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MECHANISMS OF MALIGNANT AND NON-MALIGNANT ANGIOGENESIS USING ZEBRAFISH MODELS

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Hr. Johannes Rudolph Dahl Hansen

ABSTRACT

Pathological angiogenesis significantly contribute to the onset, development and progression of most common and severe human diseases including cancer, metastatic disease, cardiovascular disease, age-related macular degeneration, diabetic retinopathy and retinopathy of prematurity. Under these pathological conditions, tissue hypoxia often acts as a trigger to switch on angiogenesis. However, there has been lacking non-invasive and clinically relevant animal models that allow us to study mechanisms of human diseases. Zebrafish, as a complementary animal model to mice, is a highly genetically and pharmacologically tractable vertebrate which is easily visualized during development. Zebrafish offers a unique opportunity to study angiogenesis under hypoxia. This thesis describes development and characterization of four novel zebrafish models in relation to hypoxia-induced angiogenesis, vascular and tumor pathology. Using these models, we demonstrate that hypoxia plays a causal role in development of retinopathy and cancer cell metastasis and thus provide important insights needed for the development of therapeutic approaches aimed at interfering with these processes. In paper I, we showed that hypoxia could induce neovascular retinopathy in zebrafish and this model is highly relevant to clinical retinopathy caused by diabetes. This zebrafish retinopathy model also allows us study the therapeutic potential of various antiangiogenic agents. In paper II, we demonstrate a novel principle that regulates blood perfusion in lymphatics as an effective defense against tissue hypoxia in zebrafish and kryptopterus bicirrhis. The arterial-lymphatic shunt is controlled by nitric oxide and the implication of this work is that NO-induced lymphatic perfusion might facilitate tumor cell spread from the blood stream into the lymphatic system. In paper III, we take advantage of the transparent nature of zebrafish embryos and availability of the transgenic strain *fli1:EGFP* to develop a zebrafish metastasis model. Using this model, we are the first to study the role of hypoxia in relation to angiogenesis in facilitating tumor cell dissemination, invasion and metastasis. To the best of our knowledge, this is the first animal model that allows scientists to study the early events of metastasis at a single cell level. In paper IV, We show that PI3 kinase is a key signaling component that mediates angiogenesis in the developing embryonic retina and in the regenerating adult fins. In conclusion, development of these zebrafish disease models have paved new avenues for studying mechanisms of pathological angiogenesis in malignant and non malignant diseases and offers unique opportunities for assessment of therapeutic potentials of known and novel drugs against these most common and lethal diseases.

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I. Cao R[*], **Jensen LD**[*], Söll I, Hauptmann G and Cao Y. Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy. PLoS ONE. 2008. 3(7):e2748

II. **Dahl Ejby Jensen L**, Cao R, Hedlund EM, Söll I, Lundberg JO, Hauptmann G, Steffensen JF and Cao Y. Nitric oxide permits hypoxia-induced lymphatic perfusion by controlling arterial-lymphatic conduits in zebrafish and glass catfish. Proc. Natl. Acad. Sci. U S A. 2009. 106(43):18408-13

III. Lee SLC[*], Rouhi P[*], **Jensen LD**, Zhang J, Ji H, Hauptmann G and Cao Y. Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invation and metastasis in a zebrafish tumor model. 2009. Proc. Natl. Acad. Sci. U S A. 106(46):19485-90

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RELATED PUBLICATIONS

V. **Jensen LD**, Cao R and Cao Y., In vivo angiogenesis and lymphangiogenesis models. Curr. Mol. Med. 2009. 9(8):982-91

VI. Rouhi P, Lee SLC, Cao Z, Hedlund EM, **Jensen LD** and Cao Y. Pathological angiogenesis facilitates tumor cell dissemination and metastasis. Cell Cycle. 2010. 9(5):913-7

VII. Rouhi P[*], **Jensen LD**[*], Cao Z[*], Hosaka K, Länne T, Wahlberg E, Steffensen JF and Cao Y. Hypoxia-induced metastasis model in embryonic zebrafish. Nat. Proc. 2010. In Press

VIII. Cao Z[*], **Jensen LD**[*], Rouhi P[*], Hosaka K, Länne T, Steffensen JF, Wahlberg E and Cao Y. Hypoxia-induced retinopathy model in adult zebrafish. Nat. Proc. 2010. In Press

IX. Wang Z[*], Xue Y[*], **Jensen LD**, Lim S, Ye X, Hedlund EM, Wu Y, Zhu Z, Cao R and Cao Y. PDGF-B modulates hematopoiesis and tumor angiogenesis by switching on hypoxia-independent erythropoietin production in stromal cells. Submitted manuscript

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CONTENTS

LIST OF ABBREVIATIONS

AMD	Age-related macular degeneration
ARNT	Aryl hydrocarbon receptor nuclear translocator
cGMP	Cyclic guanosine mono-phosphate
CRA	Central retinal artery
CRV	Central retinal vein
CV	Circumferential vein
Dll4	Delta-like 4
DR	Diabetic retinopathy
EC	Endothelial cell
EMT	Epithelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
Fli1	Friend leukemia virus integration 1
HIF	Hypoxia-inducible factor
HGF	Hepatocyte growth factor
HRE	Hypoxia-responsible element
HSPG	Heperan-sulphate proteoglycan
IGF	Insulin-like growth factor
LLC	Lewis lung carcinoma
LOX	Lysyl oxidase
MAP	Mitogen activated protein
MEK	MAP/ERK kinase
MET	mesenchymal to epithelial transition
NO	Nitric oxide
NRP	Neuropilin
OA	Optic artery
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PHD	Prolyl hydroxylase
PI3 kinase	Phasphatidyl inositide-3 kinase
PKB/akt	Protein kinase B/Ak transforming
PLC	Phospholipase C
PlGF	Placenta growth factor
Prox-1	Prospero homeobox protein 1
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
TAF	Tumor-associated fibroblasts
TAM	Tumor-associated macrophages
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
VHL	von Hippel Lindau
vSMC	Vascular smooth muscle cell

1 INTRODUCTION

Angiogenesis, the growth of new blood vessels from the existing vasculature, is a hallmark of many human diseases, and is often the driving force of pathology[1-2]. Angiogenesis has been recognized since the early 70ies, beginning with the seminal work by Dr. Folkman[3], as the major compartment that facilitates tumor growth. Tumor blood and lymphatic vessels are also key players of tumor dissemination and metastasis[4], the main cause of cancer-related morbidity.

In addition to promoting tumor progression and spread, angiogenesis significantly contributes to development of retinopathy[5-6]. During progression of retinopathy of prematurity (ROP), diabetic retinopathy (DR) or age-related macular degeneration (AMD), excessive growth of primitive and immature blood vessels in the retina lead to micro-hemorrhages, edema and eventually retinal detachment and blindness[6-10]. Both in cancer and AMD, there are several FDA approved drugs on the market which target pathologic angiogenesis, but more effective drugs are still needed.

In contrast to its detrimental roles in pathology, angiogenesis is also important for tissue repair[1,5]. In ischemic diseases such as myocardial infarction and stroke and under physiological conditions including wound healing, it is of pivotal importance that these tissues or organs are regenerated as quickly as possible in order to maintain their functions[10-11]. In these cases, it is desirable to develop pro-angiogenic, and in particular pro-arteriogenic therapeutic approaches[11]. These approaches seem to be very difficult to achieve, though, as functional vascular networks need extensive remodeling. Unfortunately, current angiogenic factor-induced blood vessels are immature and of poor quality. It is still an open question how best to assist the body in re-vascularizing injured tissues[12].

Hypoxia often triggers an angiogenic response in adult tissues. As mammalian cells rely on oxygen-dependent metabolism for long term energy production, prolonged tissue hypoxia causes cell stress eventually leading to cell death. This is especially true for cardiac musculature and the brain, as these tissues are particularly sensitive to hypoxia and have reduced potential for anaerobic energy production[13-14]. Thus even a transient blockade of oxygenation in these critical organs may markedly impair their functions which have dire consequences for the host.

Hypoxia induces a complex response in a tissue, aimed at protecting the cells against and counteracting the loss of oxygen. Hypoxia induces angiogenesis mostly via the hypoxia inducible factor (HIF)1α-vascular endothelial growth factor (VEGF) pathway[15]. However, there are many aspects of both this signaling pathway and other hypoxia-induced pathways that are still poorly understood, especially from the perspective of the whole organism.

Zebrafish has in the last two decades emerged as a powerful model organism to study developmental biology, including developmental angiogenesis. This animal model is widely used in biomedical research due to: 1) fast development; 2) transparent embryos; 3) ex-utero development; 4) large number of embryos produced in every breeding cycle and 5) their relatively cheap and easy maintenance and breeding compared to other fish strains. Furthermore, since development of the morpholino technology[16] sequencing of the entire genome [17], and the fact that zebrafish readily take up small amphiphillic molecules from the water [18], zebrafish have become highly amenable to genetic as well as pharmacologic manipulation. An additional benefit of the zebrafish model is that zebrafish has a much higher capacity for tissue regeneration, which occurs within a relatively short time compared to rodent models[19]. Furthermore, of particular interest to the work presented in this thesis, fish can be placed in hypoxic water, and thus the systemic response to mild, intermediate or severe hypoxia can be easily studied.

In this thesis, I describe zebrafish disease models developed with the aim of studying mechanisms of regenerative and hypoxia-induced angiogenesis, especially in the case of retinal angiogenesis and hypoxia-induced tumor cell dissemination.

In paper I, we developed a model of hypoxia-induced retinal angiogenesis in the adult zebrafish, and used this model to show that hypoxia induce neovascularization primarily in the capillary region of the retina. This angiogenic response is dependent on VEGF signaling. We further found that blockade of the Notch signaling pathway shifted the angiogenic response from the capillary region to the arterial region, and only in synergy with hypoxia was able to induce a robust arteriogenic response in the retina.

In paper II, we investigated the systemic effects of hypoxia on the distribution of blood in the fish vasculature. We found that fish lymphatic vessels connect to arteries in a

structure we call arterial-lymphatic conduits (ALCs). These structures are closed under normal physiological conditions, but open under hypoxia in response to NOS-induced local NO production and signaling. We hypothesize that similar conduits may be present in mammals, and could be important in hypoxia-induced tumor cell dissemination from the blood stream to the lymphatics during tumor metastasis.

In paper III, we developed a tumor cell implantation protocol in zebrafish embryos, which allowed us to study the early events of tumor cell invasion, dissemination and metastasis. Next we studied mechanisms behind hypoxia-induced tumor cell dissemination. We found that hypoxia-induced VEGF production by the tumor cells act on VEGFR2 on the host blood vessels to induce tumor angiogenesis, which lead to tumor cell invasion into the blood stream and dissemination to distal regions.

In paper IV, we developed a model of angiogenesis in the regenerating adult zebrafish tail fin. This model was used to show that phosphatidyl inositide 3 (PI3) kinase, which augment retinal angiogenesis specifically during development, is also important for adult regenerative angiogenesis.

In order to set the stage for the four papers, I will in the following describe the scientific background behind these projects in detail.

1.1 THE BIOLOGY OF BLOOD VESSELS

All vertebrates have a circulatory system based on blood vessels which transport nutrients and oxygen to the cells and collect waste products. Blood vessels are thus necessary in all tissues of the body, which impose unique requirements as they needs to be able to adapt to very different environments such as high shear stress and complex composition of blood flowing on one side and all the different tissues in the body on the other. Therefore blood vessels are complicated structures build up of several different, specialized cell types.

1.1.1 Vascular cell types – endothelial cells and vascular mural cells

Blood vessels consist of several different cell types, which have different properties reflecting the functional requirements of different types of vessels. Endothelial cells

(ECs) constitute the inner lining called the endothelium, and are thus in contact with and specially adapted for communicating with circulating blood.

ECs are attached to a basement membrane, consisting of extracellular matrix proteins[20], which in turn is covered with perivascular mural cells. For capillaries and veins, these cells are collectively called pericytes. Pericytes provide mechanical stability and elasticity and are thought to be a source of vascular growth and survival factors that help maintain a healthy endothelium[21-22].

The pericyte coverage varies in different vascular beds - in the liver for example, the capillaries have few pericytes and specialized for high exchange between blood and tissue[22], whereas in the brain, the capillaries are highly covered ensuring that potentially dangerous substances or cells are not permitted to cross the endothelial barrier[22-23].

Arteries are covered with one or several layers of a specialized type of mural cells called (vascular) smooth muscle cells (vSMCs). Similar to pericytes, these cells provide elasticity and stability to the arteries which are needed to buffer and tolerate the high blood pressure experienced by this particular endothelium[24]. In large arteries there are also other cells present such as axons that may provide contraction/relaxation signals to the vascular smooth muscle cells for regulation of vascular tone[24].

Pericytes and vSMCs are in contact with many different cells types of the surrounding tissue. Thus these cells may have higher capacity for adaptation to different environments compared to for example the endothelial cells[25-26]. This has led some researchers to propose that perivascular cells may be a reservoir for stem cells outside of the bone marrow[25-26].

In zebrafish not much is known on vascular mural cells and their functions both during development and in adults. It is clear that vascular mural cells are present in adult fish vessels[27-28], and that they may be involved in regulating vascular tone[29]. However, it is difficult to stain for vascular mural cells in zebrafish, as antibodies raised against mouse epitopes, to a large extent does not cross-react with those in zebrafish. Both we and others are therefore trying to create transgenic tools to more easily study the biology of vascular mural cells in zebrafish[30-31].

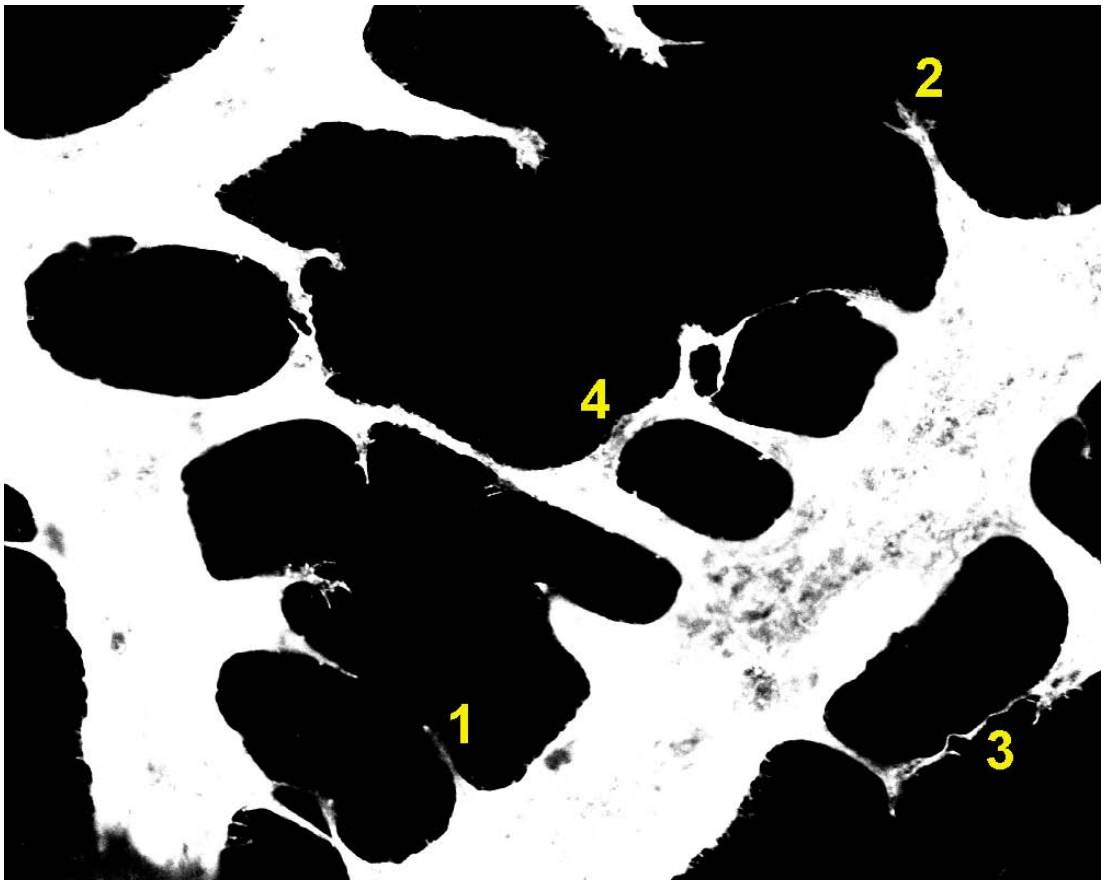
Endothelial cells are in all organisms highly specialized, and can be subdivided into arterial, capillary and venous EC families. While all ECs share expression of certain genes, such as *pecam* (CD31) and *fli1*, there are other genes which have different

expression based on the particular EC subtype[32-33]. For example, arterial EC expression of EphrinB2 ligands and venous expression of its receptor EphB4 is important for normal vascular development[34-35]. EphrinB2 is specifically induced in arteries during early development due to higher VEGF and Notch signaling in these more dorsally located cells, which indicate that different EC subtypes may have different responses to signaling factors such as Notch.

1.1.2 Development of the vasculature

The vasculature is in principle formed and expanded by two processes; vasculogenesis and angiogenesis[36]. Vasculogenesis is the *de novo* formation of blood vessels by progenitors. vasculogenesis occurs when endothelial progenitor cells, specified by the expression of the transcription factor fli1 in the bilateral posterior lateral plate mesoderm, migrate towards and meet at the midline just ventral to the notochord[37-38]. Here they coalesce and lumenize to form the dorsal aorta[39]. From the dorsal aorta, cells in the ventral floor migrate ventrally, coalesce and lumenize again to form the posterior cardinal vein[34]. Circulation is established by elongation and anastomosis of these two tubes and by establishing a connection to the heart.

The primitive blood circulation is subsequently expanded by angiogenesis, in which blood vessels branch off and grow out from existing vessels (see figure 1). Angiogenesis can be subdivided into several steps including degradation of the basement membrane, shedding of the perivascular cells, budding of endothelial cells (1 in figure 1), migration of the tip cells (2 in figure 1), proliferation of stalk cells, anastomosis with other vessels (3 in figure 1), lumenization (4 in figure 1), and finally maturation by recruitment of new perivascular cells. Thus three distinctive EC differentiation states are involved during angiogenesis; the tip cell, which is the leading cell of the growing sprout, the stalk cells, which constitute the connection to the mother vessel[40], and quiescent, differentiated cells of mature vessels. In mature vessels in fact, single cells in the endothelium are often difficult to distinguish as they are tightly connected to each other and have shared functions, dedicated to tasks such as transport, absorption or secretion of fluid, molecules or immune cells between blood and the underlying tissue[41-43].



Figur 1: Distinct steps during angiogenesis revealed in the retina of transgenic *fli1:EGFP* zebrafish. 1: EC budding. 2: Tip cell migration. 3: Anastomosis4: Lumenization. Image provided by Dr. Renhai Cao

All vertebrates have a remarkably similar blood circulation. It is therefore not surprising that developmental vasculogenesis and angiogenesis are largely regulated by the same pathways in zebrafish and mice. Even pathological or regenerative angiogenesis seems to be similarly regulated in different vertebrates[44-45].

At the core of these regulatory pathways are vascular endothelial growth factor (VEGF) which is important both for vasculogenesis, angiogenesis and vascular homeostasis[46]. VEGF, which is discussed in more detail in a separate section, is the strongest angiogenic factor in the body, and will lead to excessive angiogenesis if left unchecked. There are therefore several endogenous mechanisms of inhibiting VEGF actions, including the involvement of soluble receptors which act as decoys for their membrane bound, active analogues[47-48], and downstream inhibitory pathways such as the recently identified Dll4-Notch pathway[40,49-51].

For example, during initial angiogenic expansion of the primitive zebrafish vasculature between 22 and 32 hours post fertilization, VEGF produced in the somites drives dorsal sprouting of endothelial cells to form the intersegmental vessels[34]. This process is

negatively regulated by Dll4-Notch signaling, which limit the differentiation of endothelial cells into tip cells and protect against hyper-vascularization[40].

Angiogenesis is a highly regulated and complex process which is either positively or negatively regulated by many different factors. Positive mediators of angiogenesis are referred to as angiogenic factors and include the VEGF, fibroblast growth factor (FGF), transforming growth factor (TGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) families among others[36].
While some factors such as VEGF are more extensively studied than others, it is likely that several of these factors act together during both physiological and pathological angiogenesis[4].
Also there are several endogenous inhibitors of angiogenesis, or anti-angiogenic factors, including endostatin, vasostatin, prothrombin, thrombospondin, prolactin, osteopontin etc[52]. The relative level between angiogenic and anti-angiogenic factors in the organism determines whether angiogenesis is induced or not.
This balance is usually referred to as the angiogenic switch[53-54], which is turned on during development and tissue growth by the surplus of pro-angiogenic to anti-angiogenic factors, but turned off in quiescent, non-growing adult tissues, where there are an excess of angiogenesis inhibitors[54].

In addition to inducing angiogenesis, also the paths followed by the growing vessels are important for normal development of the vasculature, as well as the correct specification of arteries and veins.
Such path finding and specification cues are usually provided by cell membrane attached receptor-ligand signaling partners such as ephs and ephrins, uncoordinated/deleted in colorectal cancer and netins, plexins/neuropilins and semaphorins, roundabouts and slits and possibly others[55-58]. Signaling through these pathways often block formation of filopodial projections from the tip cells, thus limiting vessel growth into areas where such ligands are abundant. Intriguingly, this regulation is remarkably similar in growing axons, indicating that correct wiring of the developing nervous system and vasculature share similar regulatory pathways[55]. Recent evidence, however, indicate that these pathways may also be important for generating the chaotic architecture of blood vessels in tumors[59], for promoting angiogenesis in ischemic disease[60] or be an important anti-angiogenic and vascular normalization strategy in tumors[61].

1.1.3 Cardiovascular biology of mammals and fish

While most areas of angiogenesis and cardiovascular physiology and pathology are strikingly similar in fish and mammals, there are a few important differences, which are relevant to consider.

Primarily, as fish breathe water rather than air, their respiration has to be adapted for handling a comparatively much more viscous medium containing much less dissolved oxygen. Thus while mammals have a batch-system where oxygen is extracted from a batch of air each time, the gill system in fish is more like a continuous process where water is flowing counter-current with the blood, allowing maximal efficiency of the oxygen uptake[62]. Does this influence the cardio-respiratory response to hypoxia?

Mammalian respiration is quite inefficient compared to that in fish, so in order to maintain a high oxygen content of the blood leaving the lungs, humans have developed both cardiovascular and respiratory countermeasures to low oxygen. These include hyper-ventilation, increased heart rate (tachycardia) and higher pulmonary blood pressure and are initiated almost immediately following exposure to even slight hypoxia – such as a few kilometers above the sea surface[63]. However, if oxygen levels continue to drop, mammals cannot sustain life for long[63].

Fish on the other hand do not initiate such countermeasures at slightly reduced oxygen levels, as the oxygen uptake system is already sufficiently efficient[62]. Also, when induced, the respiratory and cardiovascular responses to hypoxia are slightly different. For example, most fish reduce their heart rate (bradycardia) when exposed to severe hypoxia, in order to increase the stroke volume[64], which is in contrast to the observed tachycardia in humans[65].

Furthermore, in normoxia usually only a part of the gill blood vessels are perfused, in order to have a lower demand on the blood pressure leaving the heart. Increased stroke volume under hypoxia, however, leads to increased blood pressure leaving the heart[64], which in turn leads to perfusion of more gill lamellae and thereby increasing the respiratory blood-water interface[66].

Also on the water side, increased frequency and amplitude of buccal (mouth) and gill movements leads to more water passing though these cavities increasing the uptake of oxygen[64].

As cardio-respiratory synchrony is of great importance in mammals, is hypothesized that it may also be beneficial in fish[67-68]. The theory is that if blood and water is perfusing the gills at a maximal rate at the same time, the oxygen uptake should be

maximized. However, it may be possible that such synchrony is only induced when fish are exposed to hypoxia, as lowering of the heart rate and increasing the ventilation frequency is required for matching of the two.

Also in the periphery, the fish cardiovascular system is slightly different from the mammalian. For example, the blood pressure in fish is lower than in mammals and the transendothelial pressure observed in both arteries (positive leading to leakage) and lymphatics (negative leading to drainage) seems to be greatly reduced in fish[69-71]. The pathological consequences of this have however not been well studied.

1.2 BENEFITS AND CONCERNS USING ZEBRAFISH IN MEDICAL RESEARCH

Most human diseases arise as a consequence of malfunctioning interplay between different cell types and tissues, and are therefore not well studied in cell-based in vitro models. Recently, zebrafish has emerged as an excellent in vivo model organism for medical and pre-clinical research and zebrafish-based models and technologies are rapidly expanding[44,72].

1.2.1 Zebrafish in general

Zebrafish (Danio Rerio) are small actinopterygian (ray finned) fish who measure 3-5 cm in length and weigh approximately 200-500 mg as adults. These fish have been used for research in vertebrate developmental biology for decades as they compared to other fishes of equal size do not need environmental enrichment in the aquaria, they are very tolerant to pollutants, have high fecundity and overall easier to handle and work with[73].

The following features in particular make zebrafish attractive as a tool in developmental research.

1) One pair of adult zebrafish can lay >200 eggs once per week.

2) Zebrafish eggs develop at room temperature but optimally at 28,5 degrees in high quality tap water, alternatively distilled water with added salts such as E3 or danieus buffer.

3) The embryos develop very rapidly compared to other vertebrates. For example

during the first 24 hours, the zebrafish embryo has developed a beating heart as well as a functional circulation loop containing blood cells. Correspondingly the human embryo has had time for one cell division in the same period of time.

4) Zebrafish embryos and juveniles are transparent.

5) Zebrafish are highly amenable to genetic and pharmacologic manipulations.

1.2.2 Genetic models in zebrafish

During the middle 90-ies the value of zebrafish as a model organism became further enhanced as two separate large-scale, un-biased ENU mutagenesis screens were carried out[74-75]. These screens identified thousands of mutants with obvious developmental phenotypes due to a mutation in a single gene. The resulting mutant libraries, which are continuously being expanded, provide researchers with new insights not only into the role of the mutated genes during development, but also the underlying causes of certain human diseases which have been found to closely resemble the phenotype of a mutant.

One example of the latter is the mutant gridlock, which harbor a mutation in the gene by the same name. In this mutant, the aorta fuse with the cardinal vein just posterior to the gill arches, thus causing blood to be shunted into the vein before being transported to the periphery[76]. Thus, even though endothelial cell specification in the peripheral parts of the fish is not affected, no blood flows to these parts.

This phenotype is also found in a congenital malformation known as aortic coarctation in humans[77]. Thus the gridlock mutant was used to identify a compound that normalizes the correct patterning and flow of blood, which potentially could be developed for use in the clinic[78].

Following the advent of the morpholino technology researchers may now functionally reduce or eliminate the expression level of any gene of interest during the first 4-6 days of development, and in this way study its physiological effects during development[79]. As, furthermore, the entire zebrafish genome have been sequenced, this is a powerful alternative to creating knock out animals, as the latter is time consuming and expensive, and furthermore only allow gene levels to be controlled at three levels; zero, half or full expression. Using morpholinos on the other hand researchers may carefully titrate the expression level of the target gene, potentially revealing other aspects of its function.

An example of this is a study done on VEGF-A. In mice, even heterozygous knock outs of VEGF-A die during early embryonic development due to a defective and malformed vasculature. However injecting different concentrations of VEGF-A morpholino in the

zebrafish embryo the effects of slightly or more severely reduced VEGF-A levels during development could be separated and studied[80]. A severe reduction of VEGF-A was found to compromise early vasculogenesis leading to no vessels being formed in the embryo at all.

Interestingly, a moderate reduction of VEGF-A levels, or the inhibition of downstream events of this pathway leads to the specific inhibition of dorsal growth of arterial vessels, whereas ventral growth of venous vessels was unaffected[34,81]. These actions of VEGF-A impinged on the ephrin-eph pathway, which is an example of cross-talk between angiogenic-factor signaling and the vascular path finding pathways.

Because the zebrafish embryo is transparent and develops in water, it is particularly beneficial to generate transgenic reporter strains expressing a fluorescent protein such as GFP under specific promoters. Such transgenic lines allow researchers to study the origin, movements, differentiation and growth of cells and tissues in single cell detail in the entire organism until the zebrafish reaches adolescence (about 1 month of age) where the skin starts to become more densely pigmented. In respect to vascular biology, using such an approach to label ECs have yielded significant contributions to our knowledge of early vasculogenesis[82-84], tube formation[85-86], the formation and origin of the first lymphatic vessels[87-90], as well as the synchronous onset of cardiac contractions exactly when the circulation have been completed[91].

1.2.3 Hypoxia and zebrafish

Fish have the unique ability to withstand very low oxygen levels for prolonged periods of time. Whereas humans risk losing consciousness at just moderately diminished oxygen levels, such as those above 4000 meters altitude (corresponding to approximately 80% of the oxygen at sea-level), zebrafish can survive for a very long time at oxygen levels less than 10% of fully air-saturated water[92-94].

This enables studies on the effects of hypoxia in a whole living animal, without having to perform surgery to restrict blood flow in a particular organ or tissue. The latter, is furthermore non-controllable, meaning that it is practically impossible to adjust the oxygen levels in the tissue precisely – it is either normoxic (in sham operated controls) or anoxic downstream of the ligation suture.

On the other hand, it is difficult to achieve localized hypoxia in only a particular tissue but not others in adult zebrafish as they do not have easily accessible, superficial blood vessels. In embryos, however, a method for localized laser-induced ligation of small

vessels was recently developed to create a small hypoxic area[95].

If such techniques can be adopted by other labs, and perhaps expanded to also covering ligation of vessels in adult fish, this could substantially promote studies on localized tissue hypoxia in zebrafish.

1.2.4 Regeneration in zebrafish

Since fish are able to regenerate cardiac muscle as well as nervous tissue[19], they may serve as valuable tools in studying the mechanisms of recovery after cardiac infarction, stroke or neurodegenerative retinopathies. For example, zebrafish models of myocardial regeneration have been used to identify a population of cells involved in regeneration of ischemic cardiac tissue, as well as the mechanism by which they de-differentiate and re-differentiate into cardiac muscle, endothelial cells of the cardiac vasculature etc[96-97].

The regenerating tail fin is particularly amenable to molecular studies, as it is possible to study the effects of pharmaceuticals simply by adding them to the water[28], or alternatively knock down or over-express genes of interest in the regenerating tissue specifically by microinjection and electroporation techniques[98-103]. As zebrafish fin regeneration also require vessel growth as well as endothelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), as do the skin wound healing models in mice, this assay may be used as a powerful alternative especially in pharmacologic or molecular studies of this process.

1.2.5 Concerns using zebrafish for vascular research

There are, however a few drawbacks which makes zebrafish unsuitable for studies in certain areas of human medicine.

Fish have a two chambered heart compared to the double set of two chambers, separated by a septum in mammals. Because of this, zebrafish are not well suited for studies on cardiac septation. Furthermore, the cardiac electrical conduction system in zebrafish may be simpler than in mammals[104-105].

The heart in fish is spongiform meaning that blood flows through the cardiac musculature. Cardiac vascularization is crucial for oxygen delivery to the thick and dense mammalian cardiac musculature, and therefore for its function. The fish heart is, however, only sparsely vascularized, and it does not seem to be as important for the cardiac function as in mammals[106-107]. In fact, some fish can even survive and are

practically asymptomatic under physiological conditions with a ligated coronary artery and therefore complete lack of coronary blood supply[106-107].

Another issue is that blood pressure is lower in fish[69-71,108] which means that fish vessels may need less mechanical support from pericytes and vSMCs than mammals. As mentioned, the area of pericyte biology in fish is quite under-investigated because better histological tools are needed before such questions can be addressed.

Also the retinal vasculature of fish and mammals differ to some extent. This is a subject that will be treated in more detail later, so suffice to say that the fish retinal vasculature is much simpler than the mammalian, which facilitate the study of retinal neovascularization in adult zebrafish.

Finally, markers specifically labeling some endothelial cell types in mammals label others or do not exist in zebrafish. One example of this is the VEGF receptor 3. VEGFR3 in mice specifically label endothelial tip cells and lymphatic endothelial cells, but have been reported to be a venous endothelial cell marker in zebrafish[109]. Furthermore, while VEGFR1 is expressed by all endothelial cell types in mammals, it specifically labels arteries in zebrafish[89-90].

1.3 HYPOXIA SIGNALING IN ANGIOGENESIS AND VASCULAR BIOLOGY

Oxygen is the primary electron acceptor in energy production for multi cellular organisms. Oxygen-mediated or aerobic metabolism, through the electron transport chain, transform sugars and lipids into CO_2 in a process that liberates close to all the Gibbs free energy of this reaction as high energy ATP. Thus this is the most efficient pathway to generate energy in the body.
Therefore, most mammalian cells rely on aerobic metabolism for sustained energy production, and certain tissues such as the heart and brain rely more heavily on electron-transport-mediated ATP generation[13-14]. Oxygen availability is therefore very important throughout our organism at all times.

However, one reason behind the high efficiency of aerobic metabolism is the reactivity

of oxygen, which also must be controlled. Thus an elaborate network of enzymes, endogenous and exogenous anti-oxidants are on constant watch for renegade oxygen radicals that may cause problems such as lipid (per)oxidation and DNA mutation[110].

1.3.1 Defining normoxic and hypoxic states to tissues

When sufficient oxygen is present in a tissue the cells may use aerobic metabolism to their preferred extent. Such a state is termed normoxia. However, normoxia is not necessarily the same for all tissues, as some tissues are less vascularized or more metabolically active than others.

For example, the spleen and brown fat has much higher blood vessel density than for example the thymus or white fat, and the latter tissues have much lower metabolism and demand less energy than the former[111-112]. Accordingly normoxia in the spleen refers to a higher oxygen concentration than normoxia in the thumus[111].

In the air we breathe, normoxia is 21 kPa (21% oxygen or 159 mmHg). However, in the arterial circulation normoxia has dropped to 13 kPa as the efficiency of the lungs is quite modest. In tissues however, normoxia can range from 0,5 to 2,5 kPa[113].

Normoxia in the tissue is not always maintained, as the level of available oxygen may change. In principle this may occur in one of three ways; the oxygen consumption in a tissue may increase (such as in active compared to passive muscles), the availability of oxygen rich arterial blood may be compromised (for example as a consequence of a blocked or ruptured artery) or the oxygen carried by the blood may be too low (for example in case of anemia).

How do we then determine if a tissue has become hypoxic? Currently many researchers rely on the pimonidazole reaction, to determine hypoxic from normoxic areas. The change from a non-immunoreactive substrate to immunoreactive adducts of pimonidazole occurs at an oxygen tension of approximately 1,3 kPa (about 10 mmHg)[113-115], which we therefore define as tissue hypoxia.

However, as mentioned above, some tissues have physiological oxygen levels at or even below this limit. Therefore, defining a relative hypoxic state to a particular tissue should be done with care, and always with knowledge of its physiological oxygen levels in mind.

1.3.2 Cellular and systemic responses to hypoxia

In all cases when a tissue becomes hypoxic it will signal to the host that more oxygen is

acutely needed. Cells are equipped with counter-measures which are aimed at sustaining homeostasis in low oxygen environments for as long as possible.
These include a shift in the metabolic profile to rely more on glycolysis (which require less oxygen) for energy production, and stopping processes which consume a lot of energy, such as cell proliferation[116-118].
They also communicate with the organism on both local and systemic levels to try and direct more oxygenated blood to the hypoxic area. This is done in four ways.

1: Tissues, such as working muscles sends signals to the brain that via sympathetic nerves up-regulate the cardio-respiratory rhythms, leading to hyperventilation and increased cardiac output[65]. This response is aimed at extracting more oxygen from the atmosphere as well as to faster exchange the blood in the tissue.
2: Arterial blood vessels in the tissue become dilated leading to higher blood perfusion[119]. This process is often carried out by eNOS-mediated NO production, as NO is a strong relaxing factor for vascular smooth muscle cells.
3: The production of EPO goes up, leading to erythropoiesis and mobilization of more red blood cells from the bone marrow, which increases the oxygen-binding capacity of the blood[120-121].
4: The tissue produce angiogenic factors – primarily VEGF-A – which stimulate angiogenic expansion of the vasculature and thus also increase perfusion but in this case through newly formed blood vessels[122-123].

1.3.3 The HIF signaling pathway

Most of the responses to hypoxia are mediated by the hypoxia-inducible factor (HIF) pathway. The HIF family of transcription factors comprise HIF1α, HIF2α, HIF3α and HIF1β or ARNT[124]. Not much is known about the actions of HIF3α, so in the following I will focus on HIF1- and HIF2α.
ARNT heterodimerize to either HIF1α or HIF2α, and the resulting dimer is termed HIF1 or HIF2 respectively. HIF1 is important for acute responses to hypoxia, as the α–subunit is constantly turning over in normoxia but immediately stabilized in hypoxia[125-126]. HIF1α remain at high levels in hypoxia for at least 24 hours, and then decrease by an unknown mechanism.
HIF2 on the other hand seems to be quite stable, in some tissues at least, even in normoxia, but is normally present at low levels[127]. HIF2α transcription is induced by

hypoxia and the levels increase over the course of 24 hours and stay high for a longer time[128].

The instability of the HIFα subunits in normoxia is due to oxygen-mediated tyrosine hydroxylation by the prolyl hydroxylase (PHD) enzymes, primarily PHD2[125]. Hydroxylated HIFα is recognized by the von Hippel Lindau (VHL) E3 ubiquitin ligase, which targets it for proteosomal degradation. Thus HIFα stability in hypoxia is primarily due to the inactivation of PHD2.

Many genes including VEGF-A and EPO, have hypoxia-responsible elements (HRE) in their promoters, to which HIF1 or HIF2 can bind and thus activate transcription. However, HIF-1 and HIF-2 may activate slightly different sets of genes[124].

Most areas of clinically detectable tumors are constantly hypoxic due to poorly perfused and low quality blood vessels, high metabolism and sometimes genetically up- or down-regulated hypoxia signaling factors or inhibitors[129-133]. Tumor hypoxia is a major driving force of tumor cell dissemination and ultimately the formation of distant metastatic nodules[134-135]. In this process it is becoming increasingly clear that HIFs play a major role[136-137].

In spite of the differences in the physiological cardio-vascular responses to hypoxia between mammals and zebrafish, mentioned previously, the molecular pathways regulating these responses seem to be identical[138-140].

If activating the HIF pathway either genetically or pharmacologically, the zebrafish answer by up-regulating VEGF, EPO and other classical HIF-target genes[140]. Also physiological effects of hypoxia such as vascular dilation, angiogenesis and erythropoiesis are conserved in zebrafish[140-144].

Thus it seems that the differences in the cardiovascular response to hypoxia between fish and mammals are more just a variation on the same theme, which is necessary for living in water rather than air. Therefore, while researchers should be mindful of the differences, I think there is no course for alarm when it comes to using zebrafish as a model system of mammalian molecular or physiological responses to hypoxia.

1.4 ANGIOGENESIS IN RETINOPATHY

Retinopathies are the leading cause of vision impairment and blindness, collectively

affecting more than 70 million people worldwide[145]. Although non-fatal, they are considered to be major debilitating diseases, and thus people living with retinopathy experience reduced quality and many problems and obstacles in their everyday lives.

Three classes of retinopathy are predominant – retinopathy of prematurity (ROP), diabetic retinopathy (DR) and age-related macular degeneration (AMD). These three diseases affect different patient groups and also different areas of the retina.

In order to understand their pathology, one should therefore keep the anatomy of the eye in mind.

1.4.1 Anatomy of the eye

The anterior part of the eye is covered by the cornea; a colorless and transparent, hard, bone-like membrane which shields the iris, lens, vitreous and retina, from the exterior. The cornea has no blood or lymph vessels, and is thus a popular model for angiogenesis and lymphangiogenesis in itself[11].

The cornea is attached to the retina, which is a multi-layered tissue responsible for most of the functions of the eye including reception and conduction of visual signals through specialized photoreceptors.

The retina is in turn covered by connective tissue known as the choroid and a pigmented epithelial cell layer know as the sclera. The space between the cornea and the retina is filled by a gel-like substance known as the vitreus.

In the human retina, blood vessels are present mostly in two layers – at the inner surface exposed to the vitreus (retinal vessels) and in outer structures close or incorporated into the choroid (choroid vessels).

The central part of the retina is called the macula, and it is usually in this area signs of retinopathy are most readily detected.

1.4.2 Retinopathy of prematurity

Retinopathy of prematurity is a common disorder in very early pre-term infants who need incubation in an oxygen enriched atmosphere after delivery in order to support life[146].

Their retinal blood vessels (as well as most other parts of their body) are not fully developed, leading to deficiency of blood in the retina. Furthermore, the hyperoxic environment in the incubators, may lead to further pruning and regression of thin retinal capillaries[147]. When such infants are brought out of the incubator and into normoxia, the lack of retinal vessels leads to retinal hypoxia and thus widespread hypoxia-induced

retinal angiogenesis[148]. These new blood vessels are of low quality, lack association with vascular mural cells and therefore give rise to retinal edema and hemorrhage[149]. As an ultimate consequence, the blood-retinal barrier may be compromised, the photoreceptors start to degenerate and accumulating edema may cause retinal detachment – collectively leading to blindness.

As retinal hypoxia is the driving force behind pathological angiogenesis in this disease, it may be treated by targeting VEGF or VEGFR1[150], which in many cases lead to normal development of retinal blood vessels.

1.4.3 Diabetic retinopathy

Diabetic retinopathy is a complication of diabetes mellitus, in which patients cannot regulate their blood sugar levels. Small capillaries, including those in the retina, seems to be particularly sensitive to high blood glucose levels, which lead to constant irritation of the endothelium. Thus after 10 years or more with the disease, 90 % of diabetic patients start to exhibit symptoms of DR[151].

The disease usually follows a course from a mild and non-angiogenic (or non-proliferative) state, into a severe state, which eventually become angiogenic.

Initially the integrity of small retinal capillaries are compromised leading to micro-hemorrhages and leakage of fluid. Compounds such as cholesterol and triglycerides in the leaked plasma may be deposited in the retina and can be observed by funduscopy as the characteristic “cotton wool-like” spots. These compounds may also aggregate inside the blood vessels, leading to blockade of flow. As more and more and also larger vessels become affected, the area downstream of the hemorrhaging or blocked vessels does not receive blood and become ischemic.

The ischemic areas start to produce VEGF, which switches on the angiogenic state of the disease[151-152]. As it was the case in ROP, the newly formed blood vessels are immature, of low structural integrity, and therefore leaky and prone to bursting – thus making matters worse.

The disease eventually follows a course similar to that described for ROP, and patients with severe disease are at risk of becoming blind.

Treatment consist of a combination of treating the diabetes, which is the underlying problem, and of anti-VEGF and other treatments aimed at reducing angiogenesis and retinal/macular edema, restoring photoreceptor functions and improving vessel quality[153].

1.4.4 Age-related macular degeneration

Age-related macular degeneration is the major course of vision impairment in the elderly[7,154]. There are, as in diabetic retinopathy, both a non-angiogenic and angiogenic (aka wet, neovascular or exudative) state of the disease, the latter being the most severe. The initial, pre-pathologic phases include the inefficient clearing of dead cell debris between the retinal pigment epithelium (sclera) and the retina, associated with old age, which accumulate in small spots known as drusen. Drusen are typical in the elderly, and is as such not a problem if they are only few and small in size. However, many and large drusen may disrupt retinal functions and lead to loss of retinal cells including photoreceptors, a state which is known as geographic atrophy.

In the dry or non-angiogenic state, geographic atrophy may expand and reach the centre of the macular, in which case the loss of cells my lead to severe vision impairment. Furthermore, many large drusen may disrupt retinal attachment to the choroid. However, the atrophy may also include endothelial and other cells of the blood vessels, which lead to a large part of the retina loosing blood flow, and thus retinal ischemia[7]. Ischemia-induced, VEGF-dependant angiogenesis ensues, similar to what was described in DR, and the disease progress along a similar path.

The affected vasculature is thus the main difference between angiogenic DR and AMD as in the former it is the retinal vessels which are affected mostly, and in the latter it is mainly the choroid vessels[155].

Choroid vessels are buried deep in the retina, thus a special anti-VEGF antibody, which is smaller and thus has higher penetration through the retina, has been developed for the treatment of AMD. It seems however, that also larger and cheaper anti-VEGF antibodies work well[156-157].

1.4.5 Comparison of the retinal vasculature in zebrafish and mammals

During development, zebrafish, as humans, have a transient hyaloid vasculature, which is attached to the lens. In humans this vasculature regresses in favor of the developing retinal vasculature, but in fish it seems instead to detach from the lens and become associated to the vitreal surface of the retina[27].

Zebrafish retinal blood vessels are histologically similar to human retinal blood vessels. They are also covered with pericytes, and irrigate the vitreal surface of the retina[27,44,141,158-159]. Zebrafish, however, does not have choroid vessels, perhaps because the outer retina may receive sufficient oxygen from cutaneous absorption.

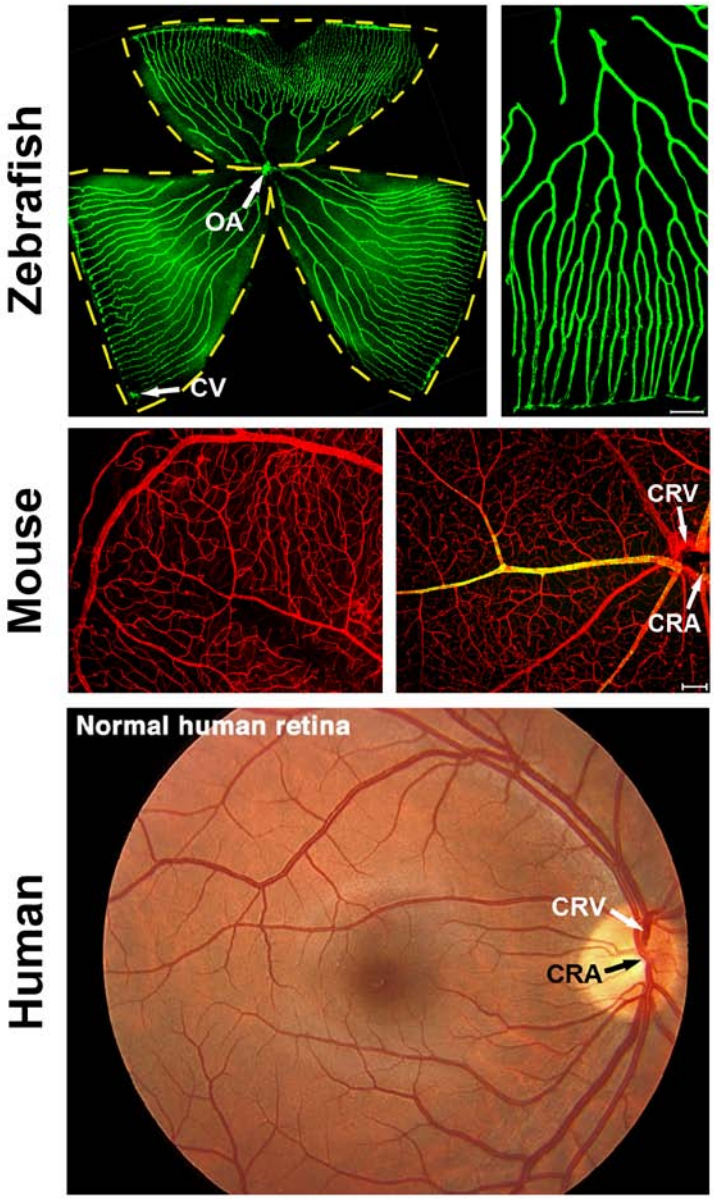


Figure 2: Comparison of the retinal vasculature in zebrafish, mice and humans. Top: Vessels in adult *fli1:EGFP* zebrafish. Middle: Immunohistochemical staining of adult mouse retina – red signals: ECs, yellow signals: arteries. Bottom: Fundoscopy of the healthy retinal vasculature in humans. OA: optic artery, CV: circumferential vein. CRA/CRV: central retinal artery/vein.

In adult zebrafish the retinal vasculature spread out from a central optic artery (OA) that, similar to the central retinal artery (CRA) in mice and humans, run alongside the optic nerve (see figure 2). 4-9 main branches (so called grade I branches) emanate from the OA and run toward the periphery, dividing 2-4 times in the process, in order to supply the entire vitreal surface of the retina[27,44,141,158].

At a region known as the capillary region, the vessels anastomose with protrusions from the circumferential vein (CV), and thus close the circulation. It is in this capillary region that blood vessels are most prone to hypoxia-induced angiogenic expansion[141]. The vitreal vasculature in the zebrafish is thus very similar both morphologically and histologically to that in mice and humans (see figure 2). Zebrafish may therefore serve

as a valuable model system for studying angiogenic retinopaties including ROP, DR and AMD.

1.5 CHARACTERISTICS AND FUNCTION OF LYMPHATIC VESSELS

The mammalian lymphatic vasculature lies in remarkable proximity to blood vessels[160-161], which is important for its ability to regulate tissue fluid homeostasis. Other important lymphatic functions include immune surveillance, lipid absorption and transport[162].

In both mammals and fish, the lymphatic circulation develops from the venous vasculature[163]. Initially, a subset of venous endothelial cells starts to express markers such as prox-1, which specify these cells as lymphatic endothelial progenitors. These cells can respond to VEGF-C and start to migrate away from the veins and form the initial lymphatic sacs[160]. These primitive structures later fuse and remodel to form vessels that are no longer physically connected to the blood vasculature[164-168], except for where the lymph flows back into circulation at the duct of Cuvier.

The fully developed lymphatic vasculature has several distinctive characteristics.

The classical view is that lymphatic vessels originate in blind ended sacs, which function to absorb fluid from the tissue. This process is achieved by two sets of lymphatic valves[169].

Primary valves exist inside the vessels, ensuring a directed flow away from the tissue and towards larger collecting lymphatics (see figure 3). These collecting lymphatic vessels are usually covered by pericytes, and have a large lumen in order to accommodate efficient drainage of large amounts of fluid with minimal fluid pressure inside the vessel[4].

Secondary valves exists between the lymphatic endothelial cells themselves, ensuring a directed flow of fluid into the vascular lumen[169] (see figure 3). Thus, lymphatic vessels are thought to exclusively exhibit unidirectional afferent flow.

The lymphatic vessels of the tissue empty into regional lymph nodes, where the lymph is screened for the presence of non-self antigens, and thus constitute an important part of the adaptive immune system[170].

Fish also have structures similar to mammalian lymph nodes, called melano-

macrophage centers or aggregates, which are handling adaptive immune functions[171-172]. They are however much simpler in structure, and not as well studied as their mammalian counterparts.

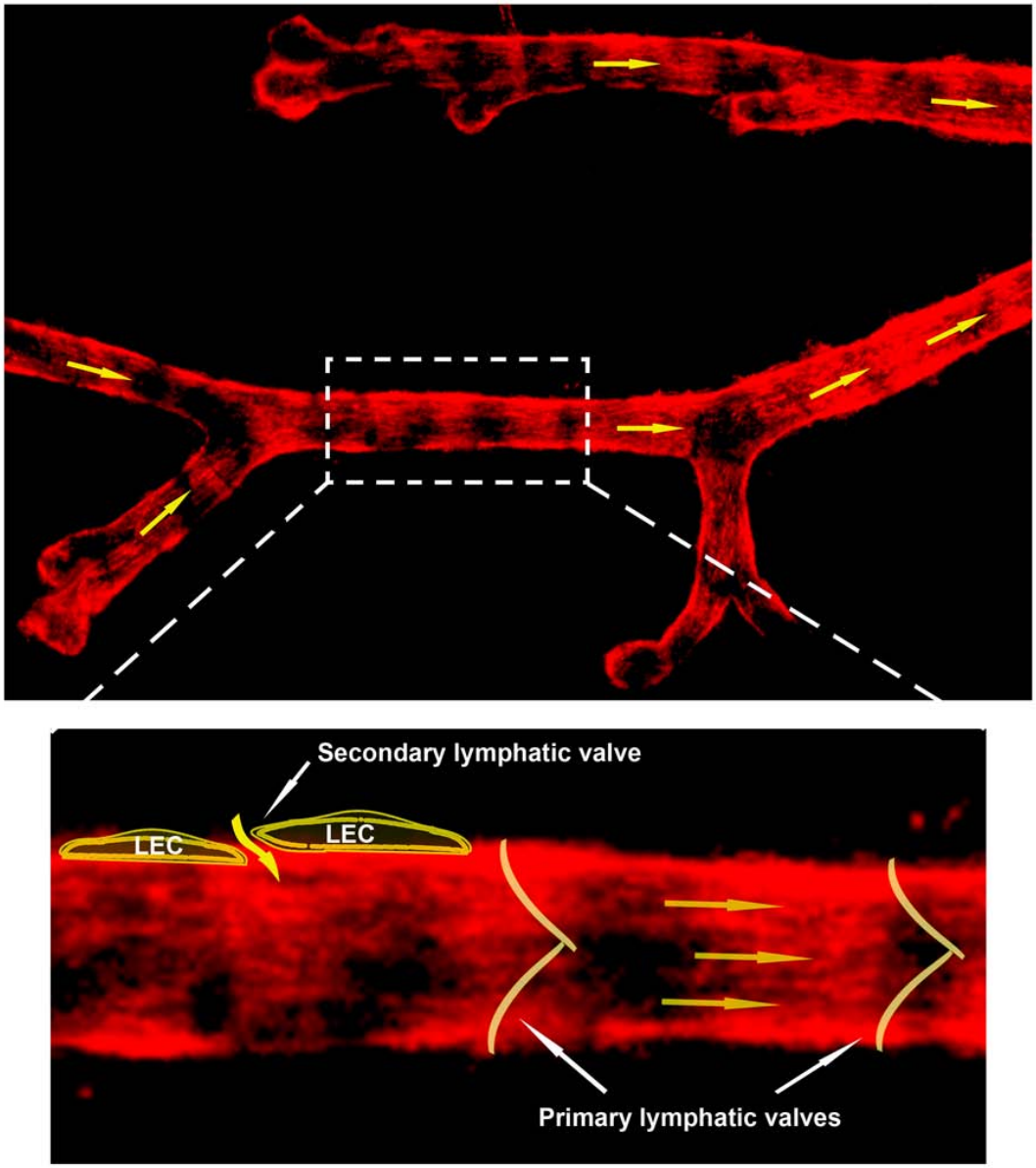


Figure 3: Characteristics of lymphatics vessels in mice revealed by immunohistochemical staining. Top: Lymphatic vessels arise in blind ended bags, and exhibit unidirectional, afferent flow. Bottom: Directionality of flow is achieved by primary and secondary lymphatic valves. Image courtesy of Mrs. Sharon Lim and Dr. Renhai Cao.

It has been a long standing debate whether fish have a "real" lymphatic vasculature or not[173-174]. Pioneering work done by Burne and Kampmeier showed that fish do have vessels with features of lymphatic vessels in mammals[174, and references therein].

However, fish anatomists and physiologists such as Vogel and Steffensen have later discovered that these so called lymphatic vessels are in fact physically connected to the

blood circulation, and fluid can be exchanged between the two compartments[29,173-176]. Thus fish lymphatics does not (at least not exclusively) originate in blind ended vessels, such as it is thought to be the case in mammals, and therefore it may be argued that, by definition, these vessels cannot be called lymphatics. Because of this, fish vascular physiologists have suggested that they are instead called secondary blood vessels. Recently the Weinstein[177-180] and the Schulte-Merker[87,89-90,181-184] laboratories have taken up this issue again, and described vessels that are histologically and functionally very similar to mammalian lymphatics, at least during the first two weeks of development. The development of this lymphatic vasculature furthermore follows the same molecular program as in mammals[180,184].

However, whether these vessels are identical to the secondary blood vessels in adult fish is still not known.

1.6 CARCINOGENESIS, METASTASIS AND THE ROLE OF HYPOXIA

Initial cell transformation into carcinogenic cells is largely a cell autonomous process, but in order for these cells to expand into a macroscopic cell mass – which is the process of tumorigenesis – the tumor requires help from the host. There are two ways in which the host unintentionally helps the tumor.

First, initial attempts of the body to clear renegade, hyper-proliferating cells involve recruitment and activation of inflammatory cells, which non-specifically kills such hyper-proliferating cells before they cause problems[185-186]. However, if these cells are able to escape killing, the inflammatory cells are thought to aid in tumorigenesis by secreting factors, such as pro-angiogenic factors, that communicate with the host[187-189].

Second, high metabolism and growth of the pre-malignant mass beyond a size where all cells can be sufficiently oxygenated by pre-existing blood vessels, lead to tumor hypoxia.

1.6.1 Tumor hypoxia and the role of the vasculature

Hypoxia is a major driving force of tumor progression as it promotes several of the hallmarks of cancer including:

- Metabolic shift from aerobic metabolism to glycolysis - known as the Warburg effect[190]

- Genomic instability both due to increased reactive oxygen species (ROS) formation and metabolically-linked acidosis[191]
- De-differentiation of the tumor cells through epithelial to mesenchymal transition (EMT)[192-193]
- Turn on the angiogenic switch, and induce formation of low quality blood vessels[122]
- Render the tumor resistant to therapy by lowering the effectiveness of radio-therapy, which rely on the presence of oxygen to generate cytotoxic oxygen-radicals[194], and by increasing blood vessel leakiness and thus increasing interstitial fluid pressure in the tumor, which reduce tumor perfusion and therefore delivery of cytotoxic agents[194]

These effects of tumor hypoxia have paved the way for a new way of thinking, in terms of targeting the tumor vasculature. Instead of eliminating tumor blood vessels, which would lead to extensive tumor hypoxia, many researchers now believe that reducing leaky tumor blood vessels by improving their pericyte coverage as well as restoring a normal arterial-venous identity and thereby improving perfusion of the tumor, will lead to better oxygenation and less pathogenic tumors[195-196].

Such changes in tumor vasculature are known as vascular normalization[197], and has been found to not only improve the effects of therapy[198], but also reduce tumor growth rate[195] and most importantly the metastatic tendency[199].

1.6.2 Epithelial to mesenchymal transition and the role of hypoxia

The majority of tumors are of epithelial origin and therefore – similar to non-transformed epithelial cells – quite immobile. However, tumor cells may increase their mobility by de-differentiation into cells with characteristics of mesenchymal cells[200-201].

The process of epithelial-to-mesenchymal transition (EMT) has been studied quite extensively and encompass both down-regulation of epithelial cell-specific genes such as E-cadherin[202], but also the up-regulation of mesenchymal genes such as snail[202], twist[203] and slug[204], which in particular promote migration and tolerance to novel environments.

The mechanism behind induction of EMT in tumor cells is still not fully understood, but it has been associated with tumor hypoxia either via HIFs directly[205-208] or indirectly as certain transcription factors which can induce EMT such as Notch and TGF-β[207,209-210], are up-regulated by HIFs[211-212]. Furthermore, de-differentiated stem-cell-like states

may be stabilized by hypoxia and hypoxia-associated ROS[213-215].

Some tumors, such as many types of renal cell carcinoma and some tumors of the central nervous system, have deletions or mutations in genes such as VHL or PHD2 and therefore up-regulated HIF signaling even in normoxia[216-217]. In these cases, however, it may be possible to target such tumor cells specifically by compounds that are non-toxic in non-malignant cells[218].

EMT is important for local invasion of peri-tumoral tissues, but also for the ability of tumor cells to penetrate the endothelium and thus disseminate via the blood stream. Trans-endothelial invasion of the blood or lymph vessels is used by tumors as the main route of seeding metastasis in distant organs[4,219].

It is therefore important also to think about the effects of tumor hypoxia on the vasculature, as this is important for developing the specific characteristics of tumor blood vessels including: poor pericyte coverage[195], loss of arterio-venous identity[220] formation of vascular plexuses and poor perfusion[195], high permeability leading to extravasation of fluid and high intratumoral fluid pressure and intravasation of tumor cells[46,221].

1.6.3 Tumor angiogenesis

VEGF contributes to the development of many of the pathological characteristics of tumor blood vessels. However, late stage tumors produce a plethora of factors at high levels[4,36], which have angiogenic potential and may be important for the above mentioned features of the tumor vasculature. For example PDGF[221-222], HGF[223], IGF[224], FGF[221] and VEGF-C[225] have all been shown to play important roles in tumor induced angiogenesis, both alone, but in particular in combination[11,221].

Thus, it is beneficial to consider the tumor microenvironment as a complex source of many growth factors and cytokines, and treatments should be designed accordingly. Indeed, specific anti-VEGF treatment in the clinic is often associated with only transient improvements at best, and patients often become refractory, as the tumor switches to depend on other growth factors[226].

As an example on how other factors may act to drive tumor angiogenesis, it has recently been shown that a combination of FGF and PDGF expressed by tumor cells result in a very strong angiogenic phenotype[221]. Similar to vessels in highly VEGF-expressing tumors this combination leads to formation of vascular plexuses and

increased metastasis by targeting both the endothelial cells and associated vascular mural cells simultaneously[221].

Thus while anti-VEGF therapy has proven to be an important therapeutic approach to target the tumor vasculature, inhibit tumor growth and prolong life expectancy in some malignancies, it would be interesting to see drugs which target other factors entering clinical evaluation as well.

Angiogenic factors are also important at sites which subsequently may be seeded by tumor cells to provide a beneficial environment for growth of metastatic nodules[227]. Such environments are known as (pre-)metastatic niches[228].

The generation of these niches can also be influenced by tumor hypoxia-induced pathways, including induction of VEGF[227] and lysyl oxidase (LOX)[136-137,229-231].

In fact, it is common to find tumor cells in the blood stream of most advanced cancer patients, even in cases where there are no signs of metastasis[232], indicating that some tumors fail in generating pre-metastatic niches and these are of major importance for metastatic growth.

Tumor-derived factors such as VEGF may also have detrimental effects on blood vessels in healthy tissues such as the liver and bone marrow[46,233]. This may lead to cancer-associated systemic syndromes, which in many cases play an important role in cancer morbidity.

It is thus important, when evaluating the effects on new anti-angiogenic compounds, to look at off-tumor targets – both when screening for therapeutic effects and toxicity.

1.6.4 The role of tumor stromal cells

Tumors are complex tissues consisting of many different cell types in addition to the tumor cells themselves. Perhaps most studied – besides the cells of the vasculature – are tumor associated fibroblasts (TAFs) and macrophages (TAMs), which are thought to be important players in tumor progression and resistance to therapy[186,189,234-236].

Such cells would also feel the effects of tumor hypoxia, and can therefore be an important source of hypoxia-induced factors such as VEGF[237].

Even endothelial cells (and peri-vascular cells) of tumor blood vessels will exhibit enhanced hypoxia signaling, at least in the poorly perfused and venous fraction.

Inducing endothelial hypoxia signaling via inhibition of PHD2 has been shown to improve the quality of the endothelium by lowering the permeability, thus making it more difficult for tumor cells to enter the vasculature[134].

It has therefore been suggested that enhancing the experienced hypoxia in the endothelium, for example by therapeutically targeting PHD2, may lead to better tumor perfusion, better delivery of chemotherapeutics and less potential for metastatic spread[134].

However, one should be careful in pursuing such an approach, as enhanced hypoxia signaling in all other cells in the tumor – including TAFs, TAMs and the tumor cells themselves presumably would lead to a more invasive phenotype, as discussed above. Thus, if one seeks to enhance hypoxia signaling in the endothelium, this has to be done in a very targeted manner.

1.7 VEGF AND VEGF-SIGNALING

VEGF is the most studied of all angiogenic factors, and is considered one of the earliest and most predominant angiogenic factors in tumors[4,238-241].

The VEGF family is a subfamily of the cystein knot super-family of secreted proteins (which also include the PDGF and bone morphogenic protein subfamilies) and is comprised of 6 members; VEGF-A, B, C, D and PlGF-1 and -2[242-244]. All these proteins act exclusively as secreted dimers, but as they are also produced in VEGF-receptor expressing cells (ie endothelial cells), they may act in an autocrine as well as paracrine and endocrine fashion.

VEGF-A is the most studied family member. The VEGF-A gene can give rise to several isoforms due to alternative splicing of the mRNA, resulting in a plethora of different sizes of the VEGF-A protein raging from 121 to 206 amino acids[245-246].

The larger isoforms have higher binding affinity to heparan sulfate proteoglycans (HSPG) and thus stay very close to, or even associated with, the plasma membrane, whereas the smallest have very limited HSPG-binding capacity and are more diffusible.

The different VEGF members activate a subset of the 5 existing VEGF receptors and co-receptors VEGFR1, VEGFR2, VEGFR3, Nrp1 and Nrp2. These receptors are transmembrane receptor tyrosine kinases, which require homo or hetero-dimerization

for signaling[247-251].

In addition to the membrane bound isoforms, at least VEGFR1[48] and VEGFR2[252] may also be produced in secreted forms by alternative splicing, the function of which seem to be to sequester VEGF and inhibit unwanted or excessive angiogenesis and lymphangiogenesis for example in the cornea[252].

The receptors have different binding affinity for their ligands. VEGF-A, for example, binds VEGFR1 with the highest affinity[253] and VEGFR3 the lowest[252].

It is still unclear today to what extent VEGFR1 participate in active VEGF signaling[244]. It seems that in cells expressing multiple VEGFRs, R1 act mainly as a decoy receptor, modulating the power of the signal transduced by the other receptors. However, in cells only expressing R1, there may be some positive signaling transmitted by this receptor as well[244].

Furthermore the choice of downstream signaling pathways also seems to differ between the different receptors. VEGFR2 primarily signal via the MAP kinases MEK and ERK2/3 as well as the PLCγ/IP3 pathway, important for cytoskeletal rearrangements involved in migration and proliferation, whereas the other receptors mostly transduce signaling via the PI3 kinase and PKB pathways, used for quiescent survival[254-255]. These different choices of pathways may be one reason for the different physiologic, pathologic and therapeutic effects of modulating one or another receptor specifically[46,112].

As VEGF-A is the most potent inducer of VEGFR2 – the primary receptor involved in angiogenesis and vascular permeability – its production is also highly regulated. The VEGF-A promoter is large, exhibiting many activation and repression domains, including hypoxia-responsible elements[256]. Thus VEGF-A is strongly induced by hypoxia.

There are indications suggesting that also VEGF-C as well as VEGFR3 may be induced, at least in some cell lines, in response to hypoxia[257], but the roles of this pathway in hypoxia-induced angiogenesis or vascular biology are not well studied.

VEGF signaling, and in particular VEGF-A/VEGFR2 and VEGF-C/VEGFR3 signaling are very important during development of the blood and lymphatic vasculatures respectively. In fact VEGF-A is one of few proteins which exhibit heterozygous embryonic lethality, indicating that the correct level of VEGF-A signaling is of crucial

importance for normal development and function of the embryonic vasculature[80,258-259].

One of the most obvious effects of VEGF-A is however not its effects on angiogenesis, rather on vascular permeability. VEGF-stimulated vessels are highly permeable due to pericyte shedding and induction of fenestrations (holes in the endothelium) which lead to increased extravasation of plasma, edema and in some cases hemorrhaging[260-261].
VEGF-A is, also an important endothelial proliferation and survival factor. Thus too low levels of VEGF lead to endothelial cell hypoplasia and apoptosis[262]. This also lead to a destabilized endothelium, edema and hemorrhaging. In adults, the physiologic levels of VEGF-A required for endothelial homeostasis are relatively low. Therefore induced VEGF-A/VEGFR2 signaling almost always leads to vascular pathologies.

Two examples of this is hypoxia-induced VEGF production in the retina leading to angiogenic retinopathy and the role of VEGF in tumor angiogenesis and metastatic spread (see earlier sections for details). In these situations it would thus be beneficial to inhibit VEGF signaling in order to treat the pathological angiogenesis.
In other cases such as during regeneration following ischemic insults to the myocardium or cerebral tissues, it would be beneficial to speed up the angiogenic response to hypoxia and thus potentially enhance VEGF signaling[11,263-266].
Efforts to use VEGF-A for therapeutic angiogenesis in cardiac ischemia have, however, not given any clinical benefits, due to the immature and poorly functional nature of VEGF-A-induced vessels[267].
Thus, in order to stimulate the growth of high quality arterial vessels one should adopt a broader view, considering the vasculature as a multi-cellular structure and thus targeting both endothelial and vascular mural cells[263,268].

1.8 NITRIC OXIDE BIOGENESIS AND SIGNALING

Nitric oxide (NO) is one of the smallest signaling molecules in the organism. It is both lipid and water soluble which makes it extremely diffusible and impossible to keep localized as it readily crosses both intracellular and plasma membranes[269].
NO is on the other hand highly reactive and has the potential to generate mutagenic and lipid oxidizing peroxy-nitrite ions, so oxidases in the cells quickly metabolize NO leading to very local and short lived signals[270].

NO can be produced in two ways – either passively by acid-catalyzed reduction of nitrite (NO_2^-), which in some cases may be a physiologically relevant pool of NO[271-273], or actively from arginine by the reductases known as nitric oxide synthase or NOS[274].

NOS exist in three different isoforms, which exhibit tissue specific localization. Neuronal NOS and endothelial NOS is expressed specifically in neurons and skeletal muscle (nNOS) and endothelial cells (eNOS) respectively, whereas the inducible form (iNOS) can be expressed by leucocytes and other cells, given the right stimulus[275].
NO acts in the central nervous system to modulate synaptic plasticity – that is in regulating which neurotransmitters should be released and in what amount[275].
However in the vasculature, the main function of NO is to stimulate guanylate cyclase activity, and thus induce cGMP formation and downstream signaling – usually leading to smooth muscle relaxation and vascular dilation[276-277].

Tissue hypoxia is known to stimulate eNOS and thus induce NO production leading to dilation of arteries[278-279]. This is believed to be the first line of defense against hypoxia leading to immediate increase of arterial perfusion in the hypoxic tissue.
This regulation of arterial vessel caliper is also important for physiologic regulation of vascular tone by increasing blood flow to active tissues such as the intestine during digestion or muscles during exercise[280-281].

Vascular dilation by NO is exploited in the treatment of obstructed arteries in the heart and other organs. In these cases nitroglycerin, an NO donor, may be given to increase arterial perfusion and thus restore oxygenation[282-283].

2 AIMS

The overall aims of this thesis were to study malignant and non-malignant angiogenesis, vascular biology and tumor cell dissemination and to develop, validate and characterize novel zebrafish angiogenesis models that could be used to this end.

The specific aims were:

I To evaluate the effects of known and previously unknown anti-angiogenic compounds in zebrafish models of angiogenesis (papers I, III and IV)

II To investigate effects of acute or chronic hypoxia on the zebrafish vasculature as well as determine the underlying mechanisms of, and find orally active molecules that can effectively either inhibit or increase these effects (papers I - III).

III To investigate mechanisms behind hypoxia-induced tumor cell dissemination in zebrafish and the role of the blood relative to the lymph circulation (papers II and III)

3 METHODS

3.1 EXPOSURE TO ACUTE HYPOXIA

Fish can tolerate very low oxygen tensions compared to mammals[92-94], and the effects of environmental hypoxia – that is hypoxia in the water – can therefore be studied. As responses to hypoxia are complicated and include both immediate physiological responses, fast but transient up-regulation of some genes as well as slower but prolonged up or down regulation of others[119-123], it is important to be able to separate acute hypoxic responses from chronic responses.

In order to study responses to acute hypoxia, we developed a semi-closed system where the water was pre-calibrated to a particular oxygen level before the fish was added. Furthermore the fish was added to a separated region of low volume in the setup, and circulation between this region and a high volume reservoir was established to continuously recycle the water [284]. These measures were taken to make sure that the oxygen levels were not changed noticeably by the addition or removal of fish in the system.
Stressed fish tend to hyperventilate and exhibit erratic swimming, which can increase their oxygen consumption and render the fish hypoxic. Therefore the part of the system that holds the fish during experimentation was hidden from the surroundings in such a way that the fish could not visually perceive any startle impulses, but the researcher could observe the fish from the top.

There are several ways to regulate the water oxygen concentration. One alternative is to perfuse the water with nitrogen gas, thus expelling dissolved oxygen[285-287]. In such a setup, an in-line oxygen sensor signals to a valve between the nitrogen tank and the air-stone submerged in the water bath, such that when the desired water oxygen tension in the water has been achieved, the valve shuts off the flow of nitrogen.
Another option is to pre-mix air or oxygen with nitrogen to the desired oxygen tension in a gas mixer, and then constantly perfuse the water with the mixed gas[288]. This approach reduces fluctuations in oxygen levels, which can be quite high with the former setup in small water volumes.

We used both methods in our acute hypoxia setup, and found no difference between the stability of the oxygen levels between the two.

3.2 EXPOSURE TO CONSTANT HYPOXIA

Prolonged or chronic hypoxia is probably more important in pathology compared to acute hypoxia, as most hypoxia-induced responses including angiogenesis, cell (de)differentiation and erythropoiesis require initiation of a gene program that may take several days or weeks to complete[14].

Thus, in order to study this in the zebrafish, we developed a setup for chronic hypoxia in which fish can be incubated, in theory, indefinitely, or at least for several weeks if needed.

Our setup was significantly simpler than the acute hypoxia setup mentioned above, and consisted of a single aquarium in which the oxygen in the water was regulated by controlled perfusion of nitrogen gas as mentioned above[141].

Compared to the two chamber system used in the acute hypoxia setup, which could only hold one fish at a time, here we focused on a system with a single larger aquarium in order to increase the number of fish that could be included in each experiment. This was important as chronic hypoxia experiments generally took at least a week to complete, whereas the acute hypoxia experiments could be done in less than an hour.

Fish as well as humans have adaptive systems that enable them to withstand hypoxia better when the oxygen levels are decreased slowly[92-94,289]. We have found that hypoxia-induced retinal angiogenesis in the adult zebrafish require oxygen levels at 10 % of fully air-saturated water or lower. However, acute exposure to such extreme hypoxia kills the fish within 15 min. Thus we acclimatized the fish by slowly lowering the oxygen levels from 20 % air-saturation, which is well tolerated, to 10 % over the course of not less than 36 hours[141], a procedure that has also been used by others[92-94,289].

Hypoxia experiments on cells are usually done in incubators, where the air inside of the incubator is constantly being exchanged with pre-mixed air of the right oxygen concentration[207,290]. When culturing adherent cells, the medium covering the cells is kept to a minimum such that the oxygen levels at the cell surface can be expected to be close to that of the air.

However, zebrafish (both embryos and adults) require more water and if without mixing oxygen gradients may form. Especially in the case of zebrafish embryos, which consume very high amounts of oxygen, diffusion of oxygen from the surface is too slow to compensate fully, and the oxygen level at the embryos will be lower than that in the air. This is especially true if several embryos are clustered together.
Therefore we used an adapted version of the chronic hypoxia setup in our experiments also on zebrafish embryos.

3.3 VASCULAR PERFUSION IN ADULT ZEBRAFISH

In most examinations of the vasculature, researchers perform histological techniques to visualize blood vessels in the tissue. However, not all blood vessels are perfused, which is particularly obvious in pathological and therapeutical angiogenesis[195]. Thus it is important to study perfusion of the vasculature, for example by injecting dyes such as fluorescently labled dextrans into the circulation.

In mice such perfusion studies are usually done by tail vein injections[195], alternatively in terminal experiments by intra-cardiac perfusion.
Zebrafish however have no major external veins that are well suited for injection, so one of the only ways to introduce compounds into the blood stream is by intra-cardiac injection[28]. This is, however, a more simple process in the fish compared to mice, as their heart is much more tolerant to the damage caused by the injection capillary[291]. When done by experienced researchers, more than 95 % of the fish survive the injection and are asymptomatic. Furthermore the incision done to open the pectoral and cardiac cavity does not need to be closed, as the tissue is elastic and close up by itself. Thus, the fish can be transferred directly back to clean water following the injection without suturing[96].

As the zebrafish heart is very small, the injections are done under a dissecting microscope with a small glass capillary mounted on a micro-manipulator. Using such a setup the fish can be injected within 2 min of being transferred to the microscope from the anesthesia, and no obvious bleeding occurs.

Smaller sized dyes (ie 70 kDa or smaller) can be used to evaluate the permeability of the vasculature. However, in order to see if cells may move through certain vessels or vascular structures, researchers may instead take advantage of available transgenic fish strains, where erythrocytes have been fluorescently labled[292].
This is a complementary tool to injecting fluorescently labeled beads or radio-active iodine labeled erythrocytes, the latter being a classical method for labeling blood cells[293]. The transgenic method is comparably much cleaner and un-invasive – and therefore preferred when available.

3.4 TUMOR CELL GRAFTING

In order to study tumor biology, there are in principle 3 ways to consider – all of which are available in zebrafish[45] as well as mice.

1) Tumorigenesis can be induced by treating the animals with carcinogens such as ENU, terpenes or other small organic mutagens[294].
2) Animals can be produced that are knock out for a particular tumor suppressor such as TP53[295], express high levels of oncogenes such as c-MYC[296-297], BRAF[298] or Ras[299], or are both deficient in tumor suppressors and express oncogenes at high levels[298].
3) Tumor cells, or pieces of tumor tissue can be grafted into the animal either in a similar position as where the original tumor is found (orthotopic grafting) or in a position where it is easily accessible, for example under the skin (xenografting)[300-303].

While possibly being the most artificial of these methods, tumor cell xenografting is a respected and widely used approach in studies on tumor biology, due to the fast assay time, amount of information that can be gathered from such experiments and the ease of either genetic or therapeutic manipulation of the tumor environment.
In zebrafish, tumor xenografting has been done both with mammalian tumor cell lines and zebrafish lines[300-303]. In adult zebrafish, syngenic zebrafish tumor cells are required in order to achieve successful grafting, as cells from other species are rejected[300-301]. However mammalian, including human cancer cells can be grafted into zebrafish embryos and immuno-suppressed juviniles[304-305] that does not yet have a fully active immune defense, and are still transparent[219,302-303,306-308]. The latter feature enables very detailed monitoring of the tumor microenvironment both in high spatial and temporal

resolution compared with mouse models, as the fish can be observed by intravital microscopy for several days[305].

When cells are grafted into zebrafish embryos, it can be done in several locations. Most commonly a subcutaneous-like area known as the peri-vitteline space between the dermis and the yolk membrane is chosen[219,303]. This area is preferred as grafting in the zebrafish brain, muscle or yolk either rarely give rise to successful grafting or cause severe side-effects to the developing embryo[309].

3.5 FIN REGENERATION

Non-malignant angiogenesis in adults are often studied in the context of repair or regeneration. In mice, a much used model of angiogenesis is the wound healing model, where a transdermal wound is created on the back of the mice, and the healing can be monitored over time[310-313].

In zebrafish, healing or regeneration can be studied by amputating a part of the tail fin and allowing it to re-grow[98-103]. Zebrafish have several fins that can be chosen, including large anal and dorsal fins, in addition to the tail fin[159]. However as the tail fin has two lobes it is possible to genetically interfere with regeneration in one lobe without affecting the other, thus having a counter-lateral, in-built control in the experiment[98-103].

Regeneration speed seems to depend on how much of the fin is amputated. In order to standardize the model, we therefore amputate just below the second last branch point of the fin rays. This corresponds to approximately the distal half of the fin.

As zebrafish are shoaling fish, it is important – especially if using males – to keep at least 6-8 fish together following the amputation. This will reduce aggression and avoid instances where one dominant fish may bite the fin of the others, thereby decreasing variability within the group and improving reproducibility.

3.6 HISTOLOGY

To visualize structures such as blood vessels in tissues, or to identify the number and identity of certain cell types and their location relative to others, histological techniques are usually done on either fixed or frozen tissue sections.

Histology can either be done with non-specific dyes such as hematoxilin, eosin or giemsa, or directed against a particular protein using antibodies raised against a specific epitope (immunohistochemistry, IHC).
The techniques are identical when using mouse or zebrafish tissues, but unfortunately many zebrafish epitopes are not sufficiently similar to those in mice or humans to allow antibody cross-reaction. Currently there are a lack of available specific antibodies which often makes IHC difficult in zebrafish[44].
The non-specific techniques usually give a broader view of the tissues, but it can often be difficult to see details such as for example the structure of the blood vessels.
Also non-specific staining is not recommended for thick, whole mounted tissue pieces used for 3D investigations.
In contrast – especially if using fluorescent probes – IHC can give very detailed information on the structures or cells targeted for investigation but do not reveal any information on other parts of the tissue.
Thus these techniques are complementary and both should be done side by side if a broad analysis of the tissue is needed.

3.7 MICROSCOPIC ANALYSIS

There are several types of microscopy available for studying zebrafish tissues both in vivo (intravital imaging) and when they have been fixed and mounted.
Usually in situ hybridization, used to detect mRNA in specific locations, as well as horse radish peroxidase IHC protocols, utilize colored probes that can be detected using non-filtered light microscopy.
However, using fluorescently labeled secondary antibodies in IHC or zebrafish lines harboring transgenically expressed fluorescent proteins, images need to be acquired using a fluorescent microscope.

In Fluorescent microscopy the exact wavelength of the light beam used to excite the sample is controlled, as is the detection of only one emitted wavelength. Therefore it is possible to distinguish signals from one fluorophore even in mixtures of several different ones.

Furthermore, light in defined wavelengths can be more highly focused meaning that flourescent microscopy gives higher spatial resolution or definition and very clear images of the labeled structures.

A more advanced type of fluorescent microscopy is confocal microscopy in which the light beams are highly focused lasers rather than incandescent light, and above all, where out-of-focus emitted light is removed by filtering through a pin-hole prior to detection. The filtration makes it possible to exclusively detect signals from a single focus plane, even in a thick tissue consisting of many focus planes.

With this technique researchers can thus obtain stacks of focused planes, which can be used to build 3D images of the samples.

This type of microscopy is therefore recommended when examining thick samples, such as whole zebrafish embryos or manually cut, whole mounted tumor samples.

3.8 VIDEO ANALYSIS

Today most cameras used for photography can also take video sequences. However, cameras specialized for obtaining video usually gives higher quality images of moving objects such as blood cells inside blood vessels[144,284].

For examination of blood flow in the adult zebrafish we therefore used a specialized digital video camera mounted on a microscope. Such video sequences had sufficiently high quality that the center of blood cells moving at high speeds could be accurately determined in still frames.

By plotting the x,y coordinates of a blood cell at different times (0.04 sec between still frames), this gave the opportunity of measuring the blood flow speed accurately. By further determining the caliber of the vessels, also flow, blood pressure, vascular resistance and other cardiovascular parameters can be calculated for each vessel.

As zebrafish are too small to be equipped with telemetry probes or similar, such videoscopic techniques are valuable substitutes for measurements of cardiovascular

physiology and even for visualizing perfused blood vessels in absence of a fluorescent or confocal microscope.

4 RESULTS

4.1 HYPOXIA-INDUCED RETINAL ANGIOGENESIS IN ADULT ZEBRAFISH (PAPER I)

Retinopathies such as ROP, DR and AMD are seriously debilitating disorders collectively affecting millions of patients worldwide[145]. As the most advanced stages of retinopathies are associated with hypoxia-induced, VEGF-mediated retinal angiogenesis, the best therapies available are anti-VEGF antibodies[145,153,314], which unfortunately, however, require regular delivery by invasive intra-occular injections[315], in order to reduce the amount of drug used per treatment occasion and to eliminate the side effects associated with systemic anti-VEGF treatment.
Identifying orally active pharmaceuticals which interfere with retinal angiogensis have been hampered by a lack of available animal models which closely recapitulate the clinical symptoms of neovascular retinopathy, especially retinal hypoxia[44,141,159].

We were interested in studying if exposure to hypoxia would result in retinal angiogenesis in the adult zebrafish, and if so, if this angiogenic response could be influenced by the addition of orally active chemical compounds to the water.
Such a model system could then possibly be exploited to discover novel orally active pharmaceuticals for treatment of neovascular retinopathies.

First we studied the retinal vasculature in the adult zebrafish, and found that it is quite similar to that of mice, although much simpler (see figure 2).
Similar to mice, zebrafish have a vasculature lying in association with the vitreal surface of the retina, originating from a central optic artery (OA) which gives rise to 4-7 primary branches which sub-divide several times to cover the entire inner surface of the retina.
Whereas in mice the vessels also penetrate into the retina, in zebrafish they are only present in one layer on the vitreal surface.
In mice, the capillary network is formed almost immediately after arteries have branched off the central optic artery, and thus capillaries are present also in the center of the optic disc. Veins are also present in the entire retina, collecting the post-capillary

blood and bringing it back to the central retinal vein (CRV) which runs alongside the central retinal artery (CRA) and the optic nerve[233] (see figure 2).
In zebrafish, however the capillary region is sharply defined by the area where the arteries anastomose with extensions of the circumferential vein at the periphery of the retina. All retinal blood supply is collected by this vein, which does not run back to the center of the retina but rather brings back the blood via another route on the outer surface of the retina.
The structure of the retinal blood vasculature is thus a remarkably simple tree-like structure, which makes this vasculature highly amenable to investigations on angiogenesis, as any small difference such as emergence of small vascular sprouts are readily detectable.

Next we developed a setup for chronic exposure of zebrafish to hypoxia, as described in the methods section. We found, using this setup, that zebrafish quickly became adapted to the hypoxic environment. Thus a hypoxic level which almost kills the zebrafish during initial exposure became well tolerated after several hours, and the oxygen level could be lowered further.
After a period of 1½-2 days we were thus able to have zebrafish surviving at only 10 % air saturation (820 ppb at 28 ^{o}C, water) which would kill the fish without the gradual adaptation.
Following this protocol we exposed adult *fli1:EGFP* transgenic zebrafish to 10 % air saturated water for 12 days and discovered that the retinal vasculature had undergone a clear angiogenic expansion, specifically in the capillary area.
This capillary angiogenesis was readily quantifiable as an increase in the number of angiogenic sprouts, branch points per vessel, inter-capillary distance and vascular density in the capillary region. At high magnifications it was even possible to see very thin fibers, known as filopodia or tips, being projected from the leading cells of the angiogenic sprouts (see figure 1). Thus this assay could also be used in the study of filopodia and tip cell dynamics in vivo.

In order to study the dynamics of this angiogenic response to hypoxia, we took fish from the hypoxic environment at different time points during the experiment.
We found that, compared to controls in normoxia, fish exhibited significant angiogenesis measured by all of the above mentioned four parameters already after three days of exposure. However after 6 days the changes were more pronounced and

not significantly different from when the fish were exposed for 12 days. Thus we decided to continue to expose the fish for 6 days in the future experiments.

We next studied what level of hypoxia is necessary for the induction of retinal angiogenesis. As in the clinical situation, angiogenesis – for example in angiogenic DR and AMD – is usually only present in advanced disease in response to retinal ischemia[7,154], we expected that severe hypoxia was needed in order to induce retinal angiogenesis. Indeed we found that 20 % air saturation was not sufficient to generate more than a few angiogenic sprouts which did not continue to mature into lumenized vessels.

One of the aims of this study was to be able to evaluate orally active chemical compounds. We thus wanted to test if known anti-VEGF compounds which have documented anti-angiogenic effects in mice would similarly have anti-angiogenic effects in our assay.

We tested two compounds with anti-VEGFR2 activity, sunitnib, which is currently used clinically in the treatment of certain tumors, and ZM323881 – the latter being the more specific. Both compounds showed strong anti-angiogenic effects at very low concentrations, 0.5 and 1.0 μM respectively.

In drug treated zebrafish, hypoxia-induced retinal angiogenesis was practically non-existing compared to fish that were exposed to vehicle alone. Both of these compounds furthermore showed no toxic effects in this assay. In fact less fish in the drug treatment groups succumbed to hypoxia during the exposure period, and the fish looked healthier and more active compared to the vehicle treated groups.

As we found that this assay was suitable to study tip cell dynamics, we next investigated the role of the Notch-signaling cascade during hypoxia-induced retinal angiogenesis.

Notch had previously been reported to inhibit tip cell formation in mice during development of the retinal vasculature[49], and in tumors[50-51], but it was not known how hypoxia influences the effects of Notch in this regard.

We found that under normoxia, inhibition of Notch signaling by the γ-secretase inhibitor DAPT indeed led to marked tip cell formation in the capillary region of the retina. These tip cells did not go on to form mature, lumenized and functional vessels, which corroborated the reported effects of blocking Notch signaling in mice[51].

However, when fish were exposed to both hypoxia and DAPT, the region of vascular tip cell formation changed radically. In this situation we found a profound induction of tip cells primarily in the arterial region which otherwise in normoxia+DAPT and hypoxia+vehicle had a smooth phenotype. The capillary region however looked like that in hypoxia+vehicle, and thus there were no extra tip cells present there. Interestingly the arterial sprouts induced by the combination hypoxia and DAPT seemed to be larger and possibly lumenized and functional (see figure 1) compared to the very thin fibers induced by DAPT in normoxia.

The details behind DAPT induced arteriogenesis in hypoxia needs further examination, and is a subject of ongoing research in the lab.

4.2 HYPOXIA-INDUCED NITRIC OXIDE OPENS A LYMPH-TO-BLOOD SWITCH IN FISH (PAPER II)

Prior to our initial investigations of the existence, function and regulation of the lymphatic vasculature in zebrafish, biomedical researchers believed that lymphatics were not present in fish. However, we found reports indicating that the vasculature in the distal parts of the fins and possibly the skin share some of the characteristics of lymphatic vessels[174-175,316, and references therein].

As the fins of the adult zebrafish are thin and transparent, we first studied the flow in the fin vasculature of anaestisized zebrafish. We found that the distal fin vessels of the zebrafish are not perfused with blood, as it was rare to find any cells in this vasculature in relaxed fish.

Furthermore when we occasionally found a cell in these vessels it was flowing very slowly compared to cells in the blood capillaries in the proximal parts of the fin. These findings indicated that these vessels may indeed be lymphatic in nature. However, in some cases where I had to chase the fish almost to exhaustion before I could get them out of the aquarium, we saw that these putative lymphatic vessels were packed with cells, flowing at a much higher speed.

Such a phenomenon had not been described in the past, so we were interested in knowing first whether these vessels were indeed lymphatic vessels and second what happened in the exhausted fish that gave rise to the putative lymphatics being perfused with blood.

To study the first point, we looked for the thoracic duct in the zebrafish. This is the major lymphatic vessel in mammals, and it is located in association with the extended aorta and the cardinal vein just ventral to the spine. We did serial cross sectioning of the analogous region in the fish and found three vessels.
The most dorsal vessel had a thick wall and is filled with blood cells, thus we identified this vessel the dorsal aorta (DA).
Just ventral to the DA, we found a blood filled large caliber vessel with thinner walls, which we identified as the posterior cardinal vein (PCV).
Finally we found a third vessel between the two with very thin walls and devoid of blood cells.
Immunohistochemical staining revealed that this vessel was positive for the mammalian lymphatic cell marker Prox1[317].
Finally electron microscopy revealed that this vessel had a very thin, single layer endothelium and scarce if any basement membrane – all characteristics of lymphatic vessels[318-319].
We thus defined this vessel as the zebrafish homologue of the thoracic duct (TD).

We were interested in investigating whether the vessels in the fins would empty into the thoracic duct, which would be proof that they belong to the same (lymphatic) vascular network. To do this, we took advantage of a different fish – the glass catfish (kryptopterus bicirrhis) – which has a completely transparent body[320].
Similar to the zebrafish, this fish also has a DA, PCV and TD which histologically are identical to the zebrafish analogues, and are located in the same region. As all vessels in the body of this fish can be accurately tracked, we were able to re-create a map of the peripheral blood and lymphatic vasculatures.
We confirmed previous findings (John Fleng Steffensen et al. Acta Zool, 67, 193-200) that all vessels which extend more than half way into the fins ultimately drain into either the thoracic duct or a second major longitudinal lymphatic vessel called the collecting lymphatic vessel.
Collectively, these studies positively identified the existence of lymphatic vessels in both the zebrafish and the glass catfish, and indicated that the vessels in the fins are in fact lymphatic in nature.

In the zebrafish we further found that the vessels in the distal part of the tail fin were

only weakly positive for VEGFR2 and stained positive for Prox1, adding to the evidence that these vessels are in fact lymphatic[321].

In order to functionally identify these vessels as lymphatic, we found that

1) There are little or no cells traveling in these vessels under normal physiological conditions

2) The flow is very slow and

3) Dye, injected into the blood stream via the heart, did not readily flow into these vessels.

Thus, both histologically and functionally, these distal fin vessels are similar to mammalian lymphatic vessels.

However, as mentioned above, in stressed fish these distal fin vessels were filled with fast flowing blood.

Initial attempts to find the stressor responsible for this phenotypic change, we found that it was not adenosine receptor mediated, as the pan-adenosine receptor antagonist theophylline[322] did not inhibit stress-induced lymphatic perfusion. We also found that neither warm nor cold water had any effect.

We then thought that hypoxia may be a factor, and instead of stressing the fish, we submitted them to acute 30 min. exposure to 15 % air saturated water, which is considered highly hypoxic for zebrafish.

We found that both blood cells and dye injected in the blood stream were found immediately in all distal fin vessels upon microscopic examination less than a minute after the fish was moved from the hypoxia chamber/injection sponge respectively. Thus hypoxia alone was able to induce the switch of lymphatic to blood-like vessels.

As we were not able to positively identify the cell type present in the lymphatic vessels under hypoxia by the examinations done under the light microscope, we utilized *fli1:EGFP;gata1:dsRed* double transgenic fish[180], where a red fluorescent protein is produced under the erythrocyte-specific promoter *gata1*, to make sure that the lymphatics were in fact perfused with erythrocytes.

We found no *gata1:dsRed* positive erythrocytes in the distal fin vessels under normoxia, but many when the fish were exposed to acute hypoxia.

Thus we identified zebrafish lymphatics as a backup circulation for blood perfusion under hypoxic stress.

Next in order to investigate the structural mechanism by which lymphatic vessels were allowed to be filled with blood, we again turned to the glass catfish.

As both these and other fish have a specialized structure previously termed inter-arterial anastomoses, linking (primary) arteries to lymphatic (secondary) vessels (John Fleng Steffensen et al. Acta Zool, 67, 193-200), we thought they may be important regulators for the observed hypoxia-induced switch.

We confirmed previous findings that these structures are indeed a starting point for the lymphatic vessels in the glass catfish (John Fleng Steffensen et al. Acta Zool, 67, 193-200).

Downstream of these structures the vessels ran towards the extremities, including the fins, divided several times and ultimately gave rise to all the vessels in this tissue. Furthermore, we found that under normoxia, these structures were tightly curled up in a cork screw like shape (see figure 4), not allowing more than an odd cell or two to enter through them, and drastically reducing the flow speed in the downstream lymphatics compared to the flow speed in the arteries from which they arise.

Under hypoxia however, these curled structures became dramatically dilated and straightened and adopted a function more like that of an arterial branch, not restricting flow in any way. This led to the swift filling of the collecting lymphatics including the thoracic duct with blood cells. These vessels also became dilated, probably due to the increased intravascular blood pressure.

Due to their role as gate-keepers between arteries and lymphatic vessels we coined the term arterial lymphatic conduits (ALC) to these structures.

ALCs have never been described in zebrafish, so we looked for them in the region where they are present in the glass catfish, and found that they are in fact present in very high numbers sitting either on the posterior part of the dorsal aorta itself (see figure 4), or on primary branches in the anterior part, but close to the DA.

Also in the zebrafish these ALCs become dilated and straightened as a response to acute hypoxia, indicating a similar mechanism behind lymphatic perfusion in both fish species.

ALCs have been described to be associated with smooth muscle in the eel[29], and NO is an important mediator of arterial smooth muscle cell relaxation[323-324]. We therefore wanted to know if NO plays a role in hypoxia-induced ALC opening and lymphatic perfusion.

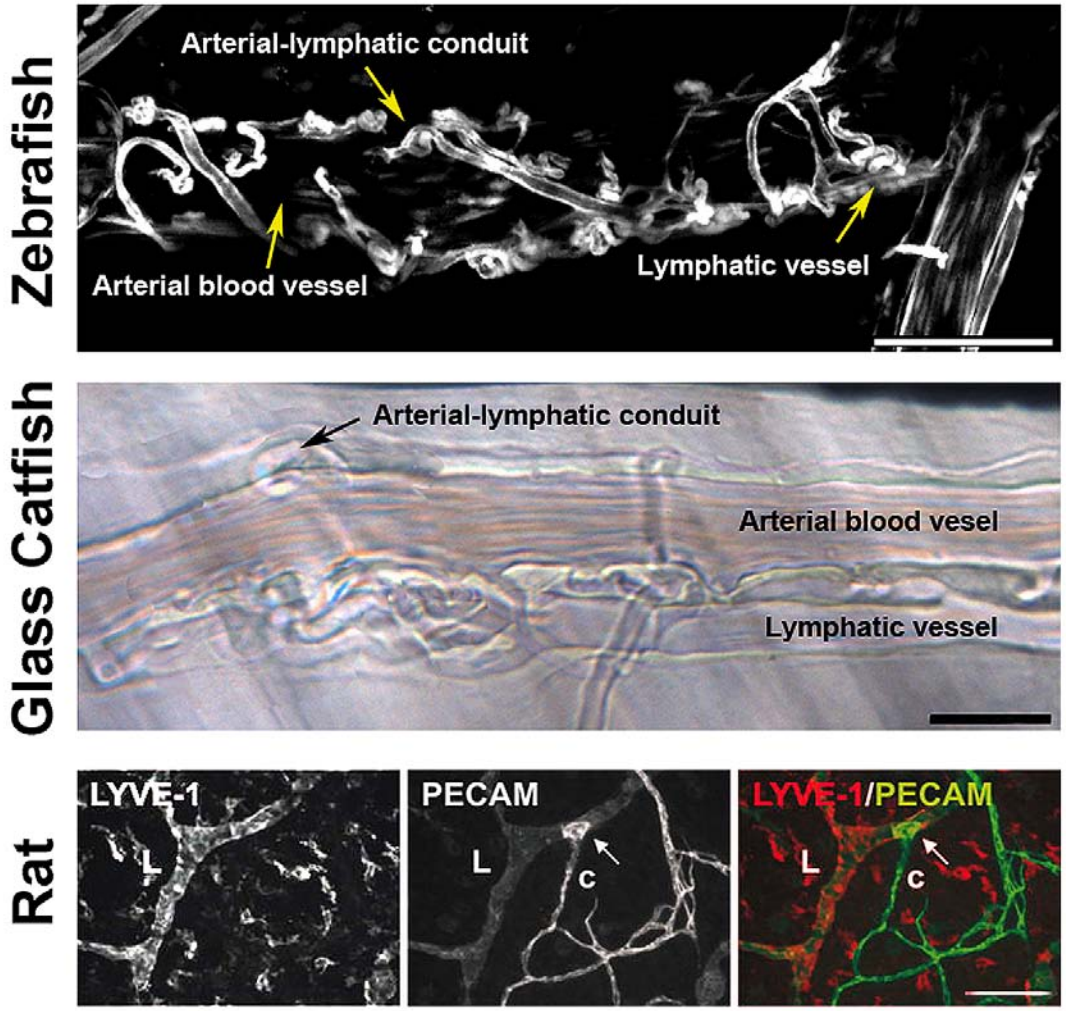


Figure 4: Arterial-lymphatic conduits (ALCs) in the zebrafish, glass catfish and rat. Top: ALCs at the posterior dorsal aorta of *fli1:EGFP* adult zebrafish. Middle: ALCs in the adult glas catfish. Bottom: connections between LYVE-1 postive lymphatic and PECAM positive endothelial cells in the rat mesentery[356].

Initially we found indications that NO production is present at the ALC region, since hypoxia-exposed glass catfish, infused with the NO-reporter DAF-DA, showed positive signals in a location that corresponds to where ALCs are present.

To study the role of NO, we undertook a pharmacologic test of small chemicals that interfere with physiological NO metabolism and signaling to probe their effects on lymphatic perfusion in the distal tail fin of the zebrafish.

In normoxia, incubation with the NO-donor sodium nitroprusside (SNP) alone led to a similar phenotype as hypoxia, whereas the NO scavenger c-PTIO blocked lymphatic perfusion in hypoxia.

This was similarly inhibited by blocking NO biogenesis via eNOS with the blockers L-NMMA and L-NAME, whereas the inactive stereo-enantiomer D-NAME had no effect. Adding back NO by co-administration of SNP restored lymphatic perfusion with

L-NMMA under hypoxia.

As most of the smooth muscle relaxation stem from a pathway where NO-induced guanylyl cyclase activity and cGMP production plays a central role[276-277], we incubated the hypoxia exposed fish with ODQ, a guanylyl cyclase-inhibitor, and found that the hypoxia-induced perfusion was blocked by this compound. In this case, SNP could not restore the phenotype.

Together these findings strongly argue that NO is the main mediator of hypoxia-induced ALC opening and lymphatic perfusion in fish.

4.3 HYPOXIA-INDUCED VEGF-VEGFR2 SIGNALING DRIVES METASTASIS IN A ZEBRAFISH XENOGRAFT MODEL (PAPER III)

Tumor hypoxia is known to be associated with a more highly metastatic phenotype[325], but the mechanism is not known as it is difficult to study in conventional mouse models. Furthermore, while tumor hypoxia can be detected as present or absent in murine models, it is not homogeneous in neither time nor space and not controllable[326].

Our aim was to develop a tumor xenograft model in transparent zebrafish embryos in which genes in either the tumor cells or the host embryo can be readily up- or down regulated, and which can be used to study the role of hypoxia on early stages of tumor cell dissemination and metastasis.

Initially we developed the tumor cell implantation protocol (see figure 5 top row). Being inspired by other published protocols on this topic[302-303,309], we chose to inject tumor cells into the peri-vitelline space of 2 days old fish embryos.

The cells were prior to injection labeled in vitro with the red fluorescent dye DiI and we found that approximately 100 cells gave a modest size tumor in embryo, which were large enough to survive and communicate with the host, but not so large that it affects its development.

Testing different murine tumor cell lines, we found that the fibrosarcoma cell line T-241 gave a rise to a coherent isolated cell mass, which was non-invasive and grew mostly in situ. The more aggressive Lewis Lung Carcinoma (LLC) cell line, however, gave rise to less coherent and much more mobile and invasive tumors. These differences mirrored the reported differences in aggressiveness and metastatic potential of these cell lines in murine xenograft models[195,327].

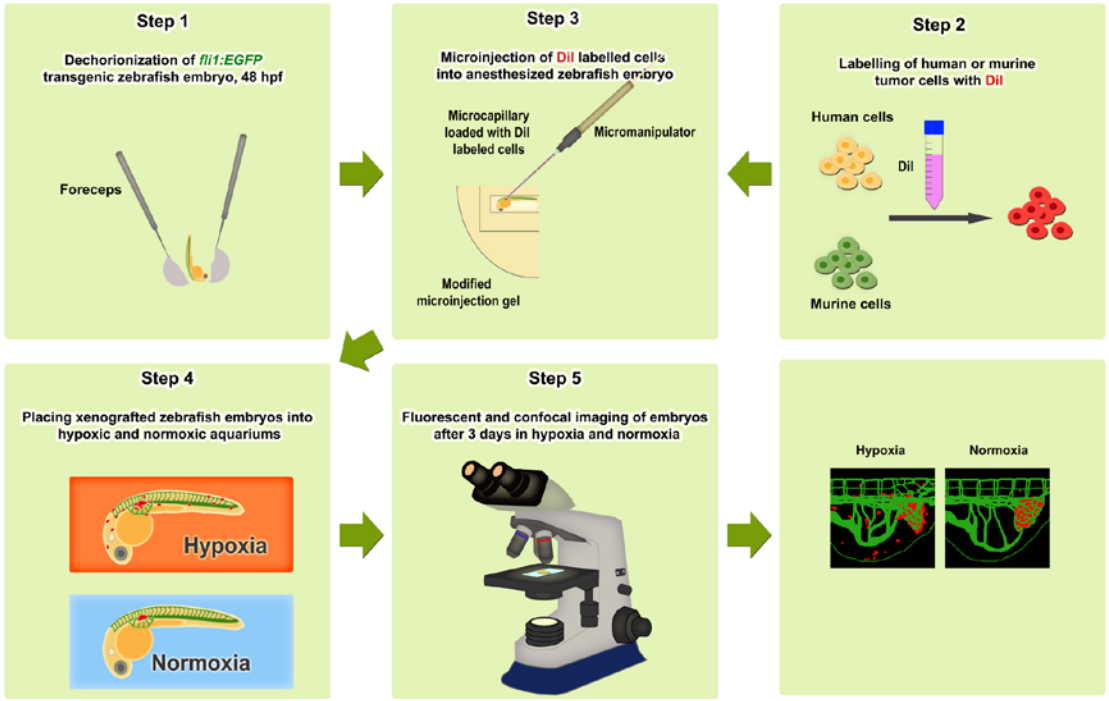


Figure 5: The tumor cell xenografting procedure. From Rouhi, P et al, Nat. Proc. In Press

Extending the use of this model to clinical samples we found that the low metastatic human ovarian carcinoma cell line OVCAR 8[328] led to tumors growing mostly in situ and send only very few cells into peripheral parts of the embryo, whereas the highly metastatic human MDA MB 231[329] breast cancer cell line hardly form a tumor mass at all, but instead most of the injected cells disseminated almost immediately after injection to all parts of the embryo.

To study the role of hypoxia in tumor cell dissemination we used the low metastatic T-241 cell line and subjected grafted embryos to either hypoxia or normoxia, in a setup that were modified from what we had previously published[141].

After submitting the embryos to 7.5 % air saturation for 3 days, we observed a drastic increase in the number of both locally but also distally disseminating cells (see figure 5 bottom row) indicating that the tumor cells disseminate both via local invasion of the surrounding tissue and by penetrating the tumor vasculature and being transported through the blood stream.

Furthermore tumor angiogenesis was also more pronounced in hypoxia, indicating that the imposed hypoxia elevated the production of angiogenic factors by the tumor cells, which could functionally activate signaling by receptors on zebrafish endothelial cells. These results were repeated with LLC cells, which gave similar results.

As VEGF is induced by hypoxia, we implanted T-241 or LLC tumor cells which have

been genetically engineered to express high levels of human VEGF-A[46] and compared their metastatic properties to control cells transfected with the empty plasmid.
We found that high expression of VEGF-A by the tumor cells phenocopied the hypoxic induction of both tumor angiogenesis and dissemination/metastasis.
Furthermore, as in hypoxia, elevated expression of VEGF-A by the tumor cells also led to pronounced pericardial and peri-vitelline edema, indicating that human VEGF-A can activate signaling by zebrafish receptors to increase the permeability of the blood vessels. Again these effects were similar in both T-241 and LLC tumor cell lines.

Looking into the dynamics of VEGF-A-induced tumor cell dissemination, we found that cells started to disseminate from the primary tumor after approximately 4 days post injection. 6 days post injection many cells had disseminated and seeded micro-metastasis in the periphery, and after longer time the mortality of the embryos increased to an extent that it was not possible to continue the experiments.

As we found that tumor-VEGF-A acted on the host vasculature, we took advantage of two unique features of the embryonic zebrafish model to further investigate the role of VEGF in tumor cell dissemination.
First we added the tyrosine kinase inhibitor sunitnib to the water, to see if inhibition of VEGF receptors (among others) could block the tumor-VEGF-A-induced dissemination. Second we injected a morpholino which specifically targeted VEGF receptor 2, immediately after fertilization of the fish eggs, to investigate if this was the only receptor important for the observed dissemination.
We found that both approaches led to a drastic reduction in the number of disseminated tumor foci – which almost returned to the level seen with non-transfected tumor cells. Also intra-tumoral blood vessels were practically ablated in the treated groups and pericardial edema was minimized, indicating that VEGF-A signaling via VEGFR2 was the primary driving force behind tumor-VEGF-A-induced tumor cell dissemination as well as systemic edema in the zebrafish.
Interestingly, the levels of sunitnib and VEGFR2 morpholino used in this study were kept low enough not to affect developmental angiogenesis as the non-tumor vasculature remained unaffected in treated compared to non-treated embryos.
Thus the tumor-induced angiogenesis seems to be more sensitive to VEGF inhibition than the growing vasculature in the developing embryo.

Finally, as hypoxia is up-regulating multiple pathways, we investigated the importance of VEGF for hypoxia-induced tumor cell dissemination. We used sunitnib to inhibit VEGF receptors under hypoxia, and found that hypoxia-induced tumor-angiogenesis, -dissemination and -metastasis as well as pericardial edema were essentially eliminated by this compound.
These results collectively indicate that hypoxia-induced VEGF-A production by tumor cells act on host endothelial VEGFR2 to induce tumor angiogenesis and this pathway is of major importance for early steps of tumor cell dissemination and metastasis.

4.4 PI3 KINASE IS AN IMPORTANT, NOVEL TARGET FOR ANTI-ANGIOGENIC THERAPY IN RETINOPATHY (PAPER IV)

As current anti-angiogenic treatments for retinopathies require repeated, invasive and expensive intra-occular injections[315], it would be of great benefit to the patients if equally potent orally active drugs could be discovered.
However, many orally active drugs are associated with systemic side effects, as currently available drugs do not target the pathologically expanding retinal vasculature specifically[158].

In an attempt to find drugs that affect retinal angiogenesis specifically, we undertook a small screen for both small molecules and proteins that were thought to interfere with retinal angiogenesis during development of the fish embryo.
Among the factors tested, we found that the PI3 kinase inhibitor LY294002 inhibited normal formation of the retinal vessels between 2 and 5 days of development, but did not have any effect on the development of the vasculature in the rest of the embryo during this time period.

The effects of LY294002 on the developing retinal vasculature were concentration dependent with increasingly inhibited retinal angiogenesis between 7.5 μM and 20 μM. Concentrations higher than 17.5 μM, however, led to systemic toxicity, so the intermediate concentration 10 μM were chosen for future experiments.

We found that LY294002 was effective mainly between day 2 and 3, as adding the drug to the water just during this period led to significant inhibition of retinal

angiogenesis when examining the embryos at day 5.
Also, even though the effects were similar when the drug were added between day 3 and 4, keeping the embryos in the drug from day 3 to the end of the experiment at day 5 did not lead to significantly inhibited angiogenesis.
Thus PI3 kinase activity in the retinal vessels is important during the second day of development, and blocking the activity only during this time window leads to inhibited retinal angiogenesis later during development.

As a control that LY294002 in fact acted by specifically inhibiting the PI3 kinase pathway, we tested the effect of blocking an important downstream signaling component – PKB/Akt – by the structurally unrelated inhibitor SH6[330]. SH6 were able to phenocopy the morphologic alterations in the hyaloid vasculature at day 5, indicating that this pathway was indeed important for correct patterning.
To further test this, ectopic hyaloid vessels which develop in the plexinD-mutant fish out-of-bounds[57], did not develop when embryos were treated with LY294002.
The PI3 kinase-Akt pathway is thus a general mechanism for angiogenesis in the retina, also in plexinD mutants.

One concern using anti-VEGF therapy for treatment of retinal angiogenesis is that VEGF also has neuroprotective functions[331] and thus blocking VEGF may lead to neuronal death[332]. To investigate if the PI3 kinase pathway also plays an important role in neuroprotection, we investigated the visual function of zebrafish larvae at 5 days post fertilization in two different ways.
First electroretinography was done to measure the function of photoreceptors following stimuli with white light at different flash intensities. We found that the b-wave amplitude was unaffected by LY294002 treatment, indicating that photoreceptors function normally.
Also, retinal histology indicated that – even though early exposure to LY294002 did induce a few apoptotic nuclei and vacuoles in the retina – when the drug were added at day two, the retina develops normally and only the retinal vasculature is affected.
Second we tested the response to perceived vision cues following intraocular administration of LY294002. Intraocular administration was chosen to eliminate weak edema that was present in fish treated by adding the drug to the water. Intraocular injection of the drug, however, did not lead to any systemic side effects, and retained its anti-angiogenic abilities on the retinal vasculature.

Following intraocular injection of LY294002, the response to vision cues in un-anaestisized fish placed in a rotating drum with alternating white and black stripes, was unaffected compared to both non-injected and vehicle-injected controls.

Zebrafish fins regenerate following partial amputation, in an angiogenesis-dependent manner[28], similar to the wound healing process in mammals. Thus blocking angiogenesis would lead to inhibition of regeneration, which would be quantifiable as the length of the regenerated fin tissue[28,333].

As a test of whether LY294002 also has anti-angiogenic effects in adult animals, we subjected fin amputated adult zebrafish to water containing 10 μM LY294002 or vehicle.

We found that in the presence of LY294002 adult zebrafish dorsal, anal or caudal fins did not regenerate as quickly as in the vehicle controls. Furthermore, the vasculature in the regenerated fin was less developed in the LY294002 group compared to controls. Interestingly we found more tip cells being formed during regeneration in the LY294002 treated group, compared to controls, but the importance and mechanism behind this phenomenon was not further studied.

These findings indicate that LY294002 indeed also have anti-angiogenic potential in adult animals.

5 DISCUSSION

Zebrafish hold several advantages over mice as an animal model system in developmental and molecular biology[334-335] and pharmacology[336-337]. Their use as models to study physiology has, however, been limited[338].
We have shown in the series of papers presented in this thesis that both adult and embryonic zebrafish are in fact very well suited to study physiological and pathological aspects of tissue regeneration and responses to hypoxia[44,122,141,159,219,284].

Adult and embryonic zebrafish alike have been frequently used for pharmacological and toxicological tests of small molecular weight compounds[339]. One example of this is the so called chemical genetics models in zebrafish[340-341].
Chemical genetics is the process of screening for new pharmaceutical leads for further development against diseases that are closely mirrored in an available zebrafish mutant line[78] or by injection of a morpholino against or mRNA overexpressing proteins important for that disease[342]. The benefit of using zebrafish in such studies is due to the ability of the fish to readily take up pharmaceuticals added to the water either transdermally or by via the water-blood interface in the gills[343].
While it is clear that zebrafish do have great qualities in terms of medium-high throughput screening in this regard[344], their pharmacodynamics such as Absorption, Distribution, Metabolism and Elimination (ADME) probably have different kinetics than in mammals[343,345]. Thus it is likely that in many cases the IC50, maximum tolerated dose and other important characteristics of new pharmaceuticals are different in zebrafish compared to mice and humans.
Another drawback is that some compounds with very bad solubility in water at neutral pH, are not taken up in zebrafish, but may never the less be very effective in mammals. As both we and others are using our disease models in the zebrafish to identify new potential drug candidates, these pharmacokinetic considerations have to be kept in mind, and the drugs should be validated in mouse models if available to increase the likelihood that they may also be active in patients.

5.1 BENEFITS AND DRAWBACKS OF THE HYPOXIA-INDUCED RETINAL ANGIOGENESIS MODEL

As tissue hypoxia is a dynamic, heterogeneous and poorly controlled state, it is very difficult to study graded hypoxia responses in mammalian disease models[326].
We have shown that zebrafish offer unique possibilities to study the effects of hypoxia on vascular physiology and pathology in a controlled manner.
Another benefit of the fish models we have presented here is the temporal control and continuous in vivo observations that are featured by either transparent embryos or transparent adult glass catfish.
Using these model systems one can see how fast a particular tissue respond to a broad range of hypoxia levels, and observe this response in real time under the microscope. Furthermore the possibilities of using microinjection techniques in the zebrafish embryo to knock down or over-express genes of interest open the door to detailed studies on the dynamics and effects of these genes in either the host or in a tumor environment. Hopefully this approach could be expanded to the hypoxia-induced retinal angiogenesis assay in the future – in a similar way as it is currently done in the regenerating tail fin[98-103] – also allowing the dissection of this response in molecular detail.

There are some important differences between the zebrafish and mammalian retinal vasculature[27,141]. Zebrafish does not have choroidal blood vessels, as it seems the outer retina can meet its oxygen demand by direct absorption from the water, which makes the retinal vasculature simpler than in mammals. This simplicity does not, however, mean that the retinal vasculature respond differently to stimuli that trigger pathological events in patients.
For example, the zebrafish retina and retinal vasculature respond to high-blood sugars in largely the same way as found in diabetic retinopathy[158]. Also our own finding is that hypoxia-induced retinal angiogenesis[141] in the zebrafish closely mirror pathological hypoxia-induced angiogenesis found in patients with AMD and DR.

The zebrafish retinal vessels are covered with mural cells, as they are in humans[27]. A hallmark of retinopathy, linked to mural cell coverage, is vascular leakage[346]. Therefore it is probable that the mural cell coverage of the vessels in retinopathic patients are impaired or disrupted.

As in the tumor situation, vascular pericyte re-installment may improve perfusion, lower hypoxia and leakage as well as the subsequent edema and hemorrhaging[233,347-348]. Such a strategy may therefore be an important step in treating retinopathy, and the zebrafish model of hypoxia-induced vascular retinopathy we have established would be well suited to study how hypoxia and hypoxia-induced signaling factors are involved in establishing mural cell coverage of new vessels, alternatively mural cell shedding of existing mature vessels prior to endothelial sprouting during retinal angiogenesis.

In this regard it would be a big improvement of the model if a zebrafish strain containing a non-green fluorescent reporter specifically in mural cells could be developed. Such a strain would significantly contribute to studies not only on the role of mural cells in retinopathy but also the dynamics of mural-endothelial cell interactions during angiogenesis and in hypoxia in general.

Also in the study of therapeutic angiogenesis for treatment of ischemic disease including myocardial and cerebral ischemia, mural cell responses in hypoxia is of great importance. As beneficial therapeutic outcome is dependent on inducing stable arterial blood vessels, mural cell and especially vascular smooth muscle cell investment of the therapy-induced vessels is critical[263-266].

However, as mentioned in the introduction, one of the first steps of angiogenesis is breakdown of the basement membrane and shedding of the mural cells before sprouting of the endothelium can occur[349].

It seems from our retinal angiogenesis model that hypoxia is not sufficient to shed the vascular smooth muscle cells from the arterial part of the vasculature, as the sprouting angiogenesis is only found in the less densely covered capillary region. However, Notch signaling may be important for endothelial-smooth muscle cell interactions, as blocking Notch suddenly allow massive arterial sprouting in response to hypoxia.

This hypothesis is strengthened by the finding that Notch signaling is important in specifying arterial endothelial cells[350]. As vascular smooth muscle cells particularly associates with arterial endothelial cells, it seems likely that these two processes are intertwined.

It will be interesting in the future to study the role of Notch in hypoxia and vascular smooth muscle cell biology as well as the implications for arteriogenesis in vivo.

A potential problem with the zebrafish hypoxia-induced retinopathy model is that hypoxia is global and not localized to specific patches in the retina. Thus, the hypoxia-induced angiogenic factors in the zebrafish model does probably not generate a gradient which implies that the angiogenesis is not directed towards an ischemic area in particular but instead relatively uniform in the entire retina.
This is an important difference from the human pathology, as angiogenesis towards a gradient of angiogenic factors may be more efficient compared to angiogenesis when no such gradient exist.
Also the disease history of retinopathy involves other steps prior to establishment of retinal hypoxia, which could influence hypoxia-induced signals[7,151,154]. These steps include edema, atrophy, inflammation and other types of immune system involvement[351-352].
Some or all of these steps are undoubtedly involved in the zebrafish model at later stages though, but it is not know if the pathological sequence of steps is important in retinopathy as it is in other pathologies such as cancer.

5.2 TO BE OR NOT TO BE A LYMPHATIC VESSEL IN FISH

The existence, anatomy, properties and function of zebrafish lymphatics is a controversial issue[173-174]. There is little doubt that fish have lymphatic-like vessels, but whether they are identical to lymphatic vessels in mammals seems to be a matter of a more or less rigorous definition of the term.
There is scattered evidence that zebrafish lymphatics to some extent may originate in blind ended vessels in the skin[180] (Schülte-Merker S, unpublished observations), but it now also seems evident that they arise from direct anastomosis, via ALCs, with the arterial blood supply (see figure 4)[29,173-176].

The lymphatic vessels we have described are morphologically, cytologically, anatomically and functionally (under physiological conditions at least) identical to those described by other groups as well as to mammalian lymphatics [88,180,182,184,353-355].
Therefore, we believe that the term is warranted, in spite of our discovery that these vessels do not exclusively originate from blind ended lymphatic bags in the periphery.

Whether the ALCs we have described in the adult fish also exist in embryos is still

unclear, but it is clear that lymphatics in the developing embryo do not receive fluid from the blood circulation[87,180,184].
Thus if the lymphatic vessels in the embryo are in fact identical to the adult lymphatic vessels, three questions needs to be addressed: When and how are ALCs established during embryogenesis and are there parallels to ALCs in mammals?
Several recent publications shed light on these issues.

It has recently been described that zebrafish lymphatics develop in parallel with the arterial blood vessels specifically[90]. In fact, lymphatic endothelial cells seem to crawl on the arterial endothelial cells and establish both transient and more long lived connections between the cell types.
It may be possible that such arterial-lymphatic endothelial cell connections can mature and develop into the arterial-lymphatic conduits we have described in adult fish, during arterial coating with smooth muscle cells.
This should be investigated later during development, as arterial coating – which presumably is an important step in ALC maturation – has not yet happened at the time points investigated by these researchers[90].

There are also indications that blood-lymphatic connections may exist in mammals. It was recently described that there are direct contact points between blood and lymphatic vessels in the rat mesentery (see figure 4), which however did not seem to transport blood into the lymphatics[356].
This is in line with our observations that ALCs are closed under normal physiological conditions. It would be interesting to see if such contacts are also present in other tissues, and perhaps in particular in tumors, and if they – as in fish – can "open up" under hypoxia.

Other researchers have also found "lymphatic-like" vessels, which seems to be in contact with blood vessels[357-358]. These vessels were slowly collecting dye injected in the blood stream, but much slower than it was distributed in the blood circulation.
They coined the term primo vessels to this subset of the vasculature, as they did not believe that they would fall under a strict definition of the term lymphatics.
However, given the growing body of evidence mentioned here, perhaps it is time to reconsider the stern definition of the lymphatic vasculature and acknowledge that lymphatic vessels may have direct connections to blood vessels in mammals as well.

As this is a young area of research, much of the recent results both in fish and rats will have to suffer the test of time and reproduction, but if the results prove to be solid, it seems like the lymphatic vessels in fish, are indeed very similar to those in mammals.
If so there may be arterial-lymphatic conduits which serve as gatekeepers for transport of cells and large macromolecules from the blood into the lymphatics even in mammals[357] – at least in hypoxic tissues. If such connections are present in or close to tumors for example, it is likely that this may be a mechanism of hypoxia induced transport of tumor cells from the blood to the lymph.
Such a mechanism would explain why lymph node metastases are often observed prior to metastases in blood-filled organs such as the liver, lungs or bone marrow – even in cases where intra-tumoral lymphatics have not been found[359].
As we have found that NO is the major driving force of these hypoxia-induced movements of blood cells into the lymphatic circulation, this may raise the possibility that anti-NO treatment such as c-PTIO or ODQ could be used as an anti-metastatic agent in combination with traditional therapeutics.

One matter of particular importance related to hypoxia and lymphatics from the fish perspective is their ability to absorb oxygen directly through the skin[360-364]. In zebrafish, this mechanism seems to contribute most of the consumed oxygen during embryogenesis[362-364], which is illustrated by zebrafish mutants such as cloche[365]. In homozygous cloche mutant embryos hemangioblast differentiation is blocked and thus neither blood nor blood vessels are formed during development.
Such a severe phenotype should result in early embryonic lethality in mice, but the fish embryos survive until day 4-5. As the larvae hatch from the egg at around day 2-3, this would correspond to post partum stages in mouse development. The oxygen needed for tissue development in this period thus have to come from cutaneous absorption.
It seems that in adult fish, the cutaneous route of oxygen uptake may primarily be important for supplying the skin and fins with oxygen, but probably not muscles or other tissues under normal physiological conditions[366].
However, our finding that the lymphatic vessels, which are the main vessel type in skin and fins, may be perfused with blood under hypoxia specifically, points to this system as an important backup or reserve for extra oxygen extraction from the water during hypoxia. Blood flowing though skin lymphatics may thus increase the cutaneous

oxygen uptake significantly thereby improving oxygenation of critical tissues such as the brain and heart under hypoxia.

5.3 HYPOXIA-INDUCED METASTATIC BEHAVIOR STUDIED IN ZEBRAFISH EMBRYOS

In mice, current metastasis models do not allow detection of tumor micro-metastatic lesions smaller than several thousand cells, which are already established and growing in the metastatic niches. Such micro-metastases thus represent the ultimate consequence of the metastatic cascade[122,219]. Investigating such late-stage metastatic clusters may not give insight into signaling factors and cell processes which are important earlier during the metastatic cascade, but at the time of settlement and growth in the new location are no longer are evident.

For example the early events of tumor-cell transformation to an invasive phenotype as well as the invasion of the peritumoral tissue and intratumoral vasculature are difficult to study in traditional mouse xenograft models.

Furthermore, while most researchers today accept that tumor hypoxia plays a role in the pathogenesis of the disease; several important issues regarding hypoxia and tumor cell invasiveness are still not well addressed.

Does hypoxia lead to tumor cell EMT?

Are the effects of hypoxia exclusively derived from effects on the host cells such as the vasculature?

What are the most important molecular pathways, and what levels of hypoxia are needed?

Many so called cancer stem cells are cells which have undergone EMT and have acquired an invasive phenotype[367-368]. These cells are often found in the vascular niche in tumors[369-372]. As the vascular niche may be presumed to be the best oxygenated region in the tumor, how does this add up with the evidence supporting a role of hypoxia in the induction and maintenance of such cells?

What are the factors involved in the dynamics of the induction and turnover of invasive, cancer stem cells?

These questions are very difficult to answer using traditional mouse models.

Mouse models are also not well suited for discovery of compounds that may interfere with the actual process of tumor cell invasion and metastasis – especially in hypoxia.

Our tumor dissemination and metastasis model in zebrafish embryos offers the possibility to investigate what happens during EMT, induction of stem-like characteristics and transformation from a non-invasive to an invasive tumor cell phenotype.
A particular strength of the model is the possibility to study the role of hypoxia and the vascular niche – as well as how to interfere either genetically or pharmacologically with this process.

In mouse tumor xenograft models, researchers usually use fast growing tumor cell lines that give rise to a large tumor within 3-4 weeks. In this short time, however, micro-metastases have not had time to develop, and this model is therefore rarely chosen for studies on the metastatic process[46,195,221].
Instead, tumor cells are injected into the blood stream, thereby circumventing the first critical steps of tumor cell invasion into the vasculature, which greatly speeds up the studies on metastatic settlement and growth[373-374].
However, as these studies are done in a situation where there is no primary tumor at all, the organism may respond differently to the circulating tumor cells.
For example, the presence of a primary tumor is probably important for development of the pre-metastatic nieces[137,227], which are therefore not present in these metastasis models.
Also, it has been reported that primary tumors may activate the growth of otherwise dormant metastatic lesions[373].
The metastasis protocol of injecting tumor cells in the blood stream directly thus seems to have several important biological drawbacks.
The zebrafish embryonic metastasis model always starts with a primary tumor in which the most aggressive cells are allowed to invade the blood stream, thus more closely resembling the clinical process of metastasis.

However, not everything that shines is gold, and there are a few points of concern with the zebrafish tumor invasion and metastasis assay, which should be taken into account.
Zebrafish embryos do not develop well at 37^{o}C, so in our assay the embryos are incubated at their optimal temperature, 28.5 ^{o}C. This is a very hypothermic environment

for the implanted tumor cells, and it is not known how the cells respond to this. We have noticed that most implanted tumor cells grow very slowly in the zebrafish, which – at least in part – may be because of hypothermia.
Also, while the zebrafish embryos do not reject the tumor cells, the mammalian tumor cells may have a different growth profile in a zebrafish environment, consisting of zebrafish serum and growth factors, compared to a native environment in a syngenic animal.
Finally, the fish generally succumb to the effects of their tumors after about 1-2 weeks, which are too short a time to study later stages of the metastatic cascade. Because of these concerns we do not recommend to use this assay for evaluation of tumor growth characteristics nor later stages of the metastatic progression.
The existing murine models are probably better suited for such studies. Thus, to get a full view of the metastatic process – especially during hypoxia – I propose that investigations should include both our zebrafish model, and the existing mouse models.

Little is known regarding to what degree hypoxia induces expression of angiogenic factors other than VEGF. There are reports that PDGF[375], Lysyl oxidase[136-137,229] and Osteopontin[373,376] may also be induced by hypoxia signaling in tumor cells.
We found that the tyrosine kinase inhibitor sunitnib, which blocks VEGF receptors, blocked hypoxia-induced tumor angiogenesis and tumor cell dissemination, adding to the evidence that VEGF is important for this process.
However, sunitnib is a non-specific drug, blocking many tyrosine kinases, including PDGF receptors and c-Kit[377], which may also be important for tumor cell dissemination and metastasis[222,378-380], thus we cannot be sure that the hypoxia-induced tumor cell dissemination we have found are entirely due to hypoxia-induced VEGF-VEGFR2 signaling.
To confirm that this is the major pathway, a more targeted approach should be taken, such as knocking down zebrafish VEGFR2 prior to tumor cell grafting and incubation in hypoxia.

5.4 IS FIN REGENERATION RELEVANT IN MEDICAL RESEARCH?

Regeneration in fish and mammals are quite different. Zebrafish have much stronger regenerative capabilities compared to mice and humans[19].

The reasons for this are not fully understood, but could be because fish have a more prominent stem/progenitor cell population in their tissues which can quickly be mobilized to rebuild damaged organs such as the heart[96-97].
Also it may be because fish cells more readily dedifferentiate into multi-potent progenitor cells, which seems to be the case in the regenerating tail fin[381].

In either case, however, regeneration is dependent on angiogenesis, which makes the regenerating tail fin model suitable for screening of anti-angiogenic compounds[32].
An example of this is that the zebrafish tail fin does not regenerate – or regenerate slower – if angiogenesis is inhibited for example by sunitnib[28].
While this assay has been widely used as an adult zebrafish model of angiogenesis, one should be cautious in drawing conclusions from such experiments, as regenerative angiogenesis may progress via a different angiogenic-factor profile compared to angiogenesis in pathologies such as cancer and retinopathy.

For example, in the zebrafish tail fin, regenerative angiogenesis seems not to be hypoxia-dependent as the fin is so thin that it receives sufficient oxygen from passive cutaneous uptake by the water.
In most cases of pathological angiogenesis, hypoxia is a major driving factor. Therefore compounds under investigation as modulators of angiogenesis in adult zebrafish should be tested in both hypoxia-dependent and independent models such as the hypoxia-induced retinal angiogenesis and hypoxia-independent tail fin regeneration to elucidate whether the compounds would target hypoxia dependent pathways or not.

6 CONCLUSIONS AND PERSPECTIVES

Hypoxia is a major driving force of pathology – especially pathological angiogenesis, vascular permeability, reduced vascular maturation and quality[195,325] as well as tumor cell invasion and metastasis[134-135]. Hypoxia is however difficult to study in traditional mouse or rat disease models as it is very difficult to control.

We have shown that zebrafish – both adults and embryos – constitute a practical model system for the studies of hypoxia-induced angiogenesis, vascular and tumor biology in vertebrates.

6.1 FURTHER DEVELOPMENT OF THE RETINAL ANGIOGENESIS ASSAY

In adult zebrafish gradual reduction of the oxygen levels allow fish to survive in as little as 10 % air saturated water. Prolonged exposure to this level of hypoxia leads to pronounced retinal angiogenesis within 6 days. This angiogenic response is VEGF dependent and requires severe hypoxia. Furthermore blockade of Notch signaling changed the hypoxia-induced angiogenesis from the capillary region to the arterial region, raising expectations that such an approach might be valid for hypoxia-induced arteriogenesis also in other tissues.

Further studies are needed to evaluate whether the hypoxia and anti-Notch induced arterial neovasculature consist of well perfused, non-leaky, mature and persistent vessels, and if so, if this is a valid strategy for treatment of myocardial and cerebral ischemia.

In order to exploit the retinal angiogenesis model to its full potential, it will be desirable to develop methods for in situ knock down or expression of genes by intra-ocular injection of morpholinos or capped-mRNA respectively, as it has been described in the regenerating tail fin[98-103].
Currently we are testing if available reagents for transfection of cells in vitro are functional in the zebrafish retina.

As electroporation seems to be a good strategy in the tail fin, it may be required also for in situ transfection in the retina. There are methods available for inserting electrodes into the zebrafish retina[159], so such an approach would be possible.

Also, we are developing procedures for injecting non-orally active pharmaceuticals such as antibodies or growth factors into the vitreus, which can then be used to study the mechanisms of angiogenesis induced by particular growth factors, in a similar way as the corneal micro pocket model is used today[223,382-383].
However, initial studies show that not all mammalian growth factors and/or antibodies may work in zebrafish, so perhaps the genetic methods described above are preferred for molecular studies in this model system.

6.2 FURTHER STUDIES ON FISH LYMPHATICS AND MAMMALIAN ALC'S

The existence and function of lymphatic vessels in fish have been a controversial issue for many years[173-174]. We have shown that fish do have lymphatic vessels, that these vessels are present in adult zebrafish and glass catfish, and that they under normal physiologic conditions do not contain blood.
Interestingly we found that fish lymphatic vessels are the predominant vessel type in the skin and fins – which lack blood circulation. These tissues rather rely on cutaneous oxygen uptake for satisfying their demands.
We further found that fish lymphatics do not exclusively begin in blind ended lymphatic bags, but also have direct connections to large arterial vessels. These connections, which are highly curled during normoxia, does not allow blood cells to enter into the lymphatic circulation, and mediate a slow flow of plasma through these vessels.
The slow plasma perfusion of these vessels may be needed to keep them from collapsing, as interstitial fluid pressure and therefore drainage is minimal in fish[69-71].
We have found that these connections dilate and unwind in response to hypoxia in an NO-dependent manner, allowing the lymphatics to be perfused with blood.
We believe this may be an important backup system for increasing the respiratory surface area of the fish, such that cutaneous oxygen uptake can be increased leading to better oxygenation of critical organs.

We also hypothesize that this system may be hijacked in pathological settings such as cancer, to mediate the flow of cancer cells into lymphatics and further to regional lymph nodes.
Further work needs to be done to identify and characterize these connections in mammals and to elucidate whether such arterial-lymphatic conduits could be important for tumor cell spreading through lymphatics.

6.3 PERSPECTIVES AND FURTHER DEVELOPMENT OF THE ZEBRAFISH XENOGRAFT ASSAY

Hypoxia is believed to change tumors to a more invasive phenotype[325], but initial stages of tumor dissemination and metastasis cannot be studied using current tumor models in mice or rats.
We have developed a tumor xenograft model in zebrafish, in which the tumors change their invasive profile when they are exposed to hypoxia. This method provides a great resource to study early events of hypoxia-induced transformation of tumors from a benign to an invasive phenotype.

We have used this model to show that hypoxia-induced tumor derived VEGF is of major importance in mediating their invasive phenotype. This is not via a direct effect of VEGF on the tumor cells, rather on VEGFR2-expressing cells in the host – probably the vascular endothelial cells, as these undergo drastic remodeling when the VEGF-VEGFR2 pathway is inhibited with either specific morpholinos against VEGFR2 or the broadly acting anti-tyrosine kinase inhibitor sunitnib.
Even under hypoxia, tumor invasion and metastasis was essentially blocked by sunitnib, indicating that the VEGF-VEGFR2 signaling by the vasculature plays a major role in this process.

This hypoxia-induced tumor invasion and metastasis model has broad applications for the study of hypoxia and malignancy, and will probably prove to be a valuable tool in the future.

The assay may also have potential as a diagnostic tool, as primary tumor samples from patients may be injected into the zebrafish embryo and screened both for their invasive

properties in vivo, their response to hypoxia and to different types of chemotherapy. Results from such screening could then be used in the choice of therapeutic intervention best suited for that particular patient.
Such a screening is also possible in SCID mice, but this is very expensive, and tumor growth and metastasis of primary cells is very slow in this system.
The zebrafish model takes less than a week to complete, and thus would return results back to the clinic much faster.

The assay could be developed further, however, by modifying the hypoxia setup to include more single chambers separated from each other such that different compounds, morpholino-injected embryos or tumor cell lines/samples can be tested in parallel.
This improvement would facilitate screening of novel compounds or genes that interfere with hypoxia-mediated processes, including invasion and metastasis.
Such screening is otherwise impossible today, and could potentially lead to the discovery of very interesting novel leads for development of anti-metastatic pharmaceuticals, or pharmaceuticals that interact with hypoxia signaling, in the future.

6.4 USING ZEBRAFISH TO FIND HIGHLY TARGETED DRUGS AGAINST RETINAL NEOVASCULARIZATION

Anti-VEGF therapies for retinopathy pose several problems and unnecessary discomforts for the patients including high cost, invasive delivery methods and potentially blocking the neurotrophic effects of VEGF[315].
Small molecules which may be taken orally targeting either VEGF or other pathways, and which have no or only very slight side effects are needed to combat these common, debilitating disorders.

We have found that zebrafish embryos show great potential in screening for compounds which effectively block retinal angiogenesis during development. Such a screen led us to identify an inhibitor of retinal angiogenesis, which did not affect angiogenesis in other parts of the fish, including the intestinal regions where the vasculature is also under development during the window this drug was active in the retinal vasculature[384].

This drug did not lead to impaired vision, indicating that neuroprotective effects of VEGF or other factors are not inhibited. The drug is furthermore effective in adult zebrafish, at the same concentration as in embryos.

It would be interesting to see if this drug interferes with hypoxia-induced or diabetic retinopathy in zebrafish and if it is active also in mouse models of retinopathy.

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Let me recommend all of you to grab an opportunity to work in either of these places should it present itself!

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