiomotin-like 2 (Amotl2) [139,140] [141]. For each member there are two known splice variants [142]. Common to all isoforms is that they contain a coiled-coil domain and a PDZ-binding domain. Lacking from the shorter isoforms is the PPXY-motif [137,139,143]. PDZ binding motifs are involved in membrane protein localization through interaction with PDZ domain containing proteins [144]. In zebrafish, the Amot PDZ-binding motif is required for Amot-mediated effects on endothelial cell migration and cell survival [145–147]. The PDZ motif functions differentially in p80 and p130 Amot (required for tight junction (TJ) localization in the former, but not the latter) [148]. The N-terminal domain contains a PPXY motif known to interact with WW domains. The Amot N-terminal region is required for localizing the p130 isoform to TJs [143].

Proper Amot function is required during mouse embryogenesis. Amot knockout (KO) in mice results in 95% embryonic lethality between embryonic day E11-E11,5. Knock out embryos displayed vascular defects such as interosmitic vascular insufficiency and brain vessel dilatation. The remaining 5% Amot KO embryos, however, survived without any vascular defects [149]. Amot controls cell migration and tight junction formation (via its PDZ-binding motif and N-terminal region). Whereas the shorter Amot (p80) isoform induces EC migration, the longer (p130) reduces migration and stabilizes actin fibres. Angiomotin (and not the other Amot family members) binds angiostatin (for which reason the protein was discovered in the first place (see above). All Amot family members (Amot, AmotL1 and AmotL2) bind to the RhoGEF Syx. Syx1 associates with Mupp1, Pals1 and Lin7, indicating that one of them connects Syx1 and angiomotin [147]. PatJ/Mupp1 is a scaffold protein with 10 PDZ domains known to be involved in TJ formation, cell polarization and directional migration. In the absence of Syx (Syx-b morpholino mediated knock down), ISV sprouting angiogenesis is perturbed – vessels arrested/delayed at the horizontal myoseptum. Patj has been found to associate with Amot in a Y2H screen [147]. Amot, and not the other family members was found to associate with Pals2, Filamin A and PTN13. Amot may bind directly to Syx, Pals1, Patj and Mupp1[147], whereas Pals2 does not bind directly to Amot but through a ternary complex [147]. Lack of Amot leads to a reduction of directional EC migration through the Patj/Mupp1/Syx1 complex [136,147,150].Amot family members have furthermore been shown to regulate cell polarity through the Par3 and Crb3 polarity protein complexes. The Crb3 complex is enriched in epithelial cell TJs and may modulate TJ formation via Pals1 [151]. Interesting clinically is tha vaccination against Amot and treating with Amot targeting antibodies [152] has been shown to inhibit tumor angiogenesis and growth *in vivo* [152].

1.5.2 Angiomotin like protein 1

AmotL1 (originally named JEAP) was originally discovered due to its propensity for TJ association. As mentioned above, AmotL1 is a member of the Amot family and share a number of characteristics with its relatives. Like the other members, AmotL1 contains the glutamine-rich region, the coiled-coil domain and the PDZ binding motif [141,153]. Prior to conducting the experiments described in Paper I, the function of AmotL1 was largely unknown. It was also not known if AmotL1 had more than one isoform. In Paper I we discovered that AmotL1 (just like its siblings) has two isoforms. Furthermore, using the zebrafish embryo as our *in vivo* model, we showed that AmotL1 is involved in EC migration regulation and cell-cell contact mediated [adhesion] *in vivo* [154]. The main finding of Paper I was that although Amot and Amotl1 overlap functionally *in vitro* (migration and TJ formation), they differ *in vivo* in that Amot controls tip cell polarity whereas AmotL1 affects stalk cell junction stability.

2 METHODS

2.1 THE ZEBRAFISH MODEL SYSTEM

From an imaging perspective, the zebrafish (Danio Rerio) embryo is very well suited for studying developmental processes *in vivo*. From each coupling a large number of offspring are produced, which means that the zebrafish researcher has a lot of embryos at his or her disposal. As the embryos can be kept transparent using PTU(1-phenyl-2-thiourea)-treated E3 water, fluorescently tagged proteins can easily be visualized *live* without the need for post mortem analysis of tissue sections. The ability to visualize live events as they unfold during development means that conclusions from descriptive research and hypothesis testing can be performed with less bias than what is risked with post hoc analysis of for example immunofluorescently stained fixed tissue samples. Zebrafish researchers typically use transgenes (Tg) expressing proteins of interest or proteins expressed in cell types of interest. In papers I-III, we have used transgenic embryos expressing fluorescent proteins under the control of the fli promoter expressed in the endothelial and primitive hematopoietic lineage. Such transgenes have been widely used as they allow the researcher to visualize the vasculature at great detail during development. Furthermore, the embryo develops rapidly which allows for studying developmental processes in a relatively shorter time span than in higher vertebrates (such as mouse). Since the early zebrafish embryo is not absolutely reliant on oxygen delivery via the cardiovascular system, functional studies in genetically manipulated embryos can be performed for extended periods of time even if the gene of interest is required for proper vessel formation. In addition to the aforementioned positive aspects of using zebrafish, the majority of the known human disease-related genes in OMIM (the Online Mendelian Inheritance in Man database) are found in zebrafish as at least one orthologue [155]. It is also possible to follow cardiovascular mutant phenotypes for several days because oxygenation of the early zebrafish embryo does not rely on blood circulation. Furthermore, functional studies by forward and reverse genetics have shown that the molecular components that regulate vascular development are conserved between mammals and fish.

2.2 IMAGING TECHNIQUES

Briefly, confocal microscopy relies on exciting fluorophores with a laser and then filtering out emission coming from areas outside the focal plane using a pinhole. Confocal imaging is used to generate high resolution images at low depth (z), the resolution diminishes quickly with increasing z distance due to increased light scattering which is filtered out by the pinhole. This is a problem when imaging thick tissues/embryos, as light scattering blurs the image. When imaging living organisms, prolonged laser light exposure increases the level of phototoxicity. Although common to both one- and two-photon microscopy, phototoxicity is an issue especially in the former. It is crucial to take phototoxicity into account when imaging living cells/tissue/embryos so that the researcher does not inadvertently cause effects that are falsely interpreted as results.

Why use live imaging? When analyzing knock down or mutant phenotypes of vascular genes in mice, one issue for the researcher is that the absence of genes/proteins important that are crucial for vascular development usually results in embryonic lethality due to lack of proper tissue

oxygenation. This provides the researcher with important information in the sense that the gene/protein is important for vascular development but limits the ability to draw conclusions about gene function.

Two-photon laser scanning microscopy (2plsm) excitation efficiency relies on the temporal and spatial distribution of the exciting light. In other words, the exciting light pulses need to be close to each other in space and time in order for the excitation event to take place. The higher the NA of the lens, the higher spatial density can be achieved. High temporal density is generated by pulses below the picosecond range. Transition is extremely unlikely at other intensities [156]. In contrast to confocal microscopy, where energy absorption occurs within the entire excitation light cone - two photon absorption occurs mainly in the perifocal region and no out-of focus signal (noise) is generated (which is the reason that no pinhole is needed to filter out out-of focus emission)[157]. In confocal microscopy, the excitation wavelength is typically between 400-650nm. Two-photon microscopy relies on the combined quantum energy of more than one photon, which means that the excitation energy is lower - ie excitation occurs at longer wavelengths (700-1,1000nm wavelengths) than the energies of the emitted photons. These longer wavelength penetrate deeper than light with shorter wavelengths and only non-scattered photons generate signal in the focal volume [158]. For these reasons 2plsm imaging has a higher depth penetration, higher signal-to-noise ratio (SNR) and is less phototoxic than one-photon confocal microscopy – making 2plsm the superior choice for live imaging (compared to one-photon confocal microscopy).

2.3 IMAGE PROCESSING

For papers II and III, data obtained through live *in vivo* imaging was post- processed using Imaris (Bitplane). For paper III – cell tracking in Imaris was performed by detecting individual cells and associated tracks. Although powerful, the software based detection algorithms routinely failed to identify cell tracks properly, which meant that extensive manual cell detection and track editing had to be performed. To reduce noise and increase spot and track detection efficiency, timelapse data stacks were deconvoluted using Autoquant X3.

3 AIMS, RESULTS AND DISCUSSION

3.1 PAPER I

"Angiomotin-Like Protein 1 Controls Endothelial Polarity and Junction Stability During Sprouting Angiogenesis"

The **aim** of this paper was to characterize the function of AmotL1 during sprouting angiogenesis, in itself and in relation to Angiomotin.

Angiomotin (Amot) is a membrane associated coiled-coil protein expressed in endothelial cells that was discovered by the Holmgren lab in a yeast-two-hybrid screen for novel Angiostatin binding proteins. Amot is essential for endothelial cell migration during mouse embryogenesis. 95% if Amot KO (knockout) mice are embryonic lethal, whereas 5% of Amot KO mice survive without any detectable vascular defects. Other members of the Amot family include Angiomotin-like 1 (Amot1) and Angiomotin-like 2 (Amotl2).

In this paper, we identify a novel isoform of Amotl1. We show that, *in vitro* - Amotl1 and Amot have similar functions. *In vivo* - Amot controls tip cell polarity, whereas Amotl1 is important for ISV cell junction stability. In addition to the previously known 100 kDa Amotl1 protein (p100), we found a novel isoform (p90) expressed by endothelial cells (ECs) in vitro that differ from its sibling only in the N-terminus (Fig 1) region. *In vitro* immunofluorescence experiments (Fig 2) showed that Amotl1 and Amot is co-localized at tight junctions (TJs) - suggesting that they may interact directly in TJs. Immunoprecipitation experiments (Fig 3) indeed showed that Amot and Amotl1 form heterooligomers independent of the PDZ domain. Using immunofluorescence and the Boyden chamber migration assay, we showed that Amot1 is required for *in vitro* polarization and migration of MAE cells (Fig 4). From the CHO cell permeability assay (Fig 5) with Amot p90/p100 being either up-regulated (stably transfected) or inhibited (siRNA), we concluded that both Amot and Amotl1 are required for proper junctional function *in vitro*. The role of Amotl1 was subsequently investigated *in vivo* using zebrafish. ECs were isolated from Fli-EGFP transgenic 26 hour post fertilization embryos using FACS. Quantitative PCR showed that both Amot and the orthologue of Amotl1 are highly expressed in $EGFP^+$ endothelial cells compared to whole embryo (Figure 6). Using confocal microscopy (Fig 7), we demonstrated that morpholino-based knock down of Amotl1 and Amot perturbs sprouting angiogenesis of ISV from the dorsal aorta. Co-injection of Amotl1 and Amot Mo had a synergistically detrimental effect on ISV formation, suggesting overlapping roles of Amot and Amotl1. The ISV defects were rescued when co-injecting embryos with mouse Amotl1 p90 and Amot p80 mRNA, but not with Amothl1 p100 (Fig 7G). Claudin 5 staining and live 2plsm imaging showed that whereas Amot Kd results in polarization defects, Amotl1 Kd exhibited perturbed cell-cell junctions between stalk cells and DA (Figure 7D and Online Movies). Claudin 5 staining verified that Amotl1 is involved in ISV migration and junction stability.

Here, we report the identification of a novel isoform of AmotL1 that controls endothelial cell polarization and directional migration. Amotl1 knock down using siRNA reduced migration significantly in mouse aortic ECs. Amotl1 co-localizes with Amot to TJs, and combined siRNA

knock down of Amotl1 and Amot in MS-1 cells synergistically increases paracellular permeability. This indicates that both Amot and Amotl1 are required for proper TJ function. Zebrafish *in vivo* knock down of Amotl1 in resulted in inter-segmental vessel migratory defects and decreased junction stability between stalk cells and dorsal aorta (DA). Zebrafish Amot knockdown, however, affected tip cell polarization. Double knockdown of Amot and Amotl1 synergistically increased tip cell depolarization and decreased the connection between stalk cells and DA. Amot and Amotl1 have similar effects on EC migration and TJ formation *in vitro*, but *in vivo* – Amot controls tip cell polarity whereas Amotl1 mainly affects cell-cell junction stability.

Amotl1 knockdown had no effect on vasculogenesis, but perturbed dorsal angiogenic sprouting s from the horizontal myoseptum. We argue that there might be alternative pathways controlling initial migration up to the horizontal myoseptum that act independent of amotl1. In absence of Amot, sprouting tip cells arrested at the myoseptum and extended multi-directional filopodia (Figure 7D, paper I). In embryos lacking Amotl1, vessels arrested at the same anatomical location but exhibited less horizontal than in the *amot* knockdown embryos. Using live 2plsm imaging and Claudin-5 staining, we showed that in *amotl1* knockdown the connections between the intersegmental vessel stalk cells and the aorta appeared destabilized. We argued that the differences between Amot and Amotl1 *in vivo* could be due to temporal expression differences of the two proteins. Although both form complexes with Syx:PatJ/Mupp1, only Amot was known to form complexes with Pals2, filamin A and PTN13. It is possible that the effect of AmotL1 on migration is exerted through the control of cell polarity. AmotL1 associates with the Pals:Path:Lin7 polarity complex. Since AmotL1 forms a ternary complex with the RhoGEF Syx and PatJ/Mupp1[147], it is possible that Syx is the RhoA activity regulating effector through which AmotL1 regulates EC migration.

3.2 PAPER II

"The Sphingosine-1-Phosphate Receptor S1PR1 Restricts Sprouting Angiogenesis by Regulating the Interplay between VE-Cadherin and VEGFR2"

The **aim** of this paper was to characterize the role of S1PR1 in sprouting angiogenesis.

Since previous reports had indicated that S1pr1 ablated mice exhibited deficient vascular pericyte coverage [131], embryonic hindbrain angiogenesis was investigated to elucidate if the phenotype was due to aberrant pericyte recruitment (as opposed to problems with subsequent maintenance). The developing subventricular plexus of S1pr1 KO embryos was stained with endothelial (IsolectinB2) and pericyte (NG2) markers. We found that pericyte recruitment and coverage was actually unaffected at E11.5 (Figure 1), but that hindbrain vessels were "hypersprouting" - i.e. had an increased number of tip cells and filopodial protrusions. Hypersprouting was also observed in the retina where S1pr1 is expressed in ECs (as seen using retinal S1pr1-lacZ analysis). Hypersprouting, increased cell density and retained mural cell coverage was observed also in retinal EC specific S1pr1 gene ablation, which led us to conclude that S1PR1 acts cell autonomously. The hypothesis was that loss of S1PR1 induces a tip cell like phenotype in ECs. The previously reported loss of mural cells upon S1P depletion had been around the nascent aorta. Imaging immunofluorescently stained (IB4 (EC), NG2 (pericytes) and

βSMA (vSMCs)) tissue sections from S1pr1 KO embryos revealed defects in aorta formation between E11,5 and E13,5. Massive endothelial hyperplasia was observed around the aorta. Major aortic branches as well as the microvasculature surrounding the aorta were defective (ectopic connections to the aortic lumen in the latter). We confirmed the previously reported abnormal vSMC coverage of the aorta. The phenotype was shown to occur due to EC autonomous effects. Hyperbranching of the aorta was not seen in $Ramp2^{-/-}$, $Dll4^{+/-}$ or in EC specific RBP-J (Notch target) depleted embryos – excluding that the phenotype was the effect of systemic vascular abnormalities or involved in notch signaling. RT-QPCR analysis revealed that the other two S1P receptors (S1pr2 and S1pr3) were downregulated in $S1pr1^{-/-}$ mice. More importantly – the VE-cadherin target gene GAS1 was also downregulated – implying a crosstalk with not only the other S1P receptors but also with VE-cadherin. The phenotype seen in the genetic ablation experiments was repeated pharmacologically in retina and aortic ring explants (using the S1PR1 antagonist (R)-W146 and the agonist SEW2871). In a bead sprouting assay using HUVEC or mouse MS-1 coated fibrin gels, with or without added VEGF we observed that S1PR1 activation inhibited VEGF-induced sprouting, that S1PR1 antagonism increased EC sprouting and that the effects of S1PR1 were VEGF dependent. In summary, S1PR1 inhibition promotes VEGF-induced sprouting (in vivo, ex vivo, and in vitro) and S1PR1 activation promotes endothelial cells aggregation and assembly into cords - inhibiting VEGF-induced effects.

As in the absence of S1pr1; EC specific VE-cadherin ablation in the postnatal retina exhibited angiogenic hypersprouting, increased tip cell numbers, filopodia and vascular density. The phenotype was in fact even stronger in iEC-Cdh5KO than in iEC-S1pr1KO mice. S1PR1 was furthermore shown to regulate VE-cadherin at endothelial junctions. S1P is known to induce association of VE-cadherin to endothelial junctions, whereas VEGF destabilizes junctional VE-cadherin and triggers its internalization. Absence of S1PR1 decreased VE-cadherin in EC junctions and tip cells *in vivo* (retinal KO) and *in vitro* (siRNA mediated S1pr1 Kd). Whereas junctional VE-cadherin (in MS-1 cells) was increased upon S1P stimulation, it decreased when treating with VEGF. S1P or SEW2871 blocked the VEGF-mediated reduction of junctional VE-cadherin in MS-1 cells, indicating an antagonistic function of S1PR1 on VEGFR2. S1PR1 antagonism ((R)-W146) on the other hand potentiated VEGFA mediated VE-cadherin internalization. Since the amount of functional VE-cadherin (assayed using blocking antibodies) is critical for the anti-angiogenic S1PR1 effects, we concluded that VE-cadherin acts downstream of S1PR1.

My contribution to the paper was the live 2plsm imaging on zebrafish S1PR1, Sphk1, VE-cadherin (cdh5) and tnnt2 (Silent heart) morphant embryos. Endothelial hypersprouting had been observed in $S1pr1^{-/-}$ mice embryos. The initial aim was to investigate if S1PR1 knockdown affects hindbrain sprouting angiogenesis in a manner similar to what was seen in S1pr1 ablated mice. Initial quantification on fixed hindbrain isolates from S1PR1 Kd embryos had indicated that hypersprouting was present also in the zebrafish embryos. Tg(fli:eGFP)y1 zebrafish embryos were injected with morpholino (MO) oligonucleotides at one to two cell stage and imaged between 52-58 hpf. Embryos were dechorionated and anesthetized in PTU. Since were interested in imaging the hindbrain vessels, embryos were mounted in low melting agarose with the dorsal side facing the objective. Since the objective was to investigate the presence or absence of hypersprouting and get a snapshot of sprouting dynamics during this time period, fish were imaged for a relatively short time of 20 minutes at two minute intervals. This rather short time exposure allowed me to use a relatively high laser power without risking significant

phototoxicity, thus increasing SNR and being able to visualize the dynamics of small filopodia. I showed that the CtAs of both S1PR1 and VE-cadherin knockdown embryos exhibit vascular hypersprouting. Whereas CtAs in control embryos are fully lumenized with low filopodial activity, vessels in the knockdown embryos exhibit significant sprouting activity during the imaged time period. Similar results were obtained when knocking down sphk1 and silent heart (tnnt2) morphants (which lack circulation). These experiments demonstrated that S1PR1 and VE-cadherin knockdown produces a vascular hypersprouting phenotype as was observed in mouse. Importantly - co-injection of low-dose s1pr1 and cdh5 MOs phenocopied the high dose injections of each single morpholino, suggesting that S1PR1 and VE-cadherin act in a common pathway *in vivo* that regulate hindbrain angiogenesis.

In order to test if S1pr1 (*s1pr1*) and VE-cadherin (*cdh5*) interact on a molecular level, morpholinos at subtherapeutic concentrations were combined and phenocopied the high dose morpholino effects – suggesting a possible molecular interaction. In an *in vitro* sprouting assay, treatment with VE-cadherin blocking antibody increased angiogenic sprouting and enhanced the effects of S1PR1 antagonism. The blocking antibody furthermore counteracted pharmacological S1PR1 agonism (SEW2871). We concluded that S1PR1 and VE-cadherin cooperate to limit VEGF mediated sprouting angiogenesis and that VE-cadherin and junctional stability are critical downstream components in the negative regulation of angiogenic sprouting exerted by S1PR1. We also concluded that the angiogenic hypersprouting phenotype in absence of S1PR1 is VEGF driven. Additionally, we found that S1P may inhibit VEGF/R2 signaling, since stimulation decreases VEGF-induced phosphorylation of VEGFR2 as well as ERK1/2 and AKT (downstream of VEGFR2). Whether S1P inhibits of VEGF/R2 signaling directly or via VE-cadherin is not clear. In conclusion, we show that S1PR1 suppress endothelial hypersprouting through stabilization of junctional VE-cadherin and inhibition of VEGFR2 signaling. During vascular development, blood borne S1P may function to limit excessive VEGF dependent vascular sprouting.

As mentioned in the introduction, vessel stability is generally ensured by the basement membrane mural cells (vSMCs and pericytes). The phenotype (especially the perforated aortal wall) in S1pr1 KO embryos implied an underlying fault in mural cell recruitment or vessel coverage. Although we do confirm vSMC defect in S1pr1 KO embryos and observe a regional lack of vSMC markers (αSMA), we conclude that the main mechanism behind the phenotype is the vascular endothelial hyperplasia and excessive branching from the aorta and that S1P / S1PR1 signaling induces vascular stability in ECs in a mural cell independent manner. Worth noting (especially in the context of Paper III), is that the S1pr1 KO phenotype occurs after formation of the major vascular networks. Additionally, the aortic hyperbranching phenotype was not observed in genetically modified DLL4/Notch embryos – implying that S1P / S1pr1 functions after initial aortal vasculogenesis. In conclusion, we suggest that blood delivered S1P induces vascular stabilization of newly perfused vessels via S1pr1 mediated control of junctional VE-cadherin and inhibition of VEGFR2 signaling. Although zebrafish S1PR1 Kd embryos exhibit hindbrain hypersprouting, we did not detect any large perturbation's of the DA as is seen in the mouse. A more recent study showed that S1PR1 is enriched in the brain, which could explain why the phenotype we observed was outspoken in the hindbrain. It was however reported that S1PR1 Kd stunted ISV formation [159] which could present a similarity to effects in the larger vessels sprouting from the murine aorta as zebrafish intersegmental vessels initially sprout from the DA (and later are patterned into intersegmental veins/arteries). Excessive filopodial sprouting was furthermore seen in the caudal vein plexus (CaVP) [159].

3.3 PAPER III (MANUSCRIPT)

"Arterial-venous and hematopoietic specification during zebrafish vasculogenesis - differences in precursor migration speed, guidance and potency"

The **aim** of this paper was to study how the zebrafish axial vessels are formed from mesodermally derived precursors. Recently, seemingly conflicting results have been presented regarding the events during arterial-venous angioblast specification. Here we show that pre-arterial and pre-venous angioblasts differ in migration speed, guidance and potency. We corroborate recent findings that second wave (lagging) venous precursors migrate directly to ventral positions rather than sprouting ventrally from the population of first wave (sprinting) arterial ECs of the forming DA. We also find that while venous ECs observe left/right symmetry, arterial cells may not be restricted in such a manner. During the observed time period, the majority of cell divisions are symmetrical whereas a subset of cells divides asymmetrically - predominantly producing venous and hematopoietic fated daughter cells.

It is tempting to hypothesize that there is a population of bi-potential hemangioblasts that not only produce endothelial and primitive hematopoietic cells, but also produce definitive HSCs in the AGM. This has however not been demonstrated as of yet. The current understanding of definitive hematopoiesis is that $Runx1^{+}$ HSCs are generated from arterial ECs in the ventral floor of the DA. These are not generated through cell division, but rather a process called "lateral bending". What is not clear however is if the arterial ECs that generate HSCs divide asymmetrically prior to forming a component of the DA tube. In our experiments, the majority of asymmetrical divisions yielded predominantly venous ECs and primitive hematopoietic cells. The total number of observed asymmetrical divisions was however low and it is possible that further analysis may reveal a greater proportion of division producing arterial ECs and primitive hematopoietic daughter cells. If so, it would be interesting to see if asymmetrical division of pre-arterial angioblasts later give rise to definitive HSCs in the AGM.

Pre-arterial and pre-venous cells are seemingly stochastically dispersed in the LPM. Since the mesodermal cells segregate into a sprinting and a lagging pool, it seems likely that one or more chemokines are involved in separating the two populations. It is not completely clear whether or not Vegfa induces the actual A/V switch in the LPM or at the midline. The fact that pre-arterial blasts separate from pre-venous and hematopoietic cells in the lateral plate, indicates that arterial specification occurs in the LPM rather than at the midline.

The A/V switch could occur randomly, pre-arterial cells starting to express kdrl and possibly inhibiting arterial fate in neighboring cells through some type of lateral inhibition. This is perhaps less likely, since the number of cells migrating to the midline needs to stay relatively constant from embryo to embryo

From 10 to 20 somite stage, first wave cells have been reported to express both arterial and venous markers – suggesting that these precursors are not yet specified to an arterial fate and that A/V specification occurs at the midline rather than before. Vegfa would recruit not yet

specified immature Kdrl/Vegfr4^{+} angioblasts medially and A/V specification would occur at the midline [84]. Evidence against this theory is that from the onset of lateral-medial migration pre-arterial and pre-venous cell pools have several unique characteristics – differential adherence to left-right symmetry, migration speed, division symmetry, guidance along anterior-posterior and dorsal-ventral axes. Were it the case that A/V specification solely occurs at the midline, all angioblasts would migrate to the midline and separate into the two fates there – as suggested earlier [35,83]. If Vegf does indeed induce arterial fate in the LPM (rather than in the midline), it is not clear what determines that selection process. Our data disputes that angioblasts are divided into a medial (VEGF responsive) and a lateral pool. Kdrl/Vegfr4 is expressed from 12 hpf in the lateral mesoderm as well as in the nascent DA at 15-29 hpf. Kdr/Vegfr2 is also expressed in the DA, but not in the axial vein. The presence of Vegfa receptors in the lateral plate prior to lateral-medial migration indicates that Vegfa may function as a pro-migratory chemokine for arterial angioblasts. Opposing this explanation is that Vegfa has been shown to be important for A/V specification but not for initial vessel patterning [28] – although double knockdown of Kdr/Vegfr2 and Kdrl/Vegfr4 does perturb axial vessel formation.

Another explanation could be that second wave cells are not recruited dorsally because they are not yet fully differentiated endothelial cells. Only Vegfa responsive cells would be recruited to the dorsal midline, whereas unresponsive cells would migrate to ventral positions (attracted by other cues). The transcription factor Etv2 (angioblast specific) was recently reported to be differentially expressed in first and second wave cells [84]. We observed that the majority of observed asymmetrical cell divisions yielded one venous and one hematopoietic daughter cell, indicating that venous ECs may differentiate later than arterial cells.

Migration speed and guidance is another nut that needs to be cracked. It has been reported that the endoderm (although not required for lpm migration) might be involved in regulating mesodermal precursor migration speed. Why exactly venous cells seem to adhere to ipsilateral migration along the left-right axis, whereas arterial cell do not, is unclear. In zebrafish cardiac development, the left-right axis is translated into the dorso-ventral axis an what we see in vasculogenesis could be a similar process to that of cardiac formation.

4 GENERAL CONCLUSIONS

In papers I and II we analyzed proteins associated with cell-cell junctions. Paper II especially illustrates that it is not only mural cells that are important for maintenance of vascular integrity. During angiogenesis, the action of pro-angiogenic factors must be well balanced with stabilizing signals so that sturdy, lumenized vessels are allowed to form. In paper II we show how a blood borne signal (S1P) induces vascular stabilization via S1PR1 and junctional VE-cadherin and inhibition of VEGFR2 signaling.

Although much has been learned over the past 20 years about the events during mouse and zebrafish vasculogenesis, some aspects remain elusive. In paper III we observe that arterial-venous specification occurs in the LPM rather than at the midline. It is currently not completely understood what signals control lateromedial precursor migration. Our data seem to indicate that although there is in the hemangioblast a common origin for hematopoietic and endothelial cells during vasculogenesis, the majority of asymmetrical divisions probably occur in the LPM and that during vasculogenesis symmetrical cell division is used to rapidly expand the pools of primitive erythrocytes and angioblasts prior to circulation start. Interestingly, the data suggests that pre-venous cells retain bi-potentiality longer than the pre-arterial cells. This is in contrast to definitive hematopoiesis, known to spawn from arterial endothelial cells. To fully understand the events during zebrafish vasculogenesis, more studies need to be performed. It is surprising that although we know of many factors needed for arterial differentiation – little is known about factors driving venous specification.

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