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ROLE OF ANGIOGENESIS IN CANCER INVASION AND METASTASIS

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To my beloved family and grandmother…

ABSTRACT

Cancer metastasis is a complex process that involves an elaborate interplay between malignant cells and various host cellular and molecular components. The metastatic cascade consists of several discrete but non-separable steps, including: 1) malignant transformation of cells; 2) intravasation and dissemination of tumor cells into the circulation or lymphatic system; 3) transport of malignant cells to distal organs and tissues; 4) adherence of tumor cells to distal sites; 5) formation of the initial metastatic niches, and 6) angiogenesis-dependent re-growth of metastatic foci to clinically detectable tumors. Despite technological advances, advanced image analyses only allow detection of a relatively large tumor-mass in human cancer patients and in various animal models.

Hypoxia is one of the characteristic features of the tumor microenvironment and low oxygen is known to induce tumor angiogenesis. However, the causal link between tumor hypoxia and tumor invasion remains elusive. In this thesis work, we have developed a novel zebrafish model that allows us to investigate tumor invasion at the single cell level in living animals. Furthermore, exposure of the zebrafish in hypoxic water permits us to study the role of hypoxia in facilitating tumor invasion at the early stage of the metastatic cascade. We further investigated hypoxia-regulated genes in tumors that contribute to metastasis. In paper I, we developed a novel zebrafish model to study metastasis at the single cell level in living animals. We take advantage of the transparent nature of zebrafish embryos that are immune privileged at the early stage of development. Additionally, the availability of a transgenic zebrafish line that expresses enhanced green fluorescent protein allows us to study the interaction between tumor cells and the vasculature in a non-invasive manner. We also developed a novel hypoxia chamber in which oxygen levels in the aquarium can be adjusted to a certain desired level. Development of these novel methods allows us to study the early events of cancer metastasis, which otherwise cannot be visualized in mammalian systems. In paper II, we studied the role of hypoxia in promoting metastasis using the method described in Paper I. We have found that hypoxia induces angiogenesis in the implanted tumors and VEGF is the crucial mediator that is responsible for hypoxia-induced tumor angiogenesis. To further validate the role of VEGF in mediating tumor invasion and metastasis, blocking VEGFR signaling by tyrosine kinase inhibitors or specific morpholinos inhibits hypoxia-induced metastasis. Moreover, overexpression of VEGF in tumor cells also significantly promotes cancer metastasis through stimulation of tumor angiogenesis although VEGF lacks direct effects on tumor cells. These findings show that hypoxia plays a pivotal role in facilitating tumor cell dissemination at the early stage of the metastatic cascade and inhibition of the VEGF signaling might be an important approach for treatment of metastatic disease. In paper III, we show that a novel mechanism of hypoxia-induced angiogenesis, which involves in physical interaction between filamin A and HIF-1α. Hypoxia-induced cleavage of filamin A promotes nuclear accumulation of HIF-1α, leading to up-regulation of its target genes such as VEGF. Via this mechanism, filamin A stimulates tumor angiogenesis and tumor growth. In paper IV, we show that filamin B serves as a negative regulator for tumor invasion and metastasis. In filamin B deficient mice, increased metalloproteinase-9 (MMP-9) activity has been detected. Similarly, silencing of MMP-9 in various tumor models resulted in enhanced tumor angiogenesis and invasion by increasing the bioavailability of VEGF.

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I. **Rouhi P***, Jensen LD*, Cao Z*, Hosaka K, Lanne T, Wahlberg E, Steffensen JF, Cao Y. Hypoxia-induced metastasis model in embryonic zebrafish. Nat Protoc. 2010;5(12):1911-8.

II. Lee SL*, **Rouhi P***, Dahl Jensen L, Zhang D, Ji H, Hauptmann G, et al. Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(46):19485-90.

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Targeting filamin B induces tumor growth and metastasis via enhanced activity of matrix metalloproteinase-9 and secretion of VEGF-A.
Manuscript

*Co-first author

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III. Hosaka K, Yang Y, Seki T, Nakamura M, Andresson P, **Rouhi P**, Yang X, Jensen L, Lim S, Feng N, Xue Y, Li X, Larsson O, Ohhashi T, Cao Y. Tumor PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodeling and metastasis. Nat Commun. 2013;4:2129.

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Myosin-X promote breast cancer invasion and metastasis downstream of mutant p53.
Manuscript accepted in JCI

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LIST OF ABBREVIATIONS

ABD	Actin-binding domain
AMD	Age-related macular degeneration
ANG2	Angiopoetin-2
bHLH	Basic helix-loop-helix
CH	Calponin homology
CV	Caudal vein
CSF-1R	Colony-stimulating factor-1 receptor
Dll4	Delta-like 4
DFX	Desferrioxamine
DA	Dorsal aorta
EGFP	Enhanced green fluorescent protein
EC	Endothelial cell
eNOS	Endothelial-derived nitric oxide
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinases
EPO	Erythropoietin
Ets	Ever shorter telomere
ECM	Extracellular matrix
FLK1	Fetal liver kinase 1
FGF	Fibroblast growth factor
FIH-1	Factor inhibiting HIF-1
FLN	Filamin
Flt1	Fms-related tyrosine kinase 1
GIST	Gastrointestinal stromal tumor
GLUT	Glucose transporter
HSPG	Heparin sulfate proteoglycan
H1	Hinge domain
HRE	Hypoxia response element
HIF	Hypoxia-inducible factor
ISV	Intra segmental vessel
KDR	Kinase insert Domain Receptor
LLC	Lewis Lung Carcinoma
LOX	lysyl oxidase
MMP	Matrix metalloprotease
MEF	Mouse embryonic fibroblasts
MAPK	Mitogen-activated protein kinase
NAD	N-terminal activation domain
NP	Neuropilin
NPC	nuclear pore complexe
OSCC	Oral squamous cell carcinoma
ODD	Oxygen-dependent degradation domain
PDGFB	Platelet-derived growth factor
PVH	Periventricular heterotopia
PMA	Phorbol myristate acetate
PKC	Protein kinase C

PlGF	Placenta Growth Factors
PAE	Porcine aortic endothelial
PHD	Prolyl hydroxylase domain enzyme
PAS	Protein-ARNT-single minded protein
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RTK	Receptor tyrosine kinases
RCC	Renal cell carcinoma
RFP	Red fluorescent protein
Scl	Stem cell leukemia
shRNA	short hairpin RNA
siRNA	short interfering RNA
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinas
TAD	Transactivation domain
TCA	Tricarboxylic acid
VEGF	Vascular endothelial growth factor
vSMC	Vascular smooth muscle cell
VHL	Von Hippel-Lindau

1 INTRODUCTION

Tumor malignancy is characterized by formation and growth of metastatic foci in distal organs. Metastasis is a complex cascade consisting of several steps; dissemination of tumor cells from the primary site, degradation of the extracellular matrix (ECM), invasion to the local tissue, intravasation into the nearby blood vessels, survival and arrest in the blood stream, extravasation into the host environment and metastatic colonization (1). During metastasis, cells undergo selection and therefore targeting these selected cells is much more difficult than targeting any proliferating cell. To date, most of the drugs developed for cancer treatment, are more in favor of primary tumor growth, and improving the quality of life for cancer patients by inhibiting metastasis has been a far-fetched aim and the extension of life in severely sick cancer patients with metastases does often not exceed over a few months. Survival of cancer patients might be prolonged more significantly if disseminating cells could be detected and targeted already at very early stages of tumor cell dissemination, perhaps patients would have had better chance to survive and better life quality. Identifying pathways and genes involved in enhancement and facilitating tumor cell invasion and metastasis is desirable as the involved genes and proteins could potentially be used as biomarkers to predict the invasiveness of a specific tumor type (2). Further, based on this information, more specific drugs might be developed that inhibit tumor cell spreading throughout the body at early stages. Development of tumor cells from normal cells requires genetical abnormalities caused by mutations in oncogenes and potential malfunctions in tumor suppressor genes. However the role of epigenetic factors should not be underestimated. Malignant tumor cells express a vast variety of different cytokines and growth factors, such as vascular endothelial growth factor (VEGF). Among these factors, pro-angiogenic factors are the most crucial for cells to survive and grow. Angiogenesis induced by the tumor, allows it to establish its own blood supply system leading to accelerated growth. As the tumor grows larger, the core becomes hypoxic resulting in hypoxia-induced up-regulation of the expression of VEGF and other angiogenic factors. A VEGF-induced tumor angiogenesis promotes and facilitates tumor cell metastasis through a series of events such as increased vessel wall permeability, vessel dilation, endothelial cell (EC) proliferation, cell survival, and angiogenesis (3).

Impaired blood perfusion due to irregular and nonfunctional tumor blood vessels causes intratumoral hypoxia, which in turn will further increase angiogenesis and causes migration and dissemination of tumor cells through different mechanisms. Among these mechanisms in such a futile circle, increased level of VEGF via activation of HIF pathway and filamins has been shown to significantly promote metastasis (4).

1.1 TUMOR METASTASIS

Discovering metastatic lesions in a cancer patient commonly points at a progressed systemic stage of the disease and is associated with poor prognosis. Surgical removal and radiotherapy can be effective to eliminate the risk of regrowth of the primary tumor but do not clear the metastatic lesions that have already spread and grown in the body. Growth of the metastatic lesions can harm the patients in different ways: 1) direct damage to the affected organ, 2) paraneoplastic syndromes, and 3) complications caused by treatments, which together eventually lead to the high level of morbidity and mortality due to cancer. Increasing evidence from animal experiments show that primary tumor and metastatic lesions do not respond similarly to the same treatment. Comparing expression profiles reveals that certain genes are expressed in both primary tumor and metastatic nodules but with different expression level. This knowledge can be beneficial in finding and targeting signaling pathways essential for growth of primary tumor or metastatic lesions.

Metastatic tumor cells locally invade the regions adjacent to the primary tumor and intravasate into the blood or lymphtic system. They circulate throughout the body and survive and eventually they get arrested in the circulation and extravasate into the host tissue. Once they have reached the new location, tumor cells must colonize, generate own blood supply, grow, and survive. Unfortunately all these steps are not easy to observe in human patients and the only step that can be detected is the last step when the metastatic lesion has grown beyond the invisible size and/or has already started to harm the host tissue. Therefore it is very crucial to find a way to detect and target the tumor cells at early stages. Most of the methods that are used to detect and study metastasis are developed in rodents, and do not provide an opportunity to study

the very early events consisting of dissemination and invasion of the tumor cells into the surrounding tissue.

Dissemination and invasion of the tumor cells from the primary site, is the initiating step of the metastatic cascade. Tumor cells must pass a few barriers before entering the circulation: 1) Tumor cells must detach from the primary tumor tissue, 2) ECM remodeling is required to allow spreading tumor cells to escape from the primary site, 3) proteolytic degradation of ECM is required in order to physically create a path for tumor cells to escape (5). Invasive tumor cells undergo a central mechanism involved in inducing tumor cell invasion and metastasis, namely epithelial to mesenchymal transition (EMT) that prepares them for local invasion and also survival of the next steps in the metastatic cascade (1). During EMT, some epithelial specific genes such as E-cadherin will be down regulated while mesenchymal genes, which specifically initiate cell migration such as snail, twist, Zeb1, and slug will be up-regulated (2, 6). Intratumoral hypoxia stabilizes the HIF-1α and it has been shown that hypoxia is an important factor in the tumor microenvironment that induces expression of key regulatory factors in EMT such as twist, snail and Zeb1 (7).

Once tumor cells have reached the circulation, they need to survive in the bloodstream, which creates a hostile environment due to velocity-induced shear forces, presence of immune cells, and lack of attachment to an underlying ground (anoikis) (8). If tumor cells survive the harsh conditions in the blood stream, they must arrest in the circulation and extravasate. The general belief is that cells will non-specifically bind to the coagulation factors such as fibronectin and thrombin or they are halted due to the size restrictions get stuck in capillaries and subsequently extravasate to the new microenvironment. Tumor cells can produce factors, which cause retraction of the ECs at the extravasation site, attach to the ECM and induce structural rearrangements of capillaries leading to extravasation (9, 10).

Angiogenic factors have diverse roles in cascade of metastasis. It is believed that beside inducing and facilitating tumor dissemination and invasion from the primary site, they also have beneficial effects on the sites that metastatic tumor cells might subsequently be seeded, which are known as pre-metastatic niches (11). Hypoxia-induced pathways can also influence generation of pro-metastatic niches by induction of genes such as VEGF and lysyl oxidase (LOX). Lysyl oxidase by covalent cross-linking of the collages and elastin, increase insoluble

matrix deposition and tensile strength, allowing much better attachment and extravasation of the tumor cells (12, 13).

Metastatic colonization is a crucial stage for the extravasated tumor cells. At this stage, tumor cells should survive in the new microenvironment that is markedly different from the primary site. The metastatic niche or the new microenvironment consists of different cell types such as ECs, inflammatory cells, fibroblasts, and ECM components. To attract, activate, and induce proliferation of these cells, tumor cells secrete cytokines, growth factors, chemokines, and proteases. As the metastatic lesion starts to grow, the tumor including its microenvironment will become hypoxic. Consequently low pH level and high concentration of metabolites increase hypoxia, which in turn increases expression level of certain angiogenic factors and induces angiogenesis. Furthermore, the ECM will be degraded resulting in release of ECM bound growth factors that have an additional impact on the progression and malignancy of the tumor (14, 15).

1.2 VASCULOGENESIS

The vascular system in all organisms and tissues forms widespread networks responsible for transportation of blood containing gases, nutrients, signaling molecules, immune cells as well as removal of cell metabolites and other cell products. Since this system has such a crucial role in the body, any even small disruptions in this system can lead to disease. The vascular system develops amongst the first processes in an organism during embryogenesis due to the crucial role it has in mammalian development (16, 17). Blood and lymphatic vessels are the two networks, which are responsible for all transportations in the vertebrates and both are built by ECs.

Endothelial cells keep the homeostatic balance of the healthy organism hence even slightest malfunction in these cells can lead to pathogenesis of various diseases, therefore these networks are tightly controlled (18). Two distinct processes, vasculogenesis and angiogenesis form the vascular system.

During embryogenesis, new blood vessels are formed de novo, from mesoderm-derived endothelial precursor cells called angioblasts. In order to form primitive vessels, angioblasts should undergo differentiation and thereafter will proliferate and form the vascular luminal complex. Thereafter

angiogenesis generates a network by sprouting from the existing primitive vascular labyrinth (19-21). Insufficient growth or maintenance of the vessels leads to ischemia, which causes diseases such as stroke, myocardial infarction, and neurodegenerative disorders, while overgrowth or irregular remodeling of the vessels will cause diseases like cancer, age-related macular degeneration (AMD), and inflammatory disorders (22).

The teleost zebrafish (*Danio rerio*) is a tropical small fish found in Indian Ganga. This small animal has been extensively used in developmental and molecular biology studies for more than a decade but recently has been used in pathological studies and the interest for using it is growing. The transparent nature of the zebrafish embryo during the first few days of development is the first and most encouraging characteristic and the reason for choosing zebrafish as a model organism in many studies. This trait has meant that some experiments that have been laborious or even impossible in other model organisms became possible, such as investigation of growing blood vessels in living animal in a completely non-invasive manner by using convenient transgenic strains such as *Fli:EGFP* or *flk:EGFP* or *gata1:dsRed,* which allows researchers to study the complex mechanism of vasculogenesis in great detail using simply high resolution microscopy (23).

In zebrafish embryos the first embryonic artery and vein are formed by individual migration of angioblasts from the lateral plate mesoderm to the midline where they give rise to the dorsal aorta (DA) and caudal vein (CV) (24). VEGF is required for angioblast differentiation, which activates Notch signaling. Notch signaling activation in angioblasts defines their fate to specify into arteries or veins. Specification occurs even before segregation of the angioblasts. At an early somite stage, angioblasts form a single line vascular cord at the midline and afterwards remodeling in the cord leads to formation of the DA. Shortly after DA formation, some angioblasts sprout towards the ventral side of the DA and while staying attached to the DA, form the CV. Intra segmental vessels (ISV) also initiate from the DA just slightly later than ventral sprouts that form CV. Dorsal sprouting of the DA in order to generate the ISV occurs with regular distances along the DA and it seems to be regulated by a different mechanism than CV formation. Although DA and CV have a common origin, the formation mechanism is different and they follow different ways of lumen formation, which has been found using a

transgenic line with an RFP reporter gene linked to the erythrocytes (*Tg:gata1:dsRed*). During DA lumen formation, the vascular cord becomes hollow and forms the luminal artery; however, in CV lumen formation procedure, erythrocytes located ventrally to the DA move into the CV after initial lumenization by cord-hollowing, thereby causing additional expansion of the CV lumen (25).

VEGF-A has a stimulatory effect on the Notch signaling pathway in sprouting angioblasts. VEGF elevates expression of the Notch ligand *Delta-like 4 (Dll4)*. ISV tips cells express Dll4, activate Notch signaling (Dll4 receptor Notch1) in the neighboring cells and limit dorsal sprouting of angioblasts. Upon Notch ligand-receptor binding, the intracellular domain of Notch (NCID) is released from the membrane by a proteolytic cleavage and NCID translocates into the nucleus where it binds to its partners in order to form a transcriptional activator (26, 27). Notch signaling is one of the pathways, which is highly conserved during evolution among all organisms and has a very essential role in cell fate determination (27, 28).

1.2.1 Vascular cell types

The complex structure of blood vessels consists of various cell types due to the specific functions and requirements for this system. Blood vessels are divided into three different groups; arteries, capillaries and veins. Endothelial cells, which are the building blocks of the vascular system, are vessel type specific. Besides the ECs, there are more specific cells called mural cells, which support and accompany the endothelial layer of the vessel wall. These mural cells are also different depending on the type of the vessel.

Arteries are a specialized type of vessels, which are under heavy shear stress and in comparison to veins have smaller lumen but are covered with smooth muscle cells and elastic fibers. This particular feature allows them to stretch, contract and support the high-pressure blood flow leaving the heart. On the contrary, when blood returns to the heart, pressure is low and the relatively thin wall of venous blood vessels is sufficient to support the flow. It was believed that vessel destiny was determined by direction of blood flow, pressure and rate but recently it has been shown that due to the expression of different molecular markers, even the smallest capillaries, arterioles, and venules are easily distinguishable.

The other difference between arteries and veins is existence of the venous valves. Venous valves extend into the vessel lumen and prevent the backflow of blood (29). Pericytes and vascular smooth muscle cells (vSMCs) are the specific cells covering ECM layer of the vessels. Pericytes cover mostly capillaries and veins while smooth muscle cells are usually found on arteries and therefore these cells can be used as almost specific markers for the vessel types. Zebrafish studies show that artery or venous fate of ECs is governed and determined by transmembrane proteins EphrinB2 and its receptor EphB4. Angioblasts expressing Ephrin B2 ligand will turn into arteries and those, which express receptor EphB4 will form veins. Loss of either of the two proteins is embryonically lethal (30). Although specific ECs, express specific markers, there are also some markers, which are common for all types of ECs, e.g. CD34, *pecam-1* and *fli1*. PECAM-1 (CD31) is a cell adhesion and signaling receptor, which is expressed on hematopoietic and ECs and FLI1 is a member of the Ets family of transcription factors and has an indispensable role in regulation of genes encoding ECM components. Furthermore, regulation of cell proliferation and maturation during vasculogenesis and angiogenesis is governed by Notch signaling pathway (31, 32).

1.3 ANGIOGENESIS: PHYSIOLOGICAL AND PATHOLOGICAL

The formation of blood vessels from preexisting vessels is called angiogenesis. This process can be divided into two types, physiological and pathological angiogenesis. Physiological angiogenesis is required as a complementary process for vascular development and maturation during embryogenesis and fetal development. Physiological angiogenesis happens in adulthood as well as during wound healing, pregnancy, skeletal muscle growth, and menstrual cycle. Pathological angiogenesis is specific to diseases such as obesity, retinopathies, psoriasis, immunogenic rheumatoid arthritis, and cancer.

Physiological and pathological angiogenesis share some similarities in the involved procedures, i.e. they both are initiated in response to a requirement for more oxygen and nutrients. Well-organized vasculature with proper blood supply in a healthy organism is maintained by a fine balance between proangiogenic and antiangiogenic molecules (33). This balance is usually

disrupted in pathological angiogenesis, leading to a disorganized, poorly functional and pathogenic vasculature.

Angiogenesis is a process composed of different steps in which various cytokines and growth factors are involved. A simplified sketch of angiogenesis consists of several steps:

- Stimulation of quiescent ECs
- Detachment of mural cells
- Degradation of ECM
- Sprouting and formation of filopodia
- Formation and migration of the tip cell
- Proliferation of the stalk cell and tube formation
- Anastomosis to other newly formed vessels
- Mural cell coverage and maturation

A quiescent vessel will not undergo angiogenesis unless it is awakened and stimulated by signals induced from the environment (34). Endothelial cells, in order to be able to move and proliferate, should be stripped from mural cells so that they can be exposed to pro-angiogenic signals. Angiopoetin-2 (ANG2) is among the pro-angiogenic growth factors, which affect the ECs and rapidly detaches the mural cells (35). Vascular endothelial growth factor (VEGF) is one of the main growth factors stimulating ECs to sprout. The next barrier that must be defeated is the ECM, which has to be digested by help of matrix metalloproteases (MMPs) that are released from tip of the sprouts (36). Filopodia protrusions are located at the tip of the sprouts and are responsible for releasing the MMPs. These filopodia are very sensitive to proangiogenic signals e.g. VEGF-A. Sprouts use these filopodia to scan the surroundings for chemoattractants and repellants and in that way guide the growing sprout towards the signal. The sprout contains a tip cell and multiple stalk cells. The tip cell is the growing part of the sprouts and is highly sensitive to pro-angiogenic signals while the stalk cells are less sensitive to the signal. Stalk cells proliferate and form the vessel lumen and support the elongation of the sprout. Sprout formation and growth continue until the pro-angiogenic signal is withdrawn, which causes the vessel to go into growth arrest, become quiescent, and stabilize. VEGF and Notch signaling regulate vessel sprouting. During tumor growth or even development, blocking Notch ligand DLL4,

which is highly expressed in the tip cell, leads to exuberant tip cell formation (37). Tip cells later on will inosculate with other cells in the vicinity and create vessel loops. Once the basement membrane is built and perivascular cells cover the newly formed vessels, blood will start to circulate in the vessels (22).

Several transcription factor genes and signaling molecules from diverse signaling pathways have been found to be involved in development of the vertebrate vasculature. All these factors including vegf, vegfr1-3, tie1, tie2, angiopoetin1-2, ephrinB2, ephB4, scl, ets1, fli1, runx, semaphorins, and plexins share a similar expression pattern (38-46).

1.3.1 Role of VEGF in angiogenesis

Vascular Endothelial Growth Factors play essential roles in vascular development during embryogenesis and also physiological or pathological angiogenesis. The VEGF family in mammalians contains 5 members with distinct functions, VEGF-A, B, C, and D, Placenta Growth Factors (PlGF-1 and 2) as well as different isoforms of these factors, which arise as a result of alternative splicing sites in the mRNA (47). VEGFs preferably act in homodimeric fashion although VEGF-A and PlGF have been observed in form of heterodimers. VEGF ligands are divided into three groups due to their specific functions and receptor binding patterns. Group I consists of VEGF-B and PlGF. Their function is among the least studied VEGF family members. The second group contains the most well characterized and most potent angiogenic member of this family VEGF-A, and its 6 alternatively spliced isoforms. Finally the last group includes VEGF-C and D (and the non-vertebrate protein VEGF-E), which exhibit angiogenic and lymphangiogenic properties (48, 49).

VEGF-A is the most studied member of this family and is considered a key player in the angiogenesis process. Alternative splicing of VEGF-A also generates isoforms of this protein with distinct properties. In humans they are called VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A165b, VEGF-A189, and VEGF-A206. These isoforms exist in mice as well but they are one amino acid shorter than their human counterpart (50, 51).

Vascular permeability is defined by movement of liquids such as serum and cells from inside the vessel to the extravascular area. Vascular permeability is

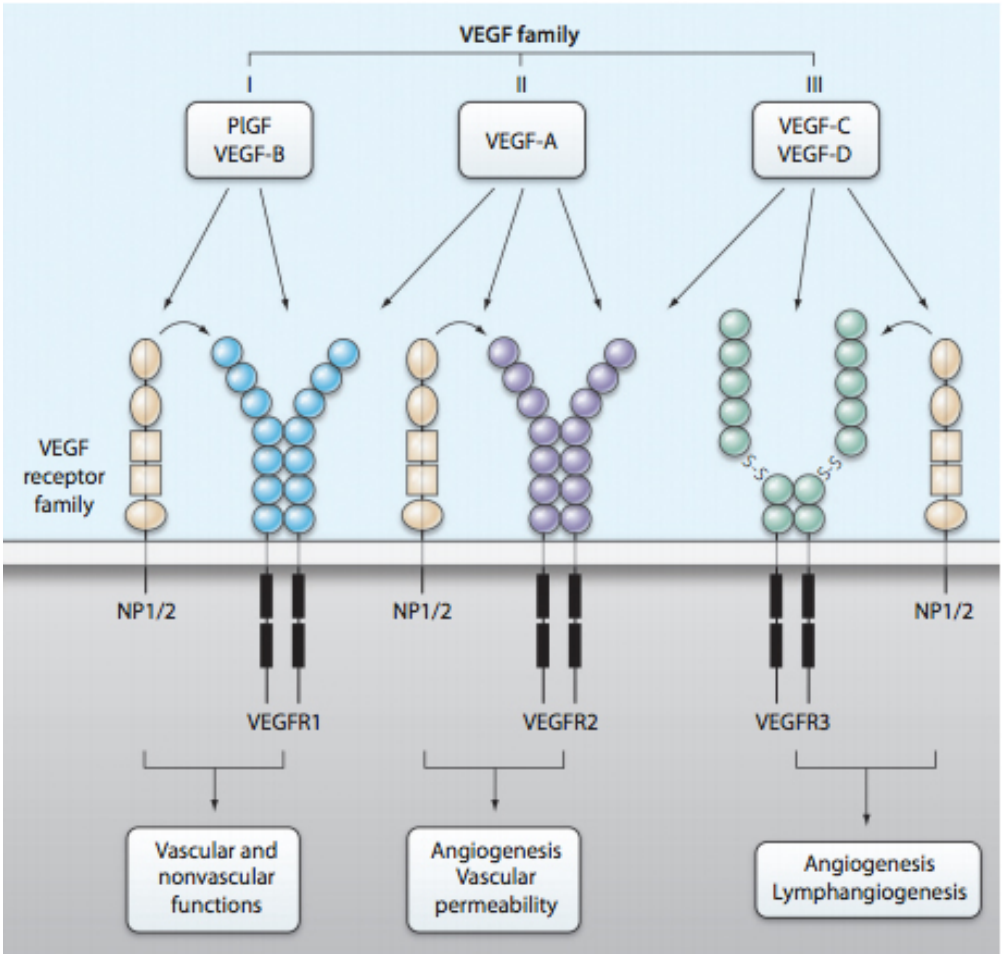

Figure 1: VEGF family (52)

governed and regulated by different factors involved in angiogenesis such as VEGF and other inflammatory mediators (53). The advanced molecular regulatory process behind the vessel leakage is dependent on a large number of variables involving physical properties of the transported molecules or the fluid (molecular size, charge, and also configuration), pressure and concentration, and type of the transportation (vesicular, through interendothelial junctions between adjacent cells, or through channels). Generally, tumor vessels have wider interendothelial junctions compared to the healthy tissue they are highly fenestrated. Fenestrations are the result of trans-endothelial channels formed by vesicles, and the non-continuous or lacking basement membrane. Due to the high permeability and leakage of the intratumoral vessels, the interstitial pressure is increased and pressure in microvessels is reduced. Oedema caused by accumulation of the cellular fluids in the extracellular space, induces swelling and increased interstitial pressure. Increased interstitial and decreased microvascular pressure together, causes reduced perfusion in the tumor and consequently decreased drug

delivery and treatment efficiency (54-56). Although it is worth remembering that vascular ECs from different vascular origins respond differently to VEGF in terms of sensitivity and mechanisms employed to induce permeability.

VEGF-A signaling mediates different activities including cell survival, proliferation, migration, vascular permeability, and angiogenesis. Distinctive functions of VEGF-A are mediated by VEGFR2 binding to different specific co-receptors and binding partners (57).

1.3.2 VEGF receptors

VEGF receptors structurally belong to the big family of Receptor Tyrosine Kinases (RTKs), and include VEGFR1, R2 and R3. VEGF receptors are mainly found on ECs (blood and lymph ECs) and their structure is composed of three parts; an extracellular domain, consisting of seven immunoglobulin (Ig) like domains, a transmembrane domain, and a short intracellular juxtamembrane region.

The extracellular domains of the receptors undergo dimerization in order to get activated and create a binding site for the ligand. Different receptors differ from each other due to their binding domains, e.g. VEGFR1 has domain 2 as a ligand-binding domain while in VEGFR3, domain no. 3 is critical domain for ligand. Except for the main VEGF receptors, there are also co-receptors such as Neuropilins (NP1-2) and Heparin Sulfate Proteoglycans (HSPGs). Different isoforms of VEGF interact with NPs as well when binding to their specific receptors. These co-receptors can influence the response mediated by the VEGFreceptor (Fig. 1).

VEGFR1 also known as Fms-related tyrosine kinase 1 (Flt1), in humans is the main receptor for type I ligands of the VEGF family, VEGF-B and PlGF although VEGF-A also bind to this receptor with high affinity.

VEGFR2 is called Kinase insert Domain Receptor (KDR) in humans and Fetal Liver Kinase 1 (FLK1) in mice. VEGFR2 is the major receptor for type II ligand, VEGF-A. This receptor has a vital role in angiogenesis and also metastasis of tumor cells throughout the body. VEGF binds to this receptor with very high efficacy but lower affinity than VEGFR1. Signals transduced by this specific receptor, induce permeability and a strong angiogenic response, leading to formation of massive quantities of leaky and immature blood vessels in a short amount of time in high rate. In pathological scenarios,

this creates a disease-promoting environment in which tumor cells proliferate, survive and undergo EMT and therefore results in dissemination and spreading of the tumor cells from the primary site to the periphery via the blood circulation.

Since VEGF ligand binds to VEGFR1 with higher affinity compared to its specific and more potent receptor VEGFR2, but that signaling through this receptor is much less or non-existing, it has been shown that VEGFR1 has a negative regulatory effect on VEGF, and blocking of VEGFR1 boosts the effect of VEGF in induction of angiogenesis (52, 58).

VEGFR3 is also known as Fms-related tyrosine kinase 4 (Flt4). It is specific for the third group of ligands, VEGF-C and D. VEGF-C and D ligands have angiogenic and lymphangiogenic effects since both have binding sites to VEGFR2 and VEGFR3. VEGF receptors are involved in angiogenesis and lymphangiogenesis during development in formation of vascular networks and therefore global knockout of these genes lead to embryonic lethality.

Blockade of VEGFRs lead to inhibition of angiogenesis, which since a long time has been a beneficial way to treat cancer patients. Among the vastly developed drugs to inhibit angiogenesis, Sunitinib has been used in combination with other drugs in many animal experiments and in human patients. Sunitinib is a multi-targeted small molecule inhibitor, which targets the VEGFR, PDGFR, RET, colony-stimulating factor-1 receptor (CSF-1R), the hematopoietic markers Flt-3, and c-kit. Sunitinib blocks the receptor tyrosine kinases (RTKs) by competing with ATP to bind the intracellular domain and consequently preventing signal transduction. Sunitinib is routinely used for treatment of gastrointestinal stromal tumor (GIST) and advanced renal cell carcinoma (RCC) (59).

VEGFR1 deficiency is embryonically lethal due to the excessive growth of blood vessels, which suggests a negative regulatory role for this receptor. Mice lacking VEGFR2 on the other hand, die from the lack of vasculogenesis and angiogenesis. And as expected, mice deficient for VEGFR3 also die at embryonic stage despite the large blood vessels formed. The lumen of these vessels is defective and mice die of accumulation of fluids in the pericardial cavity as well as cardiovascular failure.

Deletion of VEGF-A and C are embryonically lethal while the lack of one of remaining VEGF family members, affect the embryo but is not lethal. (47, 52, 60-62)

Judah Folkman showed that a piece of tumor after implantation into an avascular tissue such as rabbit cornea (63) is capable of inducing the growth of new capillaries and creating a blood supply. This fact suggests that tumors release some factors to the surroundings that are diffusible and taken up by the various neighboring cells and induces formation of new blood vessels through the complex process of angiogenesis (64). Except for only 0.01% of normal adult ECs, the rest of ECs are quiescent and cell division in order to maintain the vitality of the vasculature tissue only occurs in that small fracture (65). Angiogenesis is a process that occurs under the influence of different growth factors and cytokines, which act as stimuli and driving force. This process is known as angiogenic switch and VEGF-A has a vital role in induction of angiogenesis (66).

1.4 HYPOXIA

Lower levels of oxygen tension than the normal value in the tissue is called hypoxia. The normal level of oxygen varies between tissues due to the type of metabolism activity in each tissue and this level is lower than the air level, which is 21%. Organs such as lung parenchyma and heavily vascularized parenchymal organs such as liver, kidney, and heart have 4%-14% oxygen level while less vascularized organs like brain has 0.5%. The physiologic oxygen levels in bone marrow can be even lower and approach anoxia, 0%, which is also the case for non-vascularized tissues such as the cornea and cartilage (67).

Hypoxia occurs due to a reduction in the oxygen level and can be caused by increased altitude, an interruption of the blood supply to a region that can be a consequence of cardiovascular and pulmonary diseases or due to the growth of a tissue such as in cancer. Hypoxia gained importance when it was observed that some cancer cells showed resistance to cell-death induced by radiation (68). In the tissue, oxygen can travel to a certain distance before it is completely metabolized. It has been observed that oxygen can reach to the cells in 180 mm distance from the blood vessel and if the cells are lying

further away than this distance, oxygen cannot reach them and they will undergo necrosis both in healthy and tumor tissue. Since oxygen is a very essential molecule for the body, several molecular changes and reorganizations are required in order to adapt to its depletion.

As mentioned above, cells go through some compensatory changes in response to hypoxia, which allow them to survive and function.

One of the first consequences is the metabolic response, which leads to a switch from aerobic to anaerobic metabolism. In tumor hypoxia, cancer cells, in order to generate energy, undergo metabolic reprogramming that allows them to survive, produce the metabolites, and also helps them to maintain the same proliferation rate (69, 70). Under normoxic conditions, cells use the oxygen-dependent tricarboxylic acid (TCA) cycle to produce 38 ATP molecules from glucose, but in hypoxic conditions, they switch to an oxygen-independent metabolic pathway called glycolysis. This process converts glucose to lactate and produces two ATP for each molecule of glucose but in a shorter time and in a faster manner. In cancers, it has been shown that accumulation of lactate, a product in the glycolysis pathway, in the tumor milieu causes acidosis, which is a characteristic for the tumor environment and induces survival of tumor cells, degradation of ECM and facilitates tumor cell migration (71). In support of this hypothesis, experiments show that by buffering the pH in tumor environment, spontaneous metastasis was significantly reduced (72). Recently, it has been shown that tumor cells gain the ability to phosphorylate glycogen, which is the intracellular stored form of glucose, to produce energy. Up-regulation of glycogen phosphorylase has been observed in tumors both in *vivo* and *in vitro* (73).

Another way for the hypoxic cells to quickly cope with the lack of oxygen is to increase vessel dilation and deliver more blood without increasing the number of vessels. Vessel dilation is mediated by endothelial-derived nitric oxide (eNOS). Nitric oxide acts as a relaxing factor for vascular smooth muscle cells and allows more blood to flow into the hypoxic region and thereby increasing the oxygen level in that specific area. Increase in the number of red blood cells, which are the oxygen carriers in the body, is another way of compensating for the reduced level of oxygen in the cell milieu. Enhanced erythropoietin (EPO) production in the liver and kidney under hypoxia induces proliferation and maturation of erythroid progenitor

cells and increase the number of erythrocytes and consequently raises the oxygen delivery into the region (74). Another option of dealing with reduction of oxygen level is formation of new blood vessels in a very short time in order to deliver more oxygenated blood and compensate for the lack of oxygen. Finally, deregulation of tumor cell apoptosis is another way of helping cells to survive the hostile environment although this way per se can act as a selection for more chromosomally defected and robust cells and eventually lead to metastasis and spreading throughout the body (69).

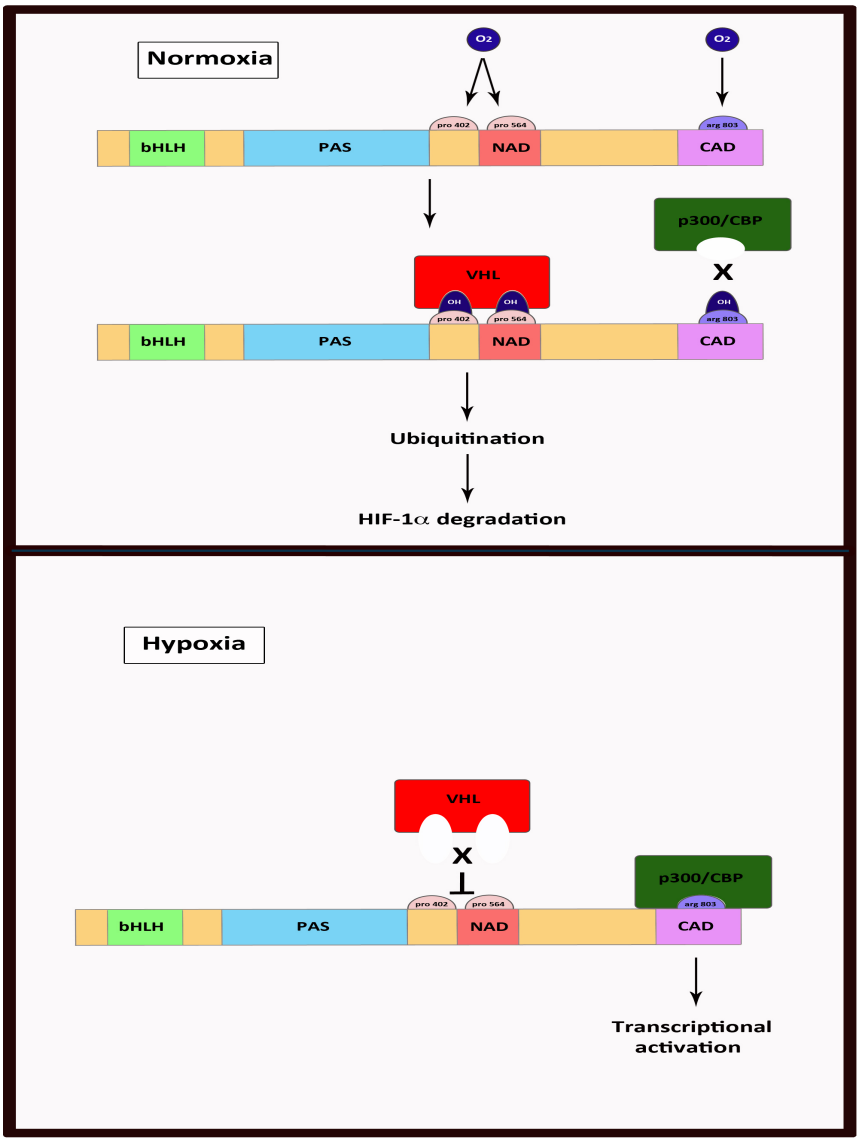

Figure 2: Oxygen sensing by hydroxylation of HIF-1α

Hypoxic response is mediated via different molecular pathways; among these molecules, hypoxia-inducible factors (HIFs) are the most studied ones. The HIF family consists of heterodimeric transcriptional factors HIFα and HIFβ, also known as aryl hydrocarbon receptor nuclear translocator (ARNT).

The HIFα family has three members, HIF-1α, HIF-2α and HIF-3α, which regulate and activate transcription of a wide range of target genes involved in cellular homeostatic reaction to altered oxygen levels.

HIF subunits have similar molecular structures and consist of two subunits. The first subunit, the amino acidic part that consists of basic helix-loop-helix (bHLH) domain and a period circadian protein-ARNT-single minded protein (PAS) domain followed by the carboxyl half of the HIF-1α, which is a transactivation domain (TAD). PAS and bHLH domains create a functional interface for heterodimerizations of the subunits and as a consequent DNA binding. TAD contains a central oxygen-dependent degradation domain (ODD). ODD contains two transactivation domains: N-terminal activation domain (NAD), responsible for targeting gene specificity and the C-terminal activation domain (CAD), which contribute to regulation of most of the HIF target genes and not all of them (75-77).
HIF-1α subunit is constitutively expressed under both normoxic and hypoxic conditions and the protein is localized in the cytoplasm. The half-life of HIF-1a is less than 5 minutes under normoxic conditions and its degradation is tightly regulated because of the very crucial role it plays in hypoxic pathways.

1.4.1 HIF regulators and activity

The main regulators of HIF-1α are a group of oxygen and iron dependent enzymes called prolyl hydroxylase domain enzymes (PHDs), which belong to the family of iron and 2-oxoglutrate-dependent dioxygenase enzymes. PHDs are known to be oxygen sensors in cells and have different expression and distribution patterns. PHD2 is mainly found in the cytoplasm and has been specifically shown to be able to hydroxylate HIF-1a at two proline residues highly conserved at position402 and 564 within the ODD. Another hydroxylation site within the ODD is asparagine 803, which is target for factor inhibiting HIF-1 (FIH-1). When the asparagine residue is hydroxylated, HIF-1a is not able to recruit its co-activators such as p300/CBP and transcription of target genes is ablated. The activity of PHDs is regulated by availability and also level of oxygen in the environment. In the cytoplasm, in presence of oxygen, PHD2 with help of the cofactors ascorbate, Fe^{2+} and 2-oxoglutrate, hydroxylate the two proline residues. The hydroxylated form of

HIF-1a subunit is exposed to the von Hippel-Lindau tumor suppressor protein (pVHL), and binds to the protein through the hydroxyl groups and becomes recognizable for the E3 ubiquitin-protein ligase for ubiquitination and undergoes proteasomal degradation (Fig. 2). PHDs activity can be inhibited by either removal of ascorbate or by agents such as desferrioxamine (DFX) that act as iron chelators and thereby impair the function of the cofactors.

On the other hand, under hypoxia, lack of oxygen and ferrous-ions impairs the function of PHDs and therefore hydroxylation of the proline residues is suspended. Consequently, the HIF-1α subunit is stabilized and translocated to the nucleus with help of importins through nuclear pore complexes (NPCs). Once the α subunit enters the nucleus, it heterodimerizes with the b subunit (ARNT) forming the HIF heterodimer. This dimer can readily bind to the specific DNA binding region of the target genes called hypoxia response element (HRE). Binding of the HIF heterodimer to the HRE of the gene of interest activates transcription and consequently expression of the corresponding gene (78, 79).

Since tissue hypoxia causes great damage, cells invest a lot of energy to constantly express HIFs. HIF-1 mRNA has been found in all human tissues such as heart, brain, lung, liver, kidney, skeletal muscle, pancreas, and placenta. Under normoxic conditions however, the amount of HIF-1β has been found to be more similar to HIF-1α. This can be attributed to the tightly regulated degradation mechanisms that exist to keep the level of this notorious transcription factor at a homeostatic level (80).

1.4.2 HIF-1 and angiogenesis

A large amount of data shows that HIF-1 has a crucial role in inducing angiogenesis during embryonic development and pathogenesis. Genes such as VEGF, Angiopoetin 1 and 2, PlGF and PDGF-B and their receptors are among angiogenic genes that are up-regulated in response to hypoxia. Expression of these genes is EC specific and limited to specify role that they have during hypoxic response, although VEGF expression is induced in various types of ECs e.g. arterial ECs, arterial smooth muscle cells, cardiac fibroblasts, and monocytes.

Vegf, which is one of the main genes involved in angiogenesis, is targeted by the HIF-1 heterodimer under hypoxia. Transcription occurs right after the

HIF-1 heterodimer binds to the HRE region of *vegf* gene (78, 80, 81). Increased levels of VEGF induce angiogenesis by stimulation of the ECs to proliferate and migrate, and together with matrix metalloproteases and other growth factors, new vessels will be formed from pre-existing ones in a short amount of time with a high pace to compensate for the lack of oxygen. In tumor condition, hypoxia is a result of high proliferation rate of tumor cells and the demand from the tumor to generate more blood vessels. The resulting increased level of VEGF induces formation of pre-mature, tortuous, and leaky vessels, which cause interstitial hypertension. Due to the interstitial hypertension, drug delivery and in general any fluid supply into the tumor will be affected and impaired and as a result, tumors will face more internal hypoxia and eventually become necrotic in the center. Acute hypoxia in the tumor, promotes some cells to adapt to the harsh conditions and become more aggressive and invasive (Fig. 3). Adapted cells that could survive this environment will escape the tumor with help of the leaky vessels and travel throughout the body via circulation (82).

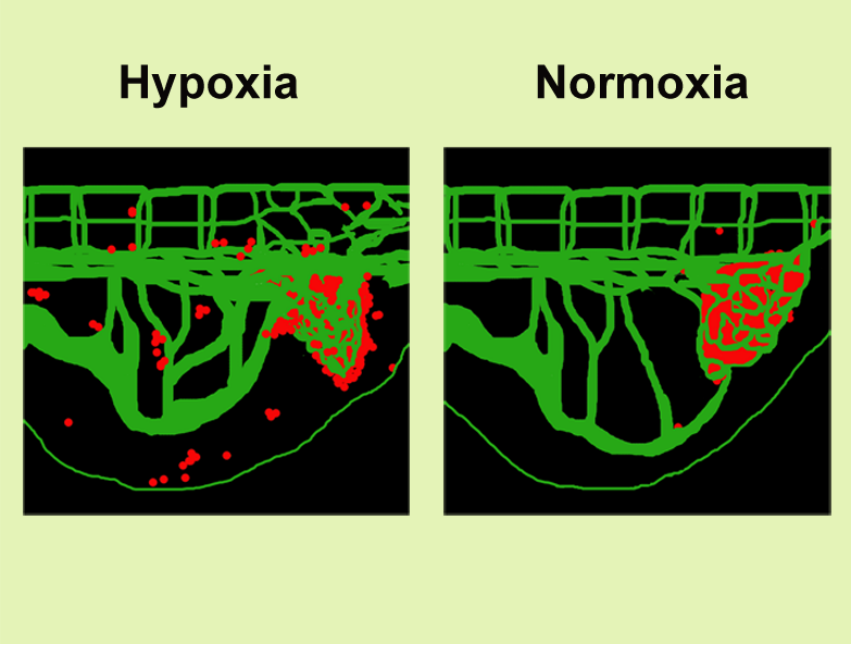

Figure 3: Tumor cell dissemination and invasion under normoxia and hypoxia. Endothelial cells are represented with green color and tumor cells are red (82).

1.4.3 Extracellular matrix and MMPs

The extracellular matrix is a complex milieu, which consists of variety of cell types involved in tissue remodeling, matrix proteins, proteases, and signaling molecules (83, 84). Irregularity in ECM proteolysis results in invasiveness of

the tumor including unregulated tumor growth, tissue remodeling, inflammation, invasion of tumor cells into the neighboring tissue, and eventually metastasis (85). Therefore, in addition to VEGF, another group of genes essential to enable the angiogenic process is the ECM metabolism genes such as MMPs, which are also up-regulated under hypoxia. MMPs are a family of zinc dependent proteases, which are in close contact with other cell types in the ECM and execute key roles in ECM homeostasis. Activity of MMPs is highly regulated at transcriptional level under physiological conditions. MMPs have different substrate specificities and based on that are classified into gelatinases, collagenases, matrilysins, and stromelysins. To date, 24 MMP genes have been identified in humans (86). The family of gelatinases has been associated to tumor aggressiveness in many cases. They have been considered to promote metastasis due to the degradation of collagen IV, which is among the most vital members of the basement membrane components. This MMP group consists of gelatinase A or MMP-2 and gelatinase B or MMP-9. It has been shown that MMP-2 is highly produced by tumor-associated fibroblasts, while quiescent fibroblasts express very low levels of this MMP (87). MMP-9 is produced by tumor cells and also inflammatory cells around the carcinoma islands in OSCC (Oral squamous cell carcinoma) and other types of tumors. MMPs are regulated at the transcriptional and post-transcriptional level with help of tissue inhibitors of metalloproteinases (TIMPs), hormones, growth factors, cytokines and cellular transformation (86). MMP-9 is secreted as inactive zymogen pro-MMP-9 which is cleaved and activated by plasmin and yields the enzymatically active isoform (88). When gelatinases become activated, they degrade the ECM and thereafter generate and release a variety of cytokines, growth factors, and chemokines trapped in the ECM and influence tumor progression and other pathogenic phenomenon such as metastasis. Factors such as VEGF-A, TGF-b, and FGF-2 released by ECM via MMP-9 activity, stimulates ECs to proliferate and migrate and consequently promote tumor growth and angiogenesis (89).

1.4.4 MAPK pathway in cancer

Mitogen-activated protein kinase (MAPK) cascades consist of protein kinases that are critically involved in transducing extracellular signals into the cells.

They thereby control fundamental cellular processes such as growth, normal cell proliferation, differentiation, migration and apoptosis and cell survival (90, 91). Various types of stimuli such as growth factors, environmental stress and cytokines activate mammalian MAPKs. ERK pathway is activated by growth factors while JNK, p38, and ERK5 pathway are stimulated by stress and growth factors (91). ERK pathway is among the most well studied pathways in mammalian MAPK pathway and is disrupted in approximately 30% of all human cancers. In ERK/MAPK pathway, under influence of growth factors and mitogens, Ras recruits Raf to the plasma membrane and activates, upon Raf activation, MEK (1 and 2) is phosphorylated, which in its turn activates ERK by phosphorylation and eventually induce cell proliferation, differentiation, apoptosis and migration (90, 92). Also it has been reported that VEGF has stimulatory effects on Ras-Raf formation, which in turn is the key combination in MAPK/ERK pathway to activate ERK1/2 and induce the specific signal (93). Some transmembrane-coupled receptors activate MAPK through a PKC-dependent pathway. Studies show that these PKC-dependent pathways are accompanied by Ras activation, which requires a Ras-Raf complex formation (94).

1.5 FILAMINS

Cell shape, locomotion, division, and phagocytosis are all processes, which are dependent on the dynamic reorganization of the actin cytoskeleton. Actin filaments can be involved in different structures from parallel bundles to gel networks and accordingly, have distinct functions. When actin filaments act as bundles, they function as cables and provide tensile strength and help to transport organelles by serving as tracks through the cell. In formation of 3D networks (gel network), actin filaments help to transport fluid, protect the cells from osmotic hydrostatic fluid flows and also function as barriers to spontaneous movements of grand organelles and also assist with localization of intracellular signals and reactions. All actin functions are organized by actin binding proteins, which via different mechanism arrange and rearrange the actin filaments structure and adjust them to the required response. Scaffolding molecules allow actin filaments to transduce a signal from the transmembrane compartment to the cytoskeleton and vice versa. Filamins are

a family of cytoskeletal proteins with high molecular weight, which serves as scaffolds to a big variety of proteins with diverse functions and in this way they can act as cell mechanics and signaling integrators. Filamins organize the filamentous actin fibers included in orthogonal networks or stress fibers in the ECM (95, 96). Embryonic development and homeostasis requires directed cell movement and any alteration and abnormality in cell migration, even in adults, will result in pathological processes and diseases such as chronic inflammatory diseases, vascular disorders, tumor formation, and metastasis amongst other diseases. The filamins that are involved in cell motility are located at the lading and rear edge of polarized motile cells in order to be in direct contact with actin cytoskeleton. Position of the filamins at the edge of the motile cell allows them to protrude and retract filopodia by directly affecting the actin cytoskeleton. The special manner of distribution, expression level, and presence of filamins at the edge of cell, defines the type of protein partners, which will interact at the membrane and promote cells to initiate or terminate migration or other actions.

Filamin molecules are found in a dimeric form with an up to 80% overall sequence homology. Vertebrate filamins are elongated V-shaped dimeric proteins with 240 and 280 kDa molecular weight polypeptide chains. Monomeric chain contains an actin-binding domain (ABD) at the N-terminus. The ABD consists of two calponin homology (CH) domains, CH1 and CH2, which are separated by a linker and two Rod domains with Ig-like repeats following the ABD. The Rod1 domain's Ig-like repeats form a single linear chain, which has the ability to bind to actin filaments while, Rod2 with its double chain structure, cannot bind to actin filaments. The two Rod domains are separated by hinge domains (H1 and H2), which are relatively long flexible loops consisting of 30 amino acids and have vital functions in maintaining flexibility of the filamins and also enable them to cross-link to actin filaments in various angles and positions. Filamin subunits undergo dimerization in order to increase the actin binding capacity and cross-linking abilities. The single Ig-like repeat, which comes after the H2 enables the subunits to dimerize (Fig. 4) (97). The filamin family in humans has three members: FLNA, FLNB, and FLNC. The three members of the family show approximately 70% overall sequence homology and with the lowest similarity (45%) found in the hinge regions. Filamins are vital for mammalian

development and it has been shown that any function-disturbing mutation in human filamins results in various congenital diseases such as brain, bone and cardiovascular system defects. FLNA and FLNB are abundantly expressed in all tissues in the body during development. Their expression level remains high later on while FLNC expression is limited to skeletal and cardiac muscles with some expression also in non-muscle cells such as in the cortical surface although at a very low level (98-100)

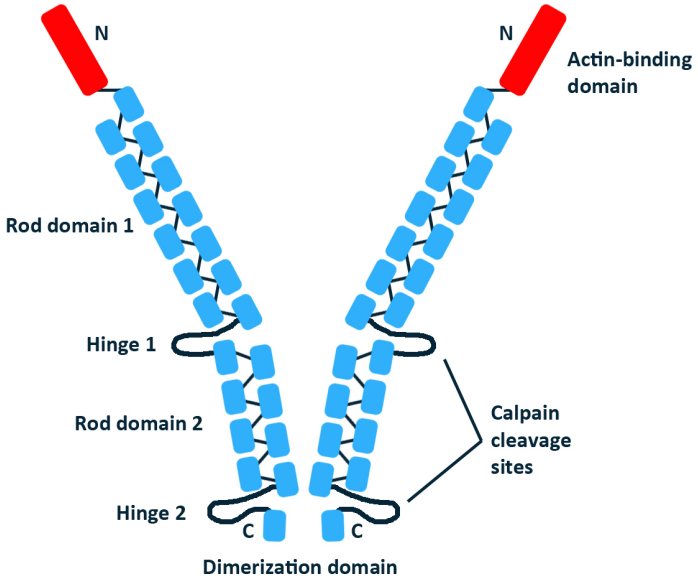

Figure 4: A schematic structure of full length FLNA molecule.

In vitro studies on human fibrosarcoma show that knockdown of filamins results in increased activity of MMPs, remodeling of the ECM and consequently elevated migratory properties of the tumor cells (101).

1.5.1 FLNA and FLNB

Cells achieving migratory properties must undergo different steps, which allow them to gain ability to sense and generate force. These steps include cell polarization, membrane protrusion, rearrangement of cytoskeleton, contractibility mediated by myosins, cycles of adhesion and de-adhesion etc. (102).

FLNA was primarily linked to cell locomotion in a human melanoma cell line, which lacked FLNA and was not able to plasma bleb and eventually migrate. This in turn, leads to debilitation of the actin structure and cortical surface

instability. The phenotype was rescued by re-expression of FLNA by which the plasma membrane regained the blebbing function and became motile again. The migratory effect of FLNA was confirmed when a nonsense mutation was found in the FLNA gene, which ablated neuronal migration and caused periventricular heterotopia (PVH). These FLNA deficient neurons instead of migrating, formed layers with distinct shape, nodules lining the ventricular surface and caused seizures. The PVH disease is X-linked and homozygous males die at embryonic stages whereas affected females survive but suffer from epilepsy and other signs of neurological malformations (103). FLNA has more than 70 binding partners and consequently has multiple functions during development. A cluster of missense mutations causes a diverse range of congenital malformations in humans (104).

FLNA depletion in cell lines also shows impaired cell spreading, migratory capacity, or initiation of migration. Reduced stress fibers and adhesion stability are among the induced cell properties caused by lack of FLNA (105-107). Overexpression of FLNs can lead to decreased migration of cell due to the increased b integrin bindings and immobilization of the cells and cause similar symptoms as of the lack of expression of filamins (108).

During development in mice, it has been shown that FLNA is expressed ubiquitously while FLNB is mostly concentrated in ECs. FLNB deficiency can lead to impaired endothelial migration during angiogenesis and formation of new vessels *in vitro*, while the effect of ablation of FLNA was a lot less pronounced. Considering the *in vitro* discoveries from ablation of FLNA and B, knockdown mouse models show that reduced level of FLNA affects the cell junctions especially VE-cadherin junctions and adherence junctions while reduction in FLNB protein expression leads to altered EC migration (109). Loss of FLNB in mice lead to impaired differentiation of chondrocyte precursors, malformation of skeleton, and ectopic bone formation in the cartilage and the skeletal disorder caused by FLNB deficiency is similar to the human condition where FLNB is mutated indicating that FLNB has a regulatory effect on cartilage and bone development (110).

FLNA contributes to mechanical stability of the cell and interacts with several proteins that regulate cell adhesion such as b1 integrin. b1 integrin is an important subunit for cell adhesion to collagen and fibronectin and therefore disturbed recycling of the b1 integrin to the membrane of the cell will prevent

directed cell migration. Reduction or deficiency of the FLNA level in cells lead to reduced surface expression level of b1 integrin, and the defect can be rescued by expression of FLNA. These data suggest that FLNA regulates expression of b1 integrin and similar cell adhesion receptors, which are the key regulators of the cell adhesion and motility (111). FLNA is abundantly expressed and is mostly localized in filopodia, lamellipodia, stress fibers and focal contacts (112). As mentioned above, FLNA is concentrated at the cell membrane when cells are spreading. This indicates the essential role of FLNA in regulation of cell adhesion and also cell adhesion strength. In contrast, FLNB is not localized in a way to be involved at focal contacts and is more incorporated into actin stress fibers.

1.5.2 Calpain

Calpain belongs to a large family of Ca^{2+} activated proteases, which cleaves the FLNA molecules with high efficiency in the H1 domain and to a lesser extent at the H2 domain (113). H1 cleavage creates two fragments with a molecular weight of 100 and 190 kDa. The 100 kDa fragment equals the 16-24 Ig repeats of the FLNA molecule and is equivalent to the C-terminus of FLNA (114). It is known that FLNA after Calpain cleavage allows enables actin cytoskeleton rearrangements. Calpain has been also been shown to be activated by hypoxia (115).

1.6 ZEBRAFISH AS A MODEL ORGANISM

The whole genome of the zebrafish has been sequenced giving us the opportunity to genetically manipulate any gene of interest in order to study its function. In addition, specific genes can be knocked down with help of Morpholinos during early development of the embryo. This method is specific to zebrafish due to the extra-uterine development and therefore much easier than doing knockouts in mammals. Genetically modified strains can also be generated in large-scale methods such as ENU (N-ethyl-N nitrosourea) and ENU mutagenesis derived TILLING (Targeting Induced Local Lesions In Genomes) (116) More recently developed methods, which use engineered nucleases such as ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases) or the CRISPR-Cas9 system. These methods introduce a break in the DNA of the target gene and this way, they can

directly target desired genes instead of causing random mutations in the genome as it is the case with ENU mutagenesis (117). Induction of temporary genetic modifications is easily done by injection of mRNA of the specific gene in order to overexpress it or by MO injections to knockdown of the gene of interest (118). Morpholinos are small synthetic RNA –analogues, which with equal efficacy as wild type RNA anneal to the host mRNA transcripts and blocks docking of the ribosome, or inhibit the maturation of the RNA before they get translated into proteins. Morpholinos are usually injected into the fish embryo at 1-4 cell stage in order to knockdown the gene of interest in all the proliferating cells and the effect will be sustained for the first 4-6 days post fertilization (119). Other physical specificities, which make zebrafish a desirable model to study disease, are high number of embryos that are produced at each pairing, the extra uterine development of the embryos, short breeding cycle, short generation time, and small space they occupy to maintaining them. All these features turn them into good candidates to perform large-scale genetic screenings and also experimental analysis. During the first days of development, zebrafish are highly penetrable. In fact up to day 5 post fertilization, the zebrafish embryo "breathes" through the skin. This amenability allows us to apply any orally administered drug or pharmacologic compound simply by adding them into the husbandry water. Furthermore by controlling the oxygen level in the water, which gives us control over different levels of hypoxia within the fish. As mentioned previously, researchers started using zebrafish not only for developmental biology studies but also to investigate pathological diseases such as cancer. Among different diseases models, an adult zebrafish model has been used to study spontaneous melanoma induced by activation of the *BRAF* oncogene. In another study, to investigate the distribution of *p53* mutations in melanoma, a specific *BRAF* construct was injected into a *p53* zebrafish mutant and when the fish reached an age of 4 months, nevi were detected and investigated by histological methods. Zebrafish embryos on the other hand create a good platform to study cancer in a different manner. Nicoli et al. established a tumor xenograft model to study angiogenesis in two days old zebrafish embryos and in our lab, we established an embryonic model, which allows us to study early events of tumor cell metastasis in single cell resolution under different oxygen concentrations (120-123). Useful strains such as *fli:EGFP, flk:EGFP,* and

gata1:dsRed simplify studying tumor angiogenesis and metastasis. Fluorescently-labeled blood vessels allow scientists to investigate any small disruption and malformation in the vessel structure with help of fluorescent or confocal microscopy without any necessity for staining. Therefore vasculogenic and angiogenic events can be monitored and recorded in the living animal over a long time window (124). Zebrafish are very useful tools for researchers to study pathological and developmental biology due to the benefits this model provides researchers. Increasing number of studies in zebrafish recently show that the growth factors and cytokines involved in the vascular development, remodeling and angiogenesis in mammals is similar to the zebrafish (125) and that physiological and pathological events in these two systems are highly similar and conserved (126-128)

Zebrafish embryo and adult models provide outstanding opportunities to study pathological and physiological angiogenesis in a non-invasive manner (129, 130).

Unfortunately lack of transgenic zebrafish lines or specific antibodies which could be used as markers for the vascular mural cells, prevents researchers from investigating the maturation and remodeling of the fish blood vessels in the same way as in mice. Although there are some markers such as SM22a and acta2 for which transgenic reporter strains have been produced, these markers do not label the majority of the cells covering the vessel wall (131). Alvarez et al shows by using electron microscopy that in embryonic and adult zebrafish many vessels are tightly covered with pericytes, which is not the impression one gets from examining these reporter strains (132).

As zebrafish embryos develop at a high speed, their cardiovascular system also develops quickly. Fish embryos develop a beating heart and a complete circulation containing circulating erythroblasts only 24 hours post fertilization. The cardiovascular system of fish differs from mammals due to the different demands and environment they live in. The respiratory system of the fish takes up the oxygen from the water unlike mammals that use air. The zebrafish heart is a spongiform two-chambered organ that pumps the venous blood into the gills for re-oxygenation, and then directly to the periphery without returning to the heart first (133). But on the other hand it has some similarities to the mammalian hearts such as containing pacemaker cells and sympathic innervations as well as cardiac valves and coronary vasculature,

which makes it a suitable model to study diseases of cardiac valves, infarction and malformation or malfunction of cardiovascular system (134).

2 AIMS

The overall aims of this thesis were to study different underlying mechanisms involved in tumor cell invasion and metastasis.

I. To establish an *in vivo* model to study tumor cell invasion and dissemination.

II. To investigate effects of hypoxia on tumor cell invasion and dissemination in zebrafish embryo.

III. To investigate regulatory effects of FLNA on HIF target genes during tumor growth.

IV. To investigate effect of FLNB in angiogenesis and tumor cell metastasis and the molecular mechanism involved.

3 METHODS

Tumor implantation in zebrafish embryo

The lack of an adaptive immune system during the first few weeks post fertilization in zebrafish embryos makes them perfect candidates for implantation of mammalian tumor cells without any possibility for transplant rejection responses. The transparent nature of zebrafish embryos allows us to trace the fluorescently labeled disseminated cells and foci at the single cell level in a completely non-invasive fashion. Although pigmentation occurs already 24 hours post fertilization adding low dose of pigmentation inhibiting drug "PTU" into the house-keeping water this can be easily prevented. Orally active drugs e.g. Sunitinib can be simply delivered to the fish embryos by adding to the water.

In order to study all disseminating single cells and foci, we use Tg*(fli1:EGFP)* zebrafish line in which all ECs express green fluorescent protein already at 16 hours post fertilization. Since disseminated tumor cells will be detected with help of fluorescent microscope, they should either permanently express a fluorescent protein or they should be dyed with a fluorescent dye which can be easily distinguished from the green fluorescent color emitted from embryos vasculature. Tumor cells are preferably implanted in non-vascularized area in the perivitelline cavity although it is crucial to have blood vessels in the vicinity of the primary tumor mass to facilitate dissemination and invasion of tumor cells into distal parts of the fish body. Transplantation of tumor cells is easily carried out using a micromanipulator, which is connected to a microinjector. Approximately 500 cells are implanted into each embryo.

Exposure of the tumor bearing zebrafish embryos to hypoxia

If the main effect of the study is to investigate the effect of acute systemic hypoxia on a specific cell, the transplanted embryos should be immediately placed into a hypoxic aquarium/chamber in which the level of oxygen is tightly regulated with a sensor placed inside the aquarium. To study dissemination and invasion of tumor cells under normoxic condition, tumor-bearing embryos will be transferred into a normoxic aquarium. In all conditions above, the fish embryos will be kept at 28**ºC** and will be investigated according to the experiment design.

Death rate is relatively high in hypoxia experiments and in order to have a sufficient number of embryos to allow for statistically correct conclusions, a relatively high number of embryos should be implanted with tumor cells and placed inside the hypoxia chamber.

Under normoxic conditions, each tumor-bearing embryo might be placed in a separate well of a multiple well plate and will be examined and investigated as preferred. Since both embryos and tumor cells have fluorescent colors investigations for tumor cell dissemination will be performed with help of fluorescence microscope.

On the contrary, in hypoxic conditions since all embryos must be kept together in the common aquarium and therefore imaging will be possible just at the last day of the experiment once embryos are collected from the hypoxic chamber.

If confocal imaging (3D) is required after fluorescent imaging, embryos should be mounted in mounting medium and kept at -20 °C until the day of imaging.

Microscopy

Number of disseminating tumor cells and foci can be easily counted with help of a fluorescent microscope in the living zebrafish embryos. The robust nature of the embryos allows the researchers to kinetically follow the invasion and dissemination pattern of the tumor cells daily or with shorter or longer intervals. Embryos will be easily anaesthetized and with help of a modified gel stage, will be fixed for imaging. Right after imaging, they will be returned into the house-keeping water where they usually gain consciousness immediately and start to swim and slide. The green signal captured represent the ECs in the fish embryo and tumor cells will be labeled with a different color such as red to be easily distinguishable from the vessels. In order to count the precise number of disseminated cells and foci, the whole fish embryo body will be captured in two parts with help of low magnification (4x). If necessary, images from different channels will be merged together to see the precise locations to which the that cells have migrated.

3D imaging can reveal more details about the structure of the vessels developed inside the tumor or host vasculature and also the form and shape of the primary tumor in sense of developing invasive or smooth edges. Also confocal imaging can be used to count the number of disseminated tumor cells and foci. Confocal (3D) imaging can be done on living fixed or dead fixed embryos. Each field will be scanned multiple times and the stack of images will be merged together to achieve a sharp image.

Morpholino injection and drug treatments

The sophisticated and precise method of MO knockdown allows the researchers to target the gene of interest and successfully ablate expression of the desired protein and induce the phenotype in order to achieve the aim of the experiment. Generally the zebrafish genome contains more than one copy of each gene and it should be kept in mind when choosing the right MO or MOs. MOs are antisense RNA-like molecules that bind to the host mRNA transcripts and blocks it and stops the protein expression. MO injection happens at the 1-4 cell stage in order to get the antisense RNA passively integrated into all the cells and stop the protein production and consequently knockdown of the gene of interest in the entire embryo throughout (early) development.

Zebrafish embryos are very permeable and penetrable during the first few days post fertilization. Blood circulation is initiated one day post fertilization but embryos do not use the blood-stream to circulate and transport oxygen. Instead they use the oxygen that has penetrated through the skin and they keep on using this way of breathing up to 5-7 days post fertilization. Not only oxygen, also many other small pharmaceutical molecules and compounds can also go through the skin and get absorbed by the fish embryo. Due to this amenability of the embryos, orally active drug in form of small molecules can also be easily administered to fish embryos by just adding into the water that fish is being kept in throughout the experiment or for the desired amount of time. Water containing drug might need to be changed and refreshed according to the

half-life and activity of the compound and evaporation of the water and possible changes in the concentration of the drug. Drug delivery through the skin will not be affected by changes in experimental settings such as various levels of oxygen and stress. Therefore this method is equally useful in normoxic conditions as in hypoxic ones.

Mouse tumor cell implantation

One of the best models to study angiogenesis and vessel structure is tumor cell implantation into mice. This model is the most similar model to the clinical situation and gives the opportunity to test different treatments if necessary. In these studies, human cell lines are subcutaneously inoculated into dorsal, subcutaneous sites on the back of SCID mice. Mice are kept in standard conditions and have free access to food and water. Tumor size will be measured when the tumor starts to grow and reach a palpable size (approximately 0.2 cm3). According to the design of the experiment, tumor size will be measured with equal intervals. When tumors reach the desired size, mice will be sacrificed and the tumors will be removed, weighed, and processed for further investigations.

Histology (Immunohistochemistry)

Histological methods are used in the labs to visualize the structure of vessels, and other elements of the tumor microenvironment, ECM compartments and etc. Specific staining of particular cell types in tumor or organ tissues is simply done by using specific primary antibodies against specific proteins or cell markers on the target cells. Secondary fluorescent antibodies against the primary antibody will help to visualize the labeled cells and with help of fluorescent or confocal (3D) microscopes, structures will be easily imaged and further investigations can be performed on the captured images. Images allow us to see the precise composition of the tissue in term of the cell types present in a quantitative way. In vessel studies specifically, 3D images of a certain width of the different types of tumor tissues, allow the researchers to easily compare vessel density, vessel number, vessel diameter and mural cell structure and other vessel related parameters. Availability of different types of primary and secondary antibodies gives researchers the opportunity of combining different antibodies at once and to stain the tissue for different cell markers and simultaneously looking at different cell types or proteins. Tissue used for IHC staining can be treated in different ways to preserve the antigens. Fresh tissue can be snap frozen and kept in -80°C and cut into thin slices prior to staining with specific markers. Alternatively, tissue can be fixed with 4% paraformaldehyde (PFA) and shortly after harvest, sliced into very thin sections and stained in a method, which is called whole mount staining. This provides the opportunity to investigate structure and gives layout of a specific field, i.e. blood vessel structure and composition of the mural cells or ECM cells and compartments. PFA-fixed tissue can be also embedded in paraffin, which in that case can be sliced into very thin sections using a microtome.

Cell culture

The formulation of the medium for culturing cells varies depending on the requirements of each cell line. Medium is usually supplemented with the regular cell requirements e.g. fetal calf serum and antibiotics penicillin/streptomycin but sometimes cells require additional

supplements such as specific antibiotics that can be used for selection and maintenance of specific gene expression. Cells are cultured at 37°C under normoxic (21% O_2) or hypoxic (1% O_2) conditions. Duration of hypoxia exposure depends on the experiment settings.

Cell transfection

To validate the effect of a specific gene on expression of another downstream gene or in a signaling pathway, a specific gene can be transfected into the cell or silenced using siRNA (short interfering RNA) or shRNA (short hairpin RNA) methods. If cells are transfected with shRNA, in order to be able to measure protein concentration and gene activity, GFP and/or luciferase reporter genes can be transfected into the cells as well and if necessary, transfected cells will be treated with the specific selection component for an amount of time before being suitable to use in experiments while siRNA transfected cells can be cultured 48 hours post transfection under hypoxic and/or normoxic conditions. Accuracy of the shRNA transfection is generally higher than siRNA transfection. Level of expression can be checked by different available methods.

Cell transformation

Cell transformation is a way of inducing immortality to the cell, which will be used in specific experiments. If there are not many suitable cell lines, which can be used for a particular aim of a study, non-cancerous cells such as mouse embryonic fibroblasts (MEF) can be transformed with an oncogene to gain cancerous properties and grow in the animal models.

RT-PCR

In order to quantify the transcription level of a particular gene, or investigate changes in expression of a gene over time, quantitative RT-PCR is performed. The RNA from cells grown and treated under different conditions will be isolated and a cDNA library generated using the isolated RNA. With help of specifically designed primers, the gene of interest is amplified. The PCR products can be analysed by running on agarose gel electrophoresis and quantified by densitometry reading. Housekeeping genes such as b-actin are used as internal control.

Enzymatic activity (Gelatin zymography) and Proteolytic activity

Gelatin zymography is a method to detect enzymatic activity. The method principles are similar to the regular gel electrophoresis or SDS-PAGE gel electrophoresis but contain a substrate, which co-polymerizes within the polyacrylamide gel matrix (e.g. gelatin) to detect the enzymatic activity.

Chromatin Immunoprecipitation (ChIP)

In order to analyze whether a protein of interest is binding to a specific DNA sequence, such as a motif within the gene promoter, one can carry out Chromatin Immunoprecipitation. Here, DNA-bound proteins in a cell are cross-linked right after the DNA is fragmented by sonication. Immunoprecipitation of a protein-DNA complex is accomplished with specific antibodies against the protein of interest. Subsequent reversing of cross-linking allows for analysis of precipitated DNA-fragments, which is performed by quantitative PCR (qPCR) using oligos amplifying specific DNA regions.

Immunoblotting

For detecting the amount of a specific protein or posttranslational modifications such as phosphorylation or cleavage, immunoblotting can provide quantitive information. Samples from total lysates of tissues or cells, or lysates after e.g. immunoprecipitation can be used. The proteins are separated based on molecular size by SDS-page, transferred to a membrane where incubation with specific primary and subsequently with labeled secondary antibodies allow for detection of proteins in a quantitative manner using e.g. HRP or IR-Dye. Housekeeping genes such as β-actin are commonly used as an internal loading control.

Matrigel invasion assay

The matrigel invasion assay is a useful in vitro tool to study the ability of tumor cells to invade and also to distinguish the cells that posses or have acquired an invasive phenotype due to different manipulations and mutations. A modified Boyden chamber membrane was coated with matrigel as basement membrane matrix. In our experiment, we investigate the invasive phenotype of OVSCR8 cells by culturing them with and without FLNB on matrigel.

4 RESULTS AND DISCUSSIONS

4.1 HYPOXIA INDUCES METASTASIS IN ZEBRAFISH EMBRYONIC TUMOR MODEL (PAPER I)

Tumor cell invasion and metastasis are among the main causes of malignancy and high mortality rates in some cancers. The molecular mechanisms of these events are under extensive investigation and can be potential novel targets for the development of anticancer drugs. Tumor cell invasion and metastasis events may occur at relatively early stages of tumor progression and development. It is well known that hypoxia promotes tumor cell invasion and metastasis by induction of angiogenesis (69-71). Unfortunately, to date, there has been no *in vivo* model allowing us to monitor the very early events of invasion of the tumor cells into neighbouring tissues and organs. In mouse models and cancer patients, metastatic nodules are detected when they have already reached a palpable size or when they are detectable with the commonly available methods such as radio imaging. At this stage, the disease is progressed relatively far and consequently, removing all the metastatic nodules and clearing the patient's body from metastasis is often an impossible task. In order to study the specific genes and molecules involved in the metastatic cascade, we aimed at developing a model system, which allows us to follow each and every cell over time as they move away from the tumor in a living animal and even follow up the invasion pattern of the cells with help of a fluorescence microscope in a non-invasive manner.

To achieve this goal, we developed a zebrafish tumor model inspired by a published method by Nicoli (120, 135). In this model, fluorescently labelled tumor cells were implanted into the perivitelline space of 2 days old zebrafish embryos. Systematic hypoxia is applied to the tumor-bearing zebrafish embryos by placing them into an aquarium containing hypoxic water. The oxygen level in the chamber is tightly regulated with help of an oxygen sensor. Zebrafish embryos were inoculated with non-invasive tumor cells and then divided into two groups, which were exposed to regular (normoxia) and low (hypoxia) level of oxygen, respectively. Embryos, which were exposed to the low level of oxygen for three days, showed significant a increase in the number of disseminated cells from the primary tumor mass while tumor cells in the

embryos kept under normal oxygen level, stayed at the primary site and did not invade the distal parts of the fish body.

Since the zebrafish body is penetrable and amenable to change in tissue oxygenation depending on the external oxygen levels, it acts as an "*in vivo* cell culture model" in which even the slightest changes in the fish environment can be directly affected the tumor mass implanted inside the fish. Therefore, implanted cells will suffer the changes in the oxygen level in the water, i.e. hypoxia, the signaling pathways involved will be activated and the resulting phenotype will be induced. Using confocal (3D) microscopy reveals details about the structure of the intratumoral vessels under different conditions and provides more further evidence that hypoxia-induced angiogenesis facilitates tumor cell invasion and metastasis.

4.2 HYPOXIA-INDUCED ANGIOGENESIS PROMOTES TUMOR CELL INVASION AND METASTASIS IN ZEBRAFISH EMBRYO MODEL. (PAPER II)

The angiogenesis pathway has stimulatory effects on the growth of the primary tumor and also a facilitating role in induction and increase of tumor cell metastasis (57). Intratumoral vasculature built under angiogenic conditions has some specific characteristics, which separates it from the regular vasculature. Angiogenesis induced by hypoxia is generally mediated by VEGF. Hypoxia activates the hypoxia inducible factor (HIF) pathway, which targets transcription of many genes as well as induces transcription of VEGF. Tumor angiogenesis induces formation of vessels that are leaky, immature, lacking mural cells, and is poorly perfused. Existence of such vessels consequently exacerbates hypoxic conditions inside the tumor, increases interstitial fluid pressure, leads to fast growth and changes in metabolic profile to compensate for the lack of oxygen (72, 74, 136).

Using the zebrafish model established in our lab, we tested various cell lines in order to identify tumor cell lines that form an isolated primary tumor. We found the murine fibrosarcoma cell line T241 suitable for the purpose of our study. Another murine cell line which was chosen to be implanted into the perivitelline space of 2 days old zebrafish embryos was Lewis Lung Carcinoma (LLC), which is more invasive in nature and in addition to generating a semi-isolated tumor mass, also gives rise to a slight dissemination in the region close

to the primary tumor mass. Both these cell lines have been previously tested in mice and the properties reported and observed were similar to those seen in zebrafish embryos. To further validate the usefulness of this method using other types of cancer cell lines, we implanted human tumor cell lines with well-known invasive and metastatic characteristics. Human ovarian carcinoma cell line OVCAR 8 cells gave rise to an in situ primary tumor that did not metastasize and the invasive human breast cancer cell line MDA MB 231 cells barely formed a primary tumor mass but instead they disseminated and invaded the surrounding and distal organs. These finding were in agreement with previous observations of the metastatic/non-metastatic behaviour of these tumors (137, 138).

The effect of hypoxia on tumor cell dissemination and metastasis was assessed using murine cell lines T241 and LLC, which were implanted into the zebrafish embryos. The metastatic profile of these cell lines has already been described. Tumor-bearing zebrafish embryos were transferred into a hypoxic chamber with a 7.5 % air saturation level post implantation and were kept in that chamber for 3 days. Non-invasive tumor cells kept under hypoxic conditions adapted an invasive and migratory phenotype and disseminated and invaded locally and distally indicating that tumor cells used various modes of dissemination to spread throughout the body. It can be assumed that those cells that invaded the neighbouring tissue, had probably adapted migratory properties. One can also speculate that due to changes in the extracellular matrix, these cells could easily move around and away from the primary site. Cells, which have disseminated to the more distal parts of the body, are likely to have used the blood circulation system to transfer from the primary site to the periphery. Existence of the single cells in the circulation provides evidence supporting the hypothesis that these cells use circulation system to travel thorough the body. Intratumoral 3D imaging of the vessels showed a drastic increase in vessel density and diameter in the hypoxia-exposed group compared to the control group kept under normoxic conditions, which suggests that VEGF-A may be involved in induction of the phenotype. Formation of invasive fronts in the hypoxic group was another metastatic and invasive property that this group has adopted under the hypoxic conditions (139). Dilated and tortuous vessels caused severe perivitelline and pericardial oedema, which is a common

sign of increased levels of VEGF-A. This will be further investigated with appropriate tools.

Expression of VEGF, which is the main growth factor in angiogenesis, is induced by hypoxia. We decided to implant T241 and LLC cells, which were genetically engineered to express high levels of human VEGF-A, and as a control, we inserted the empty vector. These cell lines were implanted into the perivitelline space of zebrafish embryos in equal cell numbers for both groups. Embryos were kept for up to 6 days post implantation in similar conditions and were imaged every other day in order to follow the metastatic events occurring post implantation. We observed that tumor cells expressing high levels of VEGF-A gained properties similar to the cells exposed to hypoxia after approximately 3 days post implantation. Invasive characteristics such as high numbers of locally and distally disseminated cells, increased intratumoral vessel density, perivitelline and pericardial oedema and development of invasive fronts were observed in the VEGF-A expressing group in both cell lines. The control group did not show any signs of malignancy and exhibited similar phenotypes as the normoxic group in the previous experiment. By looking at the host blood vessels in the vicinity of the implanted tumor mass, we realized that VEGF-A expressed by tumor cells affected the fish embryo vasculature and caused dilation, malformation and disorganization of the vessels. Since malformed and leaky vessels in the tumor environment can facilitate dissemination of the tumor cells from the primary site, we decided investigate the effect of VEGF-A on tumor cell metastasis by taking advantage of the two properties of zebrafish embryos, which make them very suitable and proper candidates to study pathological events. Since fish body is permeable, we decided to block the VEGF receptors using the commonly used small molecule, Sunitinib This compound is a tyrosine kinase inhibitor with a broad spectrum of targets including VEGF-A receptors, namely VEGFR1 and VEGFR2. The first method to investigate the effect of VEGF-A was to block the VEGFRs on the ECs in order to inhibit the signaling pathway and ablate the function of the VEGF-A ligand in the tumor environment. The concentration of Sunitinib was titrated to a level that would only block the expressed VEGF in the environment, and did not affect the native vasculature of the fish embryo. After implantation of T241-VEGF-A and LLC-VEGF-A tumor cells into the perivitelline space of fish embryos, they were transferred into the water

containing the orally active drug and one group was transferred into regular water as control. 6 days post implantation, the treated and control groups were investigated with help of fluorescence microscopy and the results were compared. The control group (non-treated) showed complete invasive phenotype compared to the experimental group, which was treated with Sunitinib. Number of local and distal dissemination of cells and foci was reduced significantly and also the tumor vasculature was markedly affected and vessel density decreased significantly.

With the goal of finding the responsible VEGFR to limit or ablate effects of VEGF-A, we chose to block VEGFR2, which is the main VEGF-A receptor. We chose a MO, which was already tested and results had been published previously. Since the zebrafish genome as opposed to the human or murine genome, contains more than one copy for most genes, MOs should be designed to target each one of the copies. Subsequently, the phenotypes resulting from targeting each specific copy must be compared and the MO against most efficient copy of the gene should be chosen for future experiments. However, sometimes it is required to knockdown more than one copy of the gene in order to achieve the accurate and proper phenotype.

To knockdown the expression of VEGFR2, we injected the specific VEGFR2 MOs into the zebrafish embryos at 1-4 cell stage. A scrambled MO, that should not affect gene expression, was injected into the control group. The concentration of the MO chosen for the experiment did not interfere with the normal development of the fish native vasculature and thereby an intact fish embryo vasculature was maintained. Implantation of T241-VEGF-A expressing cells occurred as in previous experiments, fluorescent-labelled tumor cells were implanted into the perivitelline space of fish embryos and they were kept under standard conditions for 6 days. Following the dissemination pattern of the tumor cells, we observed that cells implanted into the control group injected with scrambled MO, disseminated and invaded local and distal organs and showed the invasive phenotype as seen in the previous group. On the contrary, the VEGFR2 MO group, showed no sign of dissemination and the massive number of intratumoral vessels decreased drastically.

Hypoxia regulates many different signaling pathways and in order to study effects of hypoxia on tumor cell dissemination and metastasis via up regulating VEGF signaling pathways, we implanted non-invasive T241 tumor cells into

the fish embryo with the same setting as in previous hypoxia experiments and exposed the embryos to hypoxic water with or without Sunitinib. The tyrosine kinase inhibitor Sunitinib prevented any hypoxia-induced tumor cell invasion and metastasis and significantly reduced the intratumoral vessel density.

All the experiments performed in this work highlight the importance of the hypoxia induced VEGF expression that leads to a change in the vasculature of the tumor and the host. Changes induced by VEGF expression, promote and increase the number of disseminated tumor cells and foci at the very first steps of tumor cell invasion and metastasis. Although the effect of systemic hypoxia and VEGF expression on the extracellular matrix and tumor cannot be neglected but in this work, we did not investigate it.

4.3 HYPOXIA-INDUCED AND CALPAIN-DEPENDENT CLEAVAGE OF FILAMIN A REGULATES THE HYPOXIC RESPONSE (PAPER III)

FLNA is well known as a cytoskeleton protein, which mediates hypoxic responses. It is important to investigate the effect of FLNA in cell motility, although there is evidence derived from cancer cohorts, which show that the expression level of this protein is down regulated in some cancer types (95). Here we decided to investigate the effect of FLNA on growth of solid tumors. For this purpose, we took advantage of the human melanoma cell lines M2 (FLNA deficient cell line), and A7 (FLNA supplemented). Tumor cells were subcutaneously injected into male SCID mice. Tumor cells containing FLNA (A7) grew faster and generated larger tumors compared to the FLNA deficient (M2) group. It is known that solid tumors suffer from inner hypoxia and an increased level of hypoxia is directly related to an increase in the level of VEGF-A, which is the driving force for angiogenesis. Immunohistochemistry staining on the tumor tissue revealed increased blood vessel density, number of vessels per field, and also increased diameter of vessel lumen, all indicators of angiogenesis. The mRNA expression level in the tumors harvested from the mice, showed increased level of VEGF-A in A7 group compare to the M2 group.

To investigate the potential role of FLNA in regulation of HIF signaling pathway, an HRE-driven luciferase reporter assay in A7 and M2 cells was performed. As expected, FLNA significantly enhanced hypoxia-inducible

luciferase activity in A7 cells compare to M2. Measuring the mRNA levels of some common HIF target genes such as VEGF-A, GLUT1, GLUT3, and BNIP3, under hypoxic conditions revealed markedly increased levels of endogenous expression in A7 cells compared to M2 cells. However, under normoxia, endogenous levels of mRNA of these targets genes except for VEGF-A, were equally low in both cell lines. Additional experiments on ECs such as migration assay using conditioned medium from A7 and M2 cells, proliferation and tube formation also concluded the definitive effects of FLNA on increased level of VEGF-A expression. To further confirm the effect of FLNA on the HIF transactivation function, A7 cells were transfected with siRNA against FLNA. We discovered that by silencing FLNA, mRNA expression levels of VEGF-A and GLUT3 were significantly reduced in both hypoxic and normoxic conditions. After revealing the regulatory role of FLNA on hypoxia-induced signaling pathways, we decided to study whether HIF-1a is able to interact with FLNA. For this purpose, we used the yeast two-hybrid system, and the interaction was confirmed with Immunoprecipitation assay. The interaction domain was limited to the Ig repeats between 20 and 24 in the C-terminal of the FLNA molecule and the N- terminal of the HIF-1a protein between amino acid residues 1-390 which contains bHLH and PAS domains. FLNA as well as other filamins are very sensitive to proteolysis. Calpain protease cleaves FLNA from H1 and H2 regions and creates three fragments, 190, 90, and 10 kDa in size, respectively (114, 115). In whole cell extract of A7 and COS1, two major fragments with molecular weight of 200 and 100 kDa were found under normoxic and hypoxic conditions. The 200 kDa fragment reacted to the antibody against the N-terminal of FLNA and the smaller fragment with molecular weight of 100 kDa, was detected with help of an antibody against C-terminal of the FLNA protein. Interestingly, the cleavage activity that creates the C-terminal of FLNA ($FLNA^{CT}$) was increased once cells were treated with hypoxia. It is known that hypoxia increases level of calcium in the cytoplasm and since calpain activity requires calcium, it has been shown that hypoxia increases calpain activity. In order to validate the calpain activity to generate the cleaved fragments of FLNA rather than caspase-3/7, which could also potentially target FLNA for proteolysis, we used calpeptin and Ac-DEVD-CHO to inhibit their effects respectively. Calpeptin reduced cleavage of FLNA under hypoxic conditions and no such effect was detected in

the Ac-DEVD-CHO treated cells. These findings confirm the proteolytic effects of calpain leading to increasing levels of $FLNA^{CT}$ under hypoxia. Also it was observed that concentration of C-termianl of FLNA protein was increased in the nucleus compared to the cytoplasm under hypoxia. The effect of hypoxia-induced cleavage of FLNA by calpain on HIF-1a transactivation function was investigated by measuring mRNA levels of some of the HIF-1a target genes such as GLUT1 under calpeptin and Ac-DEVD-CHO inhibition. We observed that GLUT1 gene expression was significantly decreased in A7 cells inhibited by calpeptin but no effect was seen in M2 group. To investigate the effect of FLNA on intracellular distribution of HIF-1a, cytoplasmic and nuclear levels of HIF-1a were measured in both M2 and A7 cells. The level of HIF-1a in the cytoplasm was higher in M2 cells compared to the A7 cells. On the contrary, the nuclear level was higher in the A7 group, suggesting that FLNA facilitates nuclear accumulation of HI-1a under hypoxic conditions. Subcellular localization of a GFP-tagged HIF-1a in A7 and M2 cells under hypoxia showed a clear and exclusive nuclear localization of GFP-HIF-1a in A7 cells while M2 cells were slightly affected by hypoxia and showed a slight increase in the nuclear distribution of the GFP-HIF-1a compared to normoxic conditions. Taken these results together, we concluded that FLNA facilitates nuclear localization of GFP-HIF-1a in hypoxia. Inhibition of calpain by calpeptin under hypoxia decreased accumulation of the GFP-HIF-1a in the nucleus of the A7 and COS1 cells but had no effects on GFP-HIF-1a nuclear accumulation in M2 cells. $FLNA^{CT}$ was found increased and up regulated in other human cell lines such as U2OS and 293T, while no $FLNA^{CT}$ was seen in HEK and HeLa cells. The results achieved from various human cell lines suggest that FLNA cleavage is cell-specific and hypoxia enhances its occurrence. By silencing FLNA in U2OS and 293T cell lines under hypoxia, expression of target genes was significantly decreased, while in HeLa cells, no effect was observed. To investigate the importance of the C fragment of FLNA in regulation of nuclear translocation and transactivation function of HIF-1a, we generated cell lines stably expressing FLAG-tagged FLNA, DH1, which has been engineered to lack the H1 domain and therefore be insensitive to calpain proteolysis or $FLNA^{CT}$, and CMX as control. Subcellular distribution of GFP-HIF-1a was examined in all cell lines and those cells expressing full length of FLNA or the C-terminus of FLNA 16-24 Ig repeats showed exclusively nuclear distribution

of GFP-HIF-1a under hypoxia, while the control group as well as DH1 had predominantly nuclear distribution compared to the cytoplasmic under either condition. $FLNA^{CT}$ was able to induce the same response as full length FLNA on nuclear distribution of GFP-HIF-1a and this function was ablated by deletion of H1 domain completely. In order to assess the transactivation function of endogenous HIF-1a, we used an HRE-driven luciferase assay in all four stably transfected cell lines. Luciferase activity was several folds higher in FLNA and $FLNA^{CT}$ cell lines compared to the control and DH1. mRNA levels of GLUT1 showed increased expression in FLNA and $FLNA^{CT}$ cell lines but no difference in the control and DH1 cells. These results together indicate that calpain cleavage of the FLNA at the precise H1 domain creates a fragment of FLNA, which is essential for nuclear translocation of the HIF-1a and regulation of its transactivation function under hypoxia. A chromatin Immunoprecipitation experiment was performed to assess the requirement of FLNA in the promoter of HIF target genes. A7 and COS-1 cells had FLNA and HIF-1a in the HRE-containing region of the *VEGF-A* promoter gene under hypoxia. Even under normoxia we found HIF-1a binding to the VEGF-A promoter, in M2 cells under hypoxia, however, only HIF-1a was present in the HRE-containing region and no FLNA was detected. Based on the results from the previous experiment we can conclude that FLNA is recruited to the HIF-1a transcription complex on the HRE-containing region of the promoter of target genes.

This model suggests that FLNA has a regulatory effect on HIF-1a function and consequently VEGF-A transcription (140). HIF-1a nuclear localization is promoted by calpain proteolytic cleavage of FLNA (141) and generation of $FLNA^{CT}$ which facilitates the nuclear accumulation of the HIF-1a and drives transcription of the HIF-1a target genes including VEGF-A, similar to the situations observed in lung and peripheral cholangiocarcinoma. In those carcinomas, high level of FLNA correlates with higher expression of VEGF-A (142).

4.4 TARGETING FILAMIN B INDUCES TUMOR METASTASIS AND GROWTH VIA ENHANCED ACTIVITY OF MATRIX-METALLOPROTEINASE-9 AND SECRETION OF VEGF-A (PAPER IV)

Filamins are known to be crucial for cell motility by binding to cytoskeleton proteins such as actin or other scaffolding signaling proteins (97, 143). In the current study, we are investigating the effect of FLNB on tumor invasiveness and metastasis. We chose to use mouse embryonic fibroblasts (MEF) stably transfected with H-RAS in our *in vivo* and *in vitro* studies. To assess the effect of FLNB on tumor growth and angiogenesis, we implanted $Flnb^{+/+}$ and $Flnb^{-/-}$ MEFs transfected with H-RAS into SCID mice. Tumor growth was monitored and tumor size measured at precise intervals. Mice inoculated with $Flnb^{+/+}$ tumor cells grew, but only slightly, for a week and then tumor growth halted while the $Flnb^{-/-}$ tumors grew twice as large as in the other group. Immunohistochemistry staining of the tumor blood vessels by a PECAM antibody showed higher intratumoral vessel density and vessel number in the group deficient for FLNB compared to the FLNB containing group. In order to investigate the effects of FLNB on tumor cell dissemination and metastasis we chose the zebrafish dissemination model, which allows us to analyse the metastatic pattern of the tumor cells in both groups at single cell level. Tumor cells were labelled with fluorescent dye and implanted into the perivitelline space of 2 days old zebrafish embryos. After 6 days, embryos were imaged with help of fluorescence microscopy and subsequently quantified for the number of disseminating cells into the local and distal parts of the fish body. Although both groups showed dissemination throughout the fish body, the number of disseminated tumor cells in the MEF cells deficient for FLNB was significantly higher, indicating inhibitory effects of FLNB on dissemination and metastasis of tumor cells from the primary tumor site. Since tumor cell metastasis is a complex process, the adopted migratory phenotypes of the cells are most likely not the only cause for the observed increase in dissemination - degradation of the extracellular matrix could be important and should not be neglected (85). Therefore, we decided to investigate the effect of FLNB deficiency on extracellular matrix degradation by measuring proteolytic activity of the two MMPs (MMP-2 and MMP-9) regulated by the *Flnb* gene. $Flnb^{+/+}$ and $Flnb^{-/-}$ MEFs were treated with PMA or buffer and as results we observed that the

expression level of MMP-9 mRNA in both *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ groups was significantly increased while the increased level was much higher in *Flnb*$^{-/-}$ MEFs under influence of PMA compared to the *Flnb*$^{+/+}$group. Phorbol myristate acetate (PMA) is a phorbol ester known to substitute for DAG as a high affinity ligand for PKC and novel PKC isoforms. It has been reported that PMA strongly stimulates MMP-9 expression via PKC in some systems (113). On the contrary, levels of MMP-2 in both groups under different conditions were not changed suggesting that MMP-2 is regulated by a different mechanism compared to MMP-9. The proteolytic activity of the two MMPs was measured by the gelatin zymography assay, and although the baseline activity was similar in both cell lines, after stimulation of cells with PMA, the FLNB deficient group showed significant increase in proteolytic activity. MMP-2 proteolytic activity followed the same trend as its mRNA level and there was no significant difference in the level of activity in different cell lines with or without PMA, confirming existence of another regulatory mechanism for MMP-2. Levels of MMP-9 were tested in ECs isolated from *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ mice and as expected, the MMP-9 mRNA level was significantly higher in the FLNB deficient group compared to FLNB expressing group. In some cases (97), it has been shown that filamins compensate each other's functions. In order to eliminate the possibility of a compensatory mechanism, we used M2 human melanoma cell lines lacking FLNA. Using shRNA, we silenced the *Flnb* gene in these cells and then measured the expression level of MMP-9 mRNA. We found a significant decrease in the mRNA level in the silenced group compared to the control (non-silenced) group. The importance of the MMP-9 activity on enhancement of invasion of the tumor cells was assessed by treatment of *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ MEFs with PMA or buffer in a matrigel invasion assay. Increased numbers of invaded cells in the *Flnb*$^{-/-}$ cells treated with PMA was another indicator of the inhibitory effect of FLNB on tumor invasion and metastasis. The MAPK cascade is the responsible pathway responding to the extracellular stimuli and thereafter regulates MMP-9 expression. ERK1/2 signaling pathway is specially activated by PMA and some growth factors. Immunoblotting assays of the *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ MEFs for different molecular players involved in the MAPK cascade in response to PMA showed increased levels of RAS-GFR, phosphorylated ERK1/2 and various PKC isoforms in *Flnb*$^{-/-}$ MEFs treated with PMA. Lower level of ERK1/2 in *Flnb*$^{+/+}$ MEFs

indicates that FLNB suppresses the ERK1/2 signaling pathway and as result, reduced MMP-9 levels. And PMA stimulation of *Flnb*$^{-/-}$ MEFs leads to phosphorylation of PKC-a/b and PKC-δ/θ but not in *Flnb*$^{+/+}$ MEF. These results indicate that FLNB has a major function in regulation of the PKCs. Then we tested the proteolytic activity of MMP-9 to identify whether this phenomenon is ERK1/2 signaling dependent or not. *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ MEFs stimulated with PMA and treated with MEK inhibitor blocking ERK1/2 showed abolished proteolytic activity of MMP-9 in the *Flnb*$^{-/-}$ MEF group stimulated with PMA, while no difference in MMP-2 proteolytic activity was seen among the groups. Measuring mRNA levels of ERK1/2 and MMP-9 in the tumor samples harvested from mice also showed increased expression levels in the *Flnb*$^{-/-}$ MEF group. Since tumor invasiveness and metastatic phenotypes are highly affected by the level of angiogenesis occurring in the tumor, we decided to investigate wither VEGF-A expression in mediated by MAPK cascade. We collected conditioned medium of *Flnb*$^{+/+}$ and *Flnb*$^{-/-}$ MEFs treated with PMA or MAPK inhibitor and measured the level of VEGF-A. We found that the expression level of VEGF was significantly increased upon treatment with PMA or MAPK inhibitor, although PMA stimulation had a more pronounced effect compared to the MAPK treatment group. Silencing of the *Flnb* gene in human ovarian cancer cells also significantly increased expression level of VEGF-A. Conditioned medium collected from these cells induced elevated formation of vessel like structures in porcine aortic endothelial (PAE) cells in a matrigel tube-formation assay.

In summery our results from this work suggest that FLNB has a negative regulatory effect on tumor growth and metastasis. This is in contrast to other filamins such as FLNA that have positive effect on tumor progression and angiogenesis. Further, the effect of FLNB on tumor metastasis via MMP-9 is not affected or controlled by other filamins. FLNB inhibits tumor progression by two different mechanisms; first by reduction of VEGF-A expression, which in turn reduces angiogenesis, and second by suppressing MMP-9 expression thereby reducing extracellular matrix degradation. These processes consequently result in inhibition of tumor cell dissemination and metastasis.

5 CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, we have used the zebrafish embryo as a model organism to study invasion and metastasis of tumor cells. This *in vivo* model allows us to study the mechanism of initial steps in the metastatic cascade in a more clinically relevant manner than in commonly used *in vitro* models. However, due to the difference between the optimal temperature for maintenance of zebrafish embryos (28°C) and culturing of mammalian tumor cells (37°C), proliferation rate in some tumor cell types such as fibrosarcoma cells, which we used in some of our experiments, is low and therefore it does not reflect the true nature of the cells that they would exhibit under optimal conditions. Consequently, cellular responses can be affected. This makes the model less attractive to study tumor proliferation and growth under different conditions although green fluorescent protein produced by the mammalian tumor cells allows to confirm that the cells are alive and functional and in spite of suboptimal temperature, the tumor cells retain their intrinsic metastatic capabilities as they are metastatic potential in this model corresponds to results derived from other, previously published models. Furthermore, it is clear in my studies that tumor derived VEGF has a massive impact on the blood vessels, and that they respond to hypoxia, indicating that the tumor cells are highly metabolically active and therefore do not seem vastly affected by the lower temperature.

Another drawback of this model is that since the window to study tumor cell invasion in zebrafish is very short, the disseminated tumor cells will not have the time to regrow after colonization in the metastatic site (niche), and reach the clinically detectable size as seen in mouse models. Due to existence of a vast variety of transgenic zebrafish lines expressing different proteins, there has not been an equally big effort on developing specific antibodies for immunohistochemistry staining for zebrafish as compared to mouse. In this model, staining of proteins expressed on mammalian tumor cells is feasible since the available antibodies are developed to recognize and bind those proteins. However, staining of endogenous fish proteins is far more challenging due to the lack of fish-specific antibodies.

Applying hypoxia to tumor-bearing zebrafish embryos has a global effect on the fish, which means not only the primary tumor site is affected but also rather the whole fish body including the potential metastatic sites and niches. These circumstances prevents the researchers to study the effects of hypoxia specifically in the primary site and how hypoxia-induced cytokines and growth factors released and expressed by tumor cells

may influence non-hypoxic tissues to prepare the pro-metastatic niches. On the other hand, this model allows to exclusively study the effect of hypoxia while in the hypoxic-ischemic mouse model, induction of hypoxia by causing ischemia in a specific region/tissue, also leads to several additional events. Those, together with hypoxia will affect the outcome of the experiment. Ischemia induced in the tissue causes death of endothelial cells, lack of blood circulation and thereby nutrient supply in the affected region. Consequently, there will be a reduction in the recruitment of blood-derived inflammatory cells in the region. Inflammatory cells such as subgroups of macrophages, which to a large extent are derived from circulating monocytes, have been shown to facilitate tumor cell metastasis. Lack of blood circulation ablates these naturally occurring events in the process of tumor malignancy. Taken together, since the ischemic model contains many variables and interferes with many different mechanisms, it is not always a suitable system to study effects of hypoxia.

Since zebrafish embryo model allows us to detect the invasive nature of the tumor cells at the primary stage of invasion and dissemination, this model organism can be used to screen human cancer patients with metastatic primary tumors from those with non-metastatic ones, by implanting primary tumor cells into the fish and observe the invasion pattern. In order to study the effect of tumor environment and host inflammatory response in promoting or inhibiting tumor growth and metastasis, tumor cells can be co-implanted with other cell types, such as inflammatory cells (macrophages),fibroblasts and ECM components. Furthermore, tumor cells can be treated and educated with different cytokines, growth factors or any other substrate. They can even be co-cultured with other cell types and then implanted into the fish embryo to study the impact of other factors on invasiveness and metastatic properties of the tumor cells.

In the second part of thesis I focused my work on two different types of filamins, which although they belong to one big family are shown here to have opposing effect on angiogenesis, tumor cell invasion and metastasis.

FLNA is a protein, which interacts with a large number of genes and is widely expressed in the body. FLNA has been repeatedly seen in various types of cancer and is correlated with angiogenesis and metastasis. It has a pro-angiogenic effect by interacting with HIF-1a molecule and transferring it to the nuclei under hypoxia and induction of VEGF, which is the key growth factor in angiogenesis and consequently induction of metastasis. On the other hand, FLNB, another member of this family has been shown to have negative effect on expression of VEGF and also metastasis of

tumor cells by impairing MMP-9 function. Although it is believed that filamins may functionally compensate for each other, in this study we show that the angiogenic effect of FLNB is completely opposite of FLNA. Interestingly in contrast to FLNA, FLNB is more tissue specific and has been particularly seen expressed in ECs. Expression of FLNB specifically in EC and negative regulatory effect of FLNB on VEGF expression, propose a more specific role for this protein in EC proliferation, motility and angiogenesis, which means that FLNB might have a clear role in maintenance of EC quiescence and therefore down-regulation of FLNB leads to induction of angiogenesis and increased tumor cell metastasis.

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